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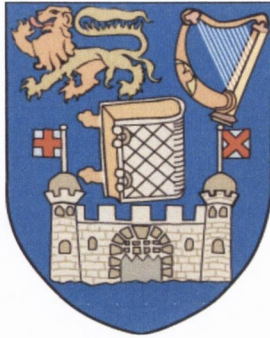
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Immune Variation in Idiopathic Bronchiectasis

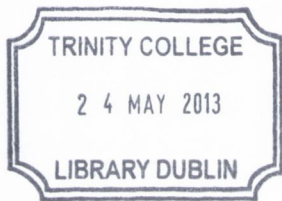
by

Dr. Niall Patrick Conlon

A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the
degree of

Doctor of Philosophy

2013



Thesis 9803

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Bronchiectasis is a chronic suppurative lung disease. It is the pathological end-point of many disease processes including cystic fibrosis and antibody deficiency. In many cases of bronchiectasis, termed 'idiopathic', no clear cause is found, even when extensive investigations are carried out. This study recruited a cohort of patients with idiopathic bronchiectasis and evaluated them for polymorphisms in candidate genes with potential modulating influences on immune function. Variation in genes coding for MBL (mannose binding lectin) and the low-affinity IgG receptor CD32A were examined in an idiopathic bronchiectasis cohort and compared with healthy Irish controls.

One hundred and one patients with idiopathic bronchiectasis were recruited from an Irish tertiary referral centre. This cohort exhibited many similarities with other published populations of idiopathic bronchiectasis in developed countries. Evaluation of the CD32A H131R polymorphism indicated a significant excess of the RR131 variant in the bronchiectasis population compared with controls ($p < 0.01$, OR 2.78, 95% CI 1.53-5.03). In contrast, CD32A heterozygosity was under-represented in the population of patients with bronchiectasis ($p < 0.03$, OR 0.53, 95% CI 0.31-0.95). These findings were suggestive of an association between bronchiectasis susceptibility and CD32A RR131.

Interrogation of clinical data from patients with idiopathic bronchiectasis revealed that the presence of CD32A RR131 was associated with expectoration of purulent sputum on a daily basis ($p < 0.001$), more frequent infective exacerbations ($p < 0.001$), and more frequent hospital admissions ($p < 0.001$) when compared with other polymorphic variants. Furthermore, patients with the RR131 polymorphism were more likely to have positive sputum culture ($p = 0.003$), particularly with encapsulated bacteria. In addition, the RR131 patient group had lung function parameters suggesting more severe disease, a finding confirmed on formal evaluation of high resolution CT scans. Interestingly, the presence of a significant smoking history was associated with worse bronchiectasis scores in the CD32A RR131 patient group alone, a finding suggestive of a possible gene-environment interaction. While RR131 variation seemed to be associated with more severe disease, heterozygous patients seemed to be less severely affected. These previously unreported findings provide compelling evidence that RR131 was not only associated with the presence of bronchiectasis, but also with disease severity.

To evaluate the functional impact of these findings, phagocytosis of opsonised pneumococcal targets by isolated neutrophils from controls of known CD32A genotype was measured by flow cytometry. It was demonstrated that significantly fewer neutrophils from RR131 donors

phagocytosed pneumococcal targets than other genotypes ($p < 0.001$). A consistent stepwise progression in target phagocytosis was observed with neutrophils from HH131 donors exhibiting the most efficient phagocytosis, and heterozygote neutrophils demonstrating intermediate phagocytosis. After stimulation, neutrophils from RR131 donors also generated reduced oxidative burst and significantly lower amounts of the azurophilic granule products elastase and myeloperoxidase. These findings were extended with the development of a whole blood opsonophagocytosis assay, allowing the examination of peripheral blood CD14 mononuclear cells simultaneously with neutrophils. In both cell species CD32A RR131 variants were consistently observed to be less efficient at phagocytosing pneumococcal targets. Examination of mononuclear cell cytokine secretion following stimulation with opsonised pneumococcal polysaccharide coated beads did not reveal any genotype dependent differences. These compelling findings reinforce our previous work, and strongly suggest that variation in CD32A H131R genotype is important in the phagocytosis of pneumococci.

In contrast, no significant difference in MBL haplotypes were observed between cases of bronchiectasis and controls in this study. Furthermore, MBL deficiency was not observed at increased frequency in the bronchiectasis population. Levels of C-reactive protein, an acute phase reactant were significantly higher in the group of patients with genotypes associated with MBL insufficiency. Patients with low serum MBL levels were more likely to produce daily purulent sputum ($p = 0.01$), but no effect was seen on other markers of disease severity. The subgroup of patients with the LYQA genotype were less likely to produce purulent sputum and less likely to be admitted to hospital. The lack of consistency in these clinic-pathological correlates is in sharp contrast to the findings with CD32A. These findings therefore do not support a major role for MBL in adult bronchiectasis. However, it is possible that polymorphisms of MBL could synergise with other uncommon subtle immune defects and predispose to bronchiectasis, but very large multi centered studies would be required to demonstrate such an effect.

This investigation highlights an important role for CD32A variation in bronchiectasis, allowing the development of a paradigm for the role of this gene in the pathogenesis of disease. These important findings may be useful in identifying patients at risk of developing bronchiectasis and may help risk stratify patients with bronchiectasis, offering practical potentially therapeutic benefit in this challenging and neglected condition. Further assessment of these findings in well-characterised international disease cohorts is necessary to fully validate these results.

For Henry Paul Conlon

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Research outputs to date

- **A common Fc receptor defect increases the risk and severity of bronchiectasis**
Poster presentation, American Thoracic Society, Denver, May 2011
Winner of an International Travel Award
- **A common Fc gamma RIIA Polymorphism increases the risk and severity of idiopathic bronchiectasis**
Oral presentation at UK Primary Immunodeficiency Network, Liverpool, Dec 2011
- **Immune variation in idiopathic bronchiectasis**
Poster presentation, ICH Immunodeficiency Winter School, Windsor, Mar 2012
- Molecular Medicine Ireland AGM oral presentation updates July 2009, July 2010 and July 2011

In preparation

- **A common polymorphism in CD32A increases the risk and severity of idiopathic bronchiectasis in an Irish cohort** N.P. Conlon, D. Ryan, I. Counihan, N. Marshall, M. Rogan, G. Murray, S. O'Neill, C. Feighery, M. Keogan, J. Jackson
- **Mannose binding lectin deficiency is not associated with idiopathic bronchiectasis in an Irish cohort** N.P. Conlon, D. Ryan, I. Counihan, N. Marshall, M. Rogan, G. Murray, S. O'Neill, C. Feighery, M. Keogan, J. Jackson

Academic achievements during the Fellowship period

- **Postgraduate Diploma in Allergy** (University of Southampton) 2009
- **Postgraduate Diploma in Statistics** (University of Dublin) 2010

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1. Introduction

Bronchiectasis is a chronic suppurative lung disease (Pasteur et al., 2010). Although often categorized as an orphan disease, evidence suggests it is a considerable burden on health care providers (Kelly et al., 2003). Patients with bronchiectasis require regular follow up and, frequently, hospital admission (Kelly et al., 2003, Sharples et al., 2002).

1.1 Historical background

Bronchiectasis as a clinical entity was first described in detail by Rene Laennec, the inventor of the stethoscope (Sakula, 1981). He was the first to correlate the auscultatory findings with the pathological changes characteristic of the disease. William Osler in the late 19th century built on this ground work with a more detailed clinical description of the disease (Wrong, 2003).

Interestingly, the great physician himself suffered from recurrent chest infections in later years and may have died from complications of bronchiectasis (Wrong, 2003). The first detailed pathological descriptions of bronchiectasis came in the mid 20th century. Reid in 1950 categorized bronchiectasis into three classical phenotypes; tubular, varicose and cystic (Reid, 1950). Further information was detailed in a large study by Whitwell, which demonstrated marked inflammation in small airways with bronchial dilatation and destruction of structural wall components in severe disease (Whitwell, 1952). Early pathological studies were hampered by the numerous causes of bronchiectasis. This heterogeneity remains a problem in studies of this condition today.

1.2 Incidence

The incidence of bronchiectasis is unknown (Pasteur et al., 2010). Medical textbooks suggest that with improved vaccination strategies and antibiotic therapy, the incidence of bronchiectasis is falling (O'Donnell, 2008). There is little recent evidence to support this idea. Many population

studies were carried out before the use of High Resolution CT scanning (HRCT), the gold standard for diagnosis. While some studies have suggested falling hospital admissions, a recent US study suggested that admissions are increasing (Seitz et al., 2010). Furthermore, recent UK data suggests that bronchiectasis mortality is increasing year on year (Roberts and Hubbard, 2010). Importantly, recent data has also given insight into the significant costs associated with bronchiectasis (Seitz et al., 2010). Bronchiectasis increases with age and is found more commonly in females. With the advent of increasingly sensitive detection methods it seems likely that the numbers of patients with bronchiectasis will rise. Therefore investigations into the causes of and risk factors for bronchiectasis are becoming increasingly necessary.

1.3 Clinical Features

Bronchiectasis has a significant impact on quality of life (Martinez-Garcia et al., 2005). The most prominent symptom is chronic productive cough (Habesoglu et al., 2011, Pasteur et al., 2010). Sputum is persistently purulent and is not infrequently blood stained (Habesoglu et al., 2011). Major haemoptysis is a rare complication of severe bronchiectasis. Infective exacerbations are common and may be debilitating. Dyspnoea is a frequently observed feature. Other respiratory co morbidities may also be observed, particularly rhinosinusitis, which is under-diagnosed, under treated and has a considerable impact on day to day living (Guilemany et al., 2009a, Guilemany et al., 2011). Finger clubbing is only rarely observed (Pasteur et al., 2010). Spirometry often shows a mild obstructive picture which may be reversible in some patients. Many patients have a preceding diagnosis of asthma. In such cases there is debate as to whether it is the asthma or the bronchiectasis that is driving the disease (Pasteur et al., 2010).

Bronchiectasis is characterized by colonization and infection with a number of different microorganisms. Non-typable *Haemophilus influenzae* is the most common infecting organism in

adults and children (Pasteur et al., 2010). Encapsulated organisms comprise the other major microbes observed (Eastham et al., 2004, Li et al., 2005, Pasteur et al., 2000). The prevalence of pathogens varies considerably across different studies. *Pseudomonas aeruginosa* is generally the second most commonly encountered micro-organism and is seen more frequently in adults than children. As in cystic fibrosis, the presence of pseudomonas is a poor prognostic factor and is associated with an accelerated decline in lung function and increased hospitalization (Ratjen, 2006). Recent paediatric data demonstrates that *Streptococcus pneumoniae* is more frequently encountered in an Irish cohort, accounting for 30% of isolates (Zaid et al.). This suggests that the pneumococcus is an important pathogen in bronchiectasis in Ireland.

Organism	% Isolation in adults	% Isolation in children
<i>Haemophilus Influenzae</i>	20-35%	24-70%
<i>Pseudomonas aeruginosa</i>	10-30%	2-11%
<i>Streptococcus pneumoniae</i>	10-20%	10-20%
<i>Moraxella cattarhalis</i>	5-20%	5-20%
<i>Staphylococcus aureus</i>	5-10%	0-5%
Atypical Mycobacteria	0-10%	0-2%

Table 1.1 showing prevalence of culture of potentially pathogenic organisms from patients with bronchiectasis. Adapted from data presented in Pasteur *et al* (Pasteur et al., 2010)

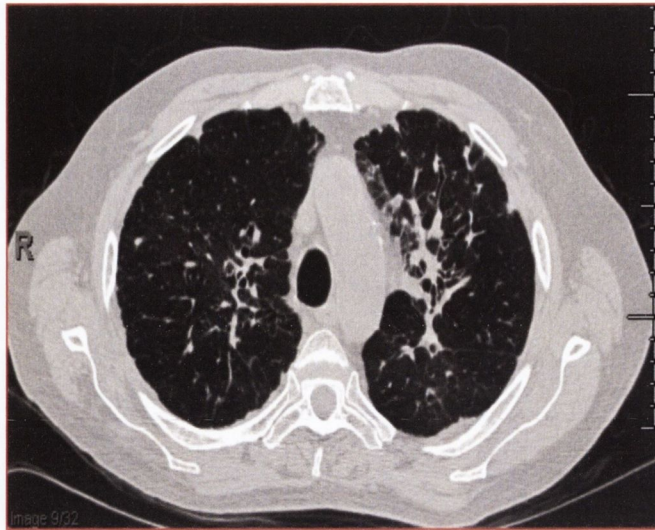
1.4 Radiological Diagnosis of bronchiectasis

Demonstration of bronchiectatic change on a high resolution CT scan is an absolute requirement for the diagnosis of idiopathic bronchiectasis (Pasteur et al., 2010) (Figure 1.1 a and b). A number of radiological features suggest the presence of bronchiectasis – irrespective of cause;

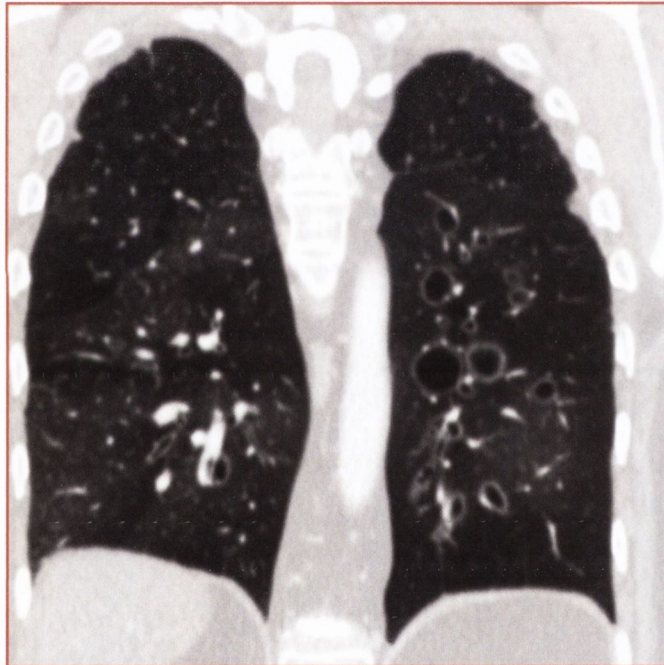
1. An increase in the bronchoarterial diameter ratio, indicating bronchial dilatation
2. Bronchial wall thickening
3. Bronchial plugging
4. Decreased attenuation of the lung parenchyma

Subtle changes suggestive of all of these factors may be seen in the lungs of smokers and asthmatics, thus the radiological evidence must be collated carefully to determine whether minor changes do indeed represent bronchiectasis.

Several scoring systems have been used to grade the radiological severity of bronchiectasis (de Jong et al., 2004, Edwards et al., 2004, Nathanson et al., 1991, Roberts et al., 2000, Smith et al., 1996, Wong-You-Cheong et al., 1992). All of these scoring systems have been validated in cystic fibrosis. To date no scoring system has been fully validated in non-CF bronchiectasis or have demonstrable superiority over other systems. Bronchiectasis scores from a number of different scoring systems confirm a link between radiological severity and more pronounced airflow limitation. Some studies also suggest a link to related clinical parameters, although the strength of the relationship is a matter of debate (Edwards et al., 2004, Smith et al., 1996, Ooi et al., 2002). At present, in the absence of significant humoral immunodeficiency, routine interval CT monitoring is not required but should be carried out as judged by clinical need (Pasteur et al., 2010).



a



b

Figure 1.1 a) showing bronchiectasis in the bases of right and left upper lobes in an adult patient. Areas of bronchiectasis are highlighted. Figure 1.1 b) showing severe cystic bronchiectasis of the left lung on sagittal section CT scan. The right lung is less severely involved in this view.

1.5 Management of idiopathic bronchiectasis

Patients with idiopathic bronchiectasis represent a significant management challenge to clinicians involved in their care. Despite the emergence of recent guidelines, management strategies are often based on expert opinion and lack clear evidence bases (Pasteur et al., 2010). Management principles for non-CF bronchiectasis are often extrapolated from studies in patients with cystic fibrosis. However, differences in the pathogenesis, severity and colonisation patterns question the validity of this extrapolation. In fact studies suggest that the medications most frequently prescribed to patients with idiopathic bronchiectasis lack any formal evidence base (Pasteur et al., 2010). Significant progress in establishing a clear evidence base for current practice is required.

1.5.1 Non-pharmacological management

Respiratory physiotherapy is a mainstay of management of bronchiectasis irrespective of the cause. Expert consensus recommends its use in patients with chronic productive cough, and in all patients during infective exacerbations (Pasteur et al., 2010). No evidence base exists for choice of physiotherapy technique. This may depend on the age and capability of the patient. Despite the lack of evidence, years of clinical experience, suggest that respiratory physiotherapy enhances airway clearance and reduces the frequency of debilitating cough (De Boeck et al., 2008, McCool and Rosen, 2006). In addition to physiotherapy regular aerobic exercise improves quality of life and prevents deconditioning. At this point, good adherence with physiotherapy and exercise programs should be recommended for all patients as part of a holistic management strategy (Ong et al., 2011).

1.5.2 Bronchodilator treatment

Almost all patients with bronchiectasis are prescribed inhaled bronchodilators (Pasteur et al., 2010). Most patients with bronchiectasis have obstructive lung function tests. However, in the presence of co morbidities, such as asthma and COPD, the process driving the disease or any bronchial hyperreactivity can be difficult to determine. A single older study indicates that the use of salbutamol in bronchiectasis improves lung function tests more than placebo (Franco et al., 2003, Sheikh et al., 2001). Despite this, there is no clear evidence for the long term use of bronchodilator therapy or indeed long acting bronchodilators in bronchiectasis (Franco et al., 2003, Sheikh et al., 2001). In practical terms, an element of reversibility or symptomatic response to bronchodilator therapy should be sought to justify prescription of these medications (Hassan et al., 1999).

1.5.3 Inhaled corticosteroids

A number of studies have addressed the use of inhaled corticosteroids in bronchiectasis (Elborn et al., 1992, Kapur et al., 2009, Martinez-Garcia et al., 2011a, Mostafapour et al., 2009, Tsang et al., 2005). Several of these studies indicated that inhaled corticosteroids reduced sputum markers of inflammation, sputum production and improved quality of life. While these studies often have relatively small numbers they do support the use of inhaled corticosteroids in difficult to control patients.

1.5.4 Antibiotics

Antimicrobial therapy is part of the mainstay of the management for infective exacerbations of bronchiectasis. The possible role of long term antibiotics, courses of prophylactic antibiotics and their use in the management of colonisation in the absence of clinical infection remains unclear. The use of antibiotics in the setting of acute infection should be tailored to sputum results. Decisions in this regard are aided by collection of sputum libraries on individual patients.

Recently updated guidelines suggest the use of amoxicillin-clavurinate, moxifloxacin or levofloxacin for acute exacerbations in patients with bronchiectasis, with ciprofloxacin reserved for those with a risk of pseudomonas infection (Pasteur et al., 2010). The duration of treatment is not clear but extended courses are frequently used irrespective of infectious agent.

Prolonged use of oral antibiotics in non-CF bronchiectasis is commonplace. A recent meta-analysis examining the use of long term antibiotics did not note improvements in exacerbation rates with long term use (Evans et al., 2007). However, more positive results were obtained from a well designed study examining the use of prophylactic azithromycin (Anwar et al., 2008). This study demonstrated reduced sputum volumes and a reduced frequency of infective exacerbations. Similar preliminary data also supports the long term use of erythromycin (Serisier and Martin, 2011). Such studies pave the way for larger, more robust randomised control trials.

Inhaled antibiotics have also been examined in bronchiectasis. Inhaled tobramycin appears to be useful in improving symptoms and lung function in bronchiectasis with pseudomonas infection (Bilton et al., 2006). New formulations combining tobramycin with fosfomycin may yield further advances but investigations are at their preliminary stages (MacLeod et al., 2009) Inhaled gentamicin is a further option (MacLeod et al., 2009). Patient selection seems likely to be critical for the choice of long term antibiotics. Studies examining optimum patient groups for individual treatment profiles are required. Inhaled DNAase has also been examined in non-CF bronchiectasis (MacLeod et al., 2009). In contrast to antimicrobial therapy, there is evidence that this type of inhaled medication has no benefit and may even cause harm (O'Donnell *et al* 1998).

1.5.5 Surgery

Surgical resection remains a treatment option for localised bronchiectasis refractory to other forms of management (Sehitogullari et al., 2011, Gursoy et al., 2010). Recent data suggests that

resection is a safe option at any age group in cases of localised bronchiectasis, with acceptable morbidity and mortality, where there is adequate pulmonary reserve (Sehitogullari et al., 2011).

Best results are found in cases where complete resection of diseased areas is possible.

Therefore, selection of appropriate candidates is important. Many patients with idiopathic bronchiectasis have diffuse disease and surgery may not be a viable option.

1.6 Pathophysiology of Bronchiectasis

The pathological characteristic of bronchiectasis, irrespective of cause, is permanent dilatation of the airways. Structural damage to the airways results from propagation of a vicious cycle of infection and inflammation (Cole, 1986, Barker, 2002). The causes of initiation of the vicious cycle are not clear, however, it seems likely that multiple mechanisms are at play. Infection is thought to play a central role in establishing a chronic inflammatory state. Despite significant advances in the knowledge of airway inflammation in conditions such as asthma and COPD, little is known about pathologic processes in bronchiectasis (King, 2009).

The predominant inflammatory cell in the airways of patients with bronchiectasis is the neutrophil. Airway neutrophil numbers are increased in sterile airways of clinically stable bronchiectatic patients compared with controls (Salva et al., 1996). Analysis of biopsy specimens from patients with bronchiectasis demonstrates an abundance of molecules which facilitate neutrophil recruitment, such as LPS (lipopolysaccharide) and TNF- α (tumour necrosis factor- α). Sputum from bronchiectatic airways exhibits increased chemotactic activity (Mikami et al., 1998). Studies also note increased levels of products of neutrophilic inflammation such as elastase, IL-8 and the matrix metalloproteinases MMP-8 and MMP-9 (Tsang et al., 2000, Sepper et al., 1995, Zheng et al., 2002). Neutrophil products may digest airway proteins affecting structural and functional integrity. Impaired airway function facilitates microbial colonization,

infective exacerbation, chronic inflammation and further damage (Barker, 2002). The role of other inflammatory cells such as macrophages in bronchiectasis is less clear, although higher levels of these cells have been reported (Zheng et al., 2001). Neutrophils, eosinophils and macrophages generate reactive oxygen species (ROS). These products of the respiratory burst are detectable in increased quantities in bronchiectasis (Loukides et al., 1998). Recurrent bacterial infections may facilitate recruitment of phagocytic cells to the lungs and result in prolonged release of toxic ROS and dysregulation of local airway homeostatic mechanisms. This excess inflammation may overwhelm host antioxidant resistance causing airway damage.

The influence of female gender and sex hormones in bronchiectasis remains to be fully elucidated (Morrissey and Harper, 2004). An important role is suggested by the female predisposition identified in many population studies. This female preponderance in unselected bronchiectasis populations may be related to an increased prevalence of bronchiectasis related diseases such as Sjogren's syndrome or rheumatoid arthritis in females (Morrissey and Harper, 2004). Alternatively, female pulmonary anatomy with smaller airways and lung volumes, may favour a relative impairment of mucociliary clearance contributing to bronchiectasis (Zeitlin, 2008). Finally female hormones themselves may play a role, with oestrogen being of demonstrable importance in mucous production, viscosity and lung function in the setting of cystic fibrosis (Tam et al., 2011).

The potential impact of non infective environmental influences on the pathophysiology of bronchiectasis is unclear. Smoking is suspected as a possible contributing factor to idiopathic bronchiectasis, although many patients are non smokers. Limited data suggests a link between smoking and indoor air pollution in developing countries with more severe disease (Bhatta et al., 2008). The association of bronchiectasis with social deprivation may suggest strong environmental influences, however further studies are required (Edwards et al., 2003).

1.7 Causes of bronchiectasis

Bronchiectasis is a pathological end-point of many disease processes (Barker, 2002) (Table 1.2). Case series suggest that specific causes can be identified in 40 - 50% of adults and 60 – 75% of children (Kim et al., 2011, Scala et al., 2000, Shoemark et al., 2007, Zaid et al., 2010, Li et al., 2005, Pasteur et al., 2010, Pasteur et al., 2000). Identification of a cause may lead to specific treatment or may limit the need for further expensive or invasive investigations. Causes are legion, but may broadly be categorized into airway/barrier problems and immune dysfunction. Some causes may represent combinations of the two.

Airway / Barrier Problems	Immune Dysfunction
Cystic fibrosis Ciliary Clearance Disorders Airway Obstruction Aspiration A1 antitrypsin deficiency Asthma/COPD	Post-infectious Hypogammaglobulinaemia Extremes of age Allergic Bronchopulmonary Aspergillosis HIV infection Malnutrition Rheumatoid arthritis Inflammatory Bowel Disease

Table 1.2 showing causes of bronchiectasis divided broadly into barrier problems and problems of immune dysfunction. Some causes such as cystic fibrosis, asthma and COPD may traverse the two groups.

A few of these causes are worth discussing in more detail.

1.7.1 Post-infectious bronchiectasis

Post-infectious bronchiectasis is the most common cause of bronchiectasis reported in the literature (Pasteur et al., 2010, Habesoglu et al., 2011). Despite this it remains an ill defined entity, especially when the temporal relationship between the suspect illness and the onset of chronic respiratory symptoms is unclear (Nicotra et al., 1995). Causes of post infectious

bronchiectasis include a variety of bacterial and viral infections, however, little firm data exists on the importance of childhood infection in the aetiology of adult bronchiectasis. Some studies note a gap of 20-30 years between the antecedent illness and the onset of symptoms, while others note persistent symptoms from childhood (King et al., 2009b, King et al., 2006a, Nicotra et al., 1995). The relationship of a single infective episode to the development of bronchiectasis may be further complicated by factors such as access to healthcare and nutrition (Grimwood, 2011). A number of studies have demonstrated post pneumonic bronchiectasis to be increased in economically disadvantaged communities (Edwards et al., 2003). This may be especially important in children. Rapid access to good quality healthcare, appropriate antibiotics and immunization programs are thought to have contributed to a reduction in post infectious bronchiectasis in many populations in developed countries (Edwards et al., 2003).

Tuberculosis is a well-known cause of localized bronchiectasis (Jordan et al., 2010).

Bronchiectasis related to mycobacterium tuberculosis infection is particularly associated with right middle lobe disease (King, 2009, Whitwell, 1952). Prolonged lymphadenopathy due to tuberculosis infection is thought to cause obstruction of the bronchus with secondary bronchiectasis. Bronchiectasis related to *mycobacterium tuberculosis* remains an important cause of bronchiectasis in the developing world and is an important complication of the resurgent disease. Non-tuberculous mycobacterial infections are becoming increasingly important as a cause of bronchiectasis in adults (Koh and Kwon, 2006). Limitations of sputum culture sensitivity for the diagnosis of non-tuberculous mycobacteria in patients with established bronchiectasis may result in an underestimation of its importance as a causative agent (Koh and Kwon, 2006, Wickremasinghe et al., 2005). Infection remains an important cause of bronchiectasis worldwide. Further studies are needed to determine a clear relationship between childhood infections and distant presentations of bronchiectasis.

1.7.2 Cystic fibrosis

Cystic fibrosis is an autosomal recessive disorder of mucociliary clearance which manifests in the lung as severe bronchiectasis (Rowe et al., 2005). Cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Regulator gene (CFTR) located on chromosome 7. The CFTR gene product is a chloride channel found in epithelial membranes. Defects in CFTR result in abnormal sodium and chloride transport across epithelial membranes (Rowe et al., 2005). This affects both the lungs and other exocrine glands, notably the pancreas. CFTR defects may result in water depletion of mucous with resultant increased viscosity and impaired clearance. The thick mucous layer favours bacterial colonization, infection and airway damage. The end-point of the infection-inflammation cycle is bronchiectasis.

The incidence of cystic fibrosis at birth in Ireland is 1/1500, the highest in the world (Devaney et al., 2003). Cystic fibrosis should always be considered as a potential cause for bronchiectasis, especially in children (Pasteur et al., 2010). However, in adult bronchiectasis homozygous mutations are found in unselected cohorts only occasionally (Divac et al., 2005, King et al., 2004). Guidelines do not recommend generalized CFTR genotyping in all cases of bronchiectasis (Pasteur et al., 2010). However, sweat testing followed by CFTR mutation screening should be carried out in cases where a diagnosis of cystic fibrosis may be deemed more likely. Examples include young age of onset, predominant upper lobe disease or where there is a suspicion of concomitant infertility especially in males.

Perhaps of greater relevance to the adult population with bronchiectasis is the emerging evidence of an increased frequency of CFTR gene single nucleotide polymorphisms or heterozygosity for known CF causing mutations (Casals et al., 2004, Tzetzis et al., 2001, Girodon et al., 1997, Bienvenu et al., 2010). However, some studies examining the prevalence of CFTR polymorphisms did not find an increased prevalence compared to control populations (Divac et

al., 2005, King et al., 2004). Recent data suggests an association between gene polymorphisms causing dysfunctional CFTR evidenced by abnormal nasal electrophysiology and the presence of diffuse non-CF bronchiectasis (Bienvenu et al., 2010). This evidence may suggest that non-CF bronchiectasis a *forme fruste* of cystic fibrosis. This data suggests that barrier abnormalities may be a factor in some cases of unexplained bronchiectasis with other factors also having a role (Boucher, 2010).

1.7.3 Immune deficiency

The link between bronchiectasis and primary immune deficiencies is well established (Notarangelo et al., 2007). Bronchiectasis is a feared complication of primary antibody deficiency disorders such as common variable immune deficiency (CVID) or X-linked agammaglobulinaemia (Tarzi et al., 2009). The heterogeneous family of primary antibody deficiencies is characterized by reduced or absent levels of pathogen specific antibody. This results in a reduction in, or absence of opsonisation, leading to impaired phagocytosis. The lack of an effective opsonophagocytic response increases susceptibility to bacterial and viral lower respiratory tract infections (Tarzi et al., 2009, Kainulainen et al., 2010). The impaired immune response permits recurrent, severe and persistent lung infections which result in bronchiectasis.

Bronchiectasis is prevalent among patients with primary antibody deficiency (Dukes et al., 1978, Martinez Garcia et al., 2001, Thickett et al., 2002). Several studies have indicated that diagnostic delay predisposes to bronchiectasis (Thickett et al., 2002, Litzman et al., 2010). Over 30% of patients with antibody deficiency have established bronchiectasis at presentation, predicting poor outcome (Thickett et al., 2002). Correction of the antibody deficiency by replacing polyclonal immunoglobulin G (IgG) by intermittent intravenous or subcutaneous administration reduces the frequency of infection (Lucas et al., 2010). Replacement therapy may also prevent

bronchiectasis in hypogammaglobulinaemic patients when instituted early, however dosing remains controversial (Llobet et al., 2009). These data emphasize the importance of excluding antibody deficiency in patients presenting with bronchiectasis (Chapel, 1994). Recent recommendations suggest routine measurement of immunoglobulin G, A and M levels with serum protein electrophoresis in all patients with bronchiectasis (Pasteur et al., 2010). Second line investigations include the measurement of IgG subclasses, assessment for specific antibody deficiency and functional tests of cellular immunity. Antibody deficiency is identified as a cause of bronchiectasis in 5-10% of unselected cases (Shoemark et al., 2007). However, in many centres local protocols ensure that such cases which are amenable to specific treatment are identified soon after presentation.

Primary immune deficiencies affecting other aspects of the immune system can also cause bronchiectasis. Some conditions, such as the severe combined immune deficiencies, predispose to recurrent lower respiratory tract infection and bronchiectasis as part of a general susceptibility to a wide variety of pathogens (Notarangelo et al., 2007). These diseases are 'immunological emergencies' requiring urgent hematopoietic stem cell transplantation. Other conditions such as chronic granulomatous disease facilitate the persistence of infections by defects in the generation of phagocyte oxidative burst (Notarangelo et al., 2007). In all such conditions the failure of the immune system to deal adequately with infection results in the damage characteristic of bronchiectasis.

The relationship of the immune system to bronchiectasis is further evidenced by incidence peaks in childhood and in the elderly. In transient hypogammaglobulinaemia of infancy the development of bronchiectasis may be enhanced by a temporary inability of the maturing adaptive immune response to deal with pathogens efficiently (Kidon et al., 2004). However, this outcome is rare especially with early management. At the other extreme of age immunosenescence may contribute to bronchiectasis in the elderly.

The term secondary immune deficiency is broad, encompassing conditions that range from medication associated immune defects and infection associated immune defects such as HIV to the ill defined infection susceptibility associated with poor nutrition (Amorosa et al., 1992). Medications such as chemotherapeutic agents, immune suppressants and anti-epileptics may rarely be an indirect cause of bronchiectasis (Morehead, 1997). Such medications may predispose to infection by inducing hypogammaglobulinaemia or by more diffuse suppressive effects on immune system components. Bronchiectasis is uncommon in HIV cohorts in the developed world given the widespread use of highly active antiretroviral therapy. It may be a more significant problem in children (Sheikh et al., 1997). Secondary immune deficiency due to nutritional defects is generally ill defined (Valery et al., 2004). A causal association between bronchiectasis and nutritional defects is not clear. The increased incidence of bronchiectasis in socially disadvantaged populations may in part be due to immune system associated nutritional defects (Valery et al., 2004). Additionally there is evidence of a relationship between poor nutrition and poor outcomes in other respiratory disease (Celli, 2010). Despite this, nutrition is unlikely to be a critical causal factor in Western populations with unexplained bronchiectasis. Further evidence to determine the impact of nutrition on infection susceptibility is required.

Immune deficiency states leave the lung open to recurrent infection and subsequent inflammation which can result in bronchiectasis. The strong association between immune deficiency and bronchiectasis serves as evidence of the importance of efficient immune function for lung health.

1.7.4 Subtle immune deficiency – complex susceptibility factors for infection

Primary immune deficiencies are traditionally characterized as rare, familial traits. However, emerging knowledge of genetic predisposing factors to infectious disease coupled with clinical data indicate that infection susceptibility due to genetic factors is common (Alcais et al., 2009). Casanova argues that the low life expectancy of humans before the end of the 19th century indicates we are inherently immunodeficient (Casanova and Abel, 2007). This susceptibility has been masked by medical progress and advances in nutrition and public health.

Emerging examples of these subtle immune deficiencies include specific susceptibility to herpes simplex virus (HSV) 1 encephalitis due to TLR3 deficiency and UNC-93B deficiency (Casrouge et al., 2006, Zhang et al., 2007). Children with deficiencies in these innate immune molecules are susceptible to spontaneous HSV encephalitis. However, this susceptibility is compensated for after puberty by the adaptive immune system (Casanova and Abel, 2007). In a similar manner, susceptibility to pneumococcus associated with IRAK4 deficiency and to environmental Mycobacteria associated with defects in Type I cytokine pathways may also become less pronounced with maturity (Casanova and Abel, 2002, Picard and Casanova, 2004). These defects in innate pathways can predispose to susceptibility to specific infections.

Specific antibody deficiency is increasingly recognized as a factor predisposing to recurrent respiratory tract infection. This disorder is characterized by a poor humoral response following stimulation with polysaccharide antigens; typically the 23-valent pneumococcal polysaccharide vaccine (Tuerlinckx et al., 2007). Specific antibody deficiency is associated with a predisposition to respiratory tract infection and otitis media (Boyle et al., 2006). However, the clinical significance of absent antibody responses and the defining laboratory characteristics of the condition are the subject of debate (Boyle et al., 2006, Tuerlinckx et al., 2007). It is notable that

10-20% of healthy adults can fail to respond to polysaccharide vaccination (Rodrigo et al., 1997). A possible role for antibody production defects in bronchiectasis has been examined (Pasteur et al., 2000, Stead et al., 2002, Vendrell et al., 2005). Specific antibody deficiency was diagnosed in 11% of patients in one series of adult patients (Vendrell et al., 2005). Other investigators failed to detect significant numbers of bronchiectatic adults with specific antibody deficiency and have debated the relevance of this type of investigation beyond the fourth decade (Stead et al., 2002). Vaccine challenge with assessment of post vaccination antibody titres is often included in the investigation of patients with bronchiectasis. However, the relevance of such results, assays used and thresholds for diagnosis and treatment are unclear.

Variation in mannose binding lectin is a further common immune deficiency that is associated with enhanced susceptibility to infection (Bouwman et al., 2006). Low levels of this innate immune system protein seem particularly important when the adaptive immune system is compromised.

1.7.5 Allergic bronchopulmonary aspergillosis (ABPA) and Asthma

ABPA is a hypersensitivity reaction to *Aspergillus fumigatus* which can colonize susceptible airways in asthma or cystic fibrosis (Patterson and Strek, 2010). ABPA is characterized by peripheral blood and sputum eosinophilia and, in severe forms, is associated with bronchiectasis of the proximal airways (Patterson and Strek, 2010). The lung damage occurs as a result of the exaggerated inflammatory response to this ubiquitous and normally harmless environmental mould. The reasons for individual susceptibility remain unclear, but a genetic background is

suspected (Balloy and Chignard, 2009). This coupled with the presence of airway epithelial damage, inflammation and excessive mucous production typical of pre-existing asthma allows persistence of aspergillus conidia and propagation of the life cycle of the fungus (Balloy and Chignard, 2009). Aspergillus antigens can then initiate inflammation via innate immune system activation with a subsequent influx of immune system cells to the airways (Balloy and Chignard, 2009). Antigen presentation to helper T cells also occurs. In the setting of ABPA a predominant Th2 response is elicited (Koth et al., 2004). The Th2 cytokines IL-4 and IL-13 promote B cell class switching to Aspergillus specific IgE production, while IL-5 facilitates a strong eosinophilic response. This exaggerated immune response causes bronchiectatic lung damage in the long term with a central pattern that is virtually pathognomonic (Patterson and Strek, 2010). Presentation with bronchiectasis may take place several years after the ABPA inflammatory insult which can complicate the diagnosis. ABPA is identified as a cause of bronchiectasis in 1-10% of cases (Bahous et al., 1985).

Asthma may also be considered as a cause of bronchiectasis. HRCT scan evidence suggests that 60% of patients with severe non allergic asthma may have varicose bronchiectasis (Paganin et al., 1996). Bronchial wall thickening may be observed in more than 80% of patients with asthma. However, sensitization to aspergillus is observed in over 60% of patients with asthma in some series (Fairs et al., 2010). Whether asthma is associated with bronchiectasis independently of aspergillus or other fungi is an unresolved question.

1.8 Genetic associations of idiopathic bronchiectasis

The association of CFTR gene mutations with cases of idiopathic bronchiectasis as previously discussed remains the subject of debate. Other groups have used a candidate gene approach to identify possible genetic associations or disease modifying loci in idiopathic bronchiectasis.

Boyton *et al* in 2005 were the first to demonstrate genetic susceptibility to idiopathic bronchiectasis (Boyton et al., 2006b). They examined a possible role for polymorphisms in HLA-C and KIR 2D genes. These genes code for an NK cell ligand/receptor pair variation in which may have functional implications. The HLA-Cw*03 allele was associated with a 2.25 fold increased risk of bronchiectasis (Boyton et al., 2006b). Homozygosity for HLA-C group 1 motifs, those with an asparagine at position 80 of the α 1 domain, was more generally associated with increased susceptibility. These findings suggested a role for NK cell dysfunction, with possible increased activity, in idiopathic bronchiectasis (Boyton et al., 2006b). Focused functional studies remain outstanding (Boyton et al., 2006b, Boyton, 2009). In addition, evidence for a role of NK cells in idiopathic bronchiectasis pathology has been enhanced by the observation that individuals with TAP deficiency develop basal bronchiectasis as part of a generalized increased susceptibility to infection associated with necrotizing granulomatous skin lesions (Boyton, 2009). TAP deficiency is caused by deletion or complete loss of function mutation of TAP1 and TAP2 genes, important in antigen processing and presentation. Such individuals often have expanded NK cell and invariant T cell populations. A small study in children with idiopathic bronchiectasis identified polymorphisms of the TAP gene to be associated with bronchiectasis, however this study was of low power and possibly confounded by linkage disequilibrium (Dogru et al., 2007).

Boyton *et al* also identified a link with idiopathic bronchiectasis and the HLA-DR1, DQ5 haplotype, suggesting a role for adaptive immune cells in the pathogenesis of the disease (Boyton et al., 2008). The authors hypothesized this could operate as a pathogen susceptibility factor. Other groups have focused the role of matrix metalloproteinases (MMP) in bronchiectatic airway destruction. Polymorphisms in MMP genes have been associated with an increased risk in bronchiectasis in some studies but not in others (Stankovic et al., 2009, Lee et al., 2007).

Idiopathic bronchiectasis is potentially a multifactorial disorder. It seems likely that genetic variation affecting infection susceptibility or regulation of inflammation will have a modifying

effect on the risk of bronchiectasis. The role of such genes and the potential for interaction with environmental factors is a fertile area for future research.

Overall Aims

- This project aimed to recruit a cohort of patients with bronchiectasis for which no other cause could be clearly identified. This would allow patient characteristics to be outlined. Additionally, the burden of disease in terms of infection frequency and hospital admissions could be explored.
- From this clinical base, a candidate gene approach was proposed. It was aimed to evaluate the importance of genetic variation in the innate and adaptive immune systems. We chose to evaluate the six polymorphisms affecting the function of mannose binding lectin, a component of the complement cascade. Additionally we examined a polymorphism in CD32A, an Fc receptor for IgG.
- Further investigation of the functional relevance of these polymorphisms was proposed with the ultimate aim of generating a paradigm for the involvement of altered immunity in the development of bronchiectasis.

Chapter 2 – Bronchiectasis Cohort

2.1 Patients, materials and methods

2.1.1 Patient identification

Patients were selected from out-patient attendees at the Department of Respiratory Medicine at Beaumont Hospital Dublin. Clinical staff, as part of the work-up for patients with proven bronchiectasis on high resolution CT scan, were invited to send samples for a 'bronchiectasis profile'. This bronchiectasis profile comprised a series of tests to exclude known or potential immunological causes of bronchiectasis (Table 2.1). Patients on whom a 'bronchiectasis profile' was ordered were identified as possible candidates for inclusion in the study. Saved samples from the bronchiectasis profile were aliquoted and stored at -80°C.

Table 2.1 – tests included in the Beaumont Hospital bronchiectasis profile

Immunoglobulins G, A and M and electrophoresis
IgG subclasses
Specific antibodies to pneumococcus
Total IgE
Aspergillus specific IgE
Alpha-1- antitrypsin
Mannose binding lectin

2.1.2. Patient consent

Patients on whom a bronchiectasis profile was ordered had their HRCT scan reports reviewed to ensure the presence of bronchiectasis, as part of the ongoing audit process in the Department of Immunology at Beaumont Hospital. Patients who did not have bronchiectasis in their high resolution CT scan report were deemed to have had a profile ordered inappropriately and were not included in the ongoing cohort. Duplicate tests were also reviewed, ensuring patients were not counted twice. Patients were then approached, by post, for consent to be included in the study, to have further genetic and immunological testing and to have their medical records reviewed. If patients did not respond to the first 'mail-shot', a second approach, again by post, for consent was made. If no response was obtained, the patient was perceived to have refused consent and was not included in the study. Patients that refused consent, or withdrew consent were not included in the study.

2.1.3 Ethics Approval

This study, and the manner in which consent was sought from patients and controls, was approved by the research ethics committees of Beaumont Hospital, Dublin and St. James's Hospital, Dublin.

2.1.4 Inclusion and Exclusion Criteria

The demonstration of bronchiectasis on HRCT scan images was the only inclusion criteria for the study. If significant bronchiectasis was not observed then patients were not included in the cohort.

However, we established a number of exclusion criteria, aimed at removing patients with a defined cause for bronchiectasis, leaving the residual 'idiopathic' cohort. The following were considered as exclusion criteria:-

1. Barrier abnormalities – cystic fibrosis, immotile cilia syndrome, stenotic lesions
(not all patients were tested for these problems. The decision to test was made by the clinical team and based on clinical need, e.g. age at presentation, distribution of bronchiectasis, organisms cultured)
2. Immunoglobulin abnormalities – IgG deficiency, IgG1 and IgG2 subclass deficiency
(isolated IgA, IgM, or IgG3 and IgG4 subclass deficiencies were not considered to be of enough significance to immune function to warrant exclusion from the study. Response to pneumococcal vaccination did not form part of the assessment for inclusion in the study as the threshold for diagnosing abnormal responses and the importance of such abnormalities remains controversial)
3. Hypersensitivity pneumonitis – clinical, serological or radiological evidence of ABPA
4. Significant contributing co morbidities including solid-organ and haematological malignancy, clinically significant autoimmune disease (warranting immunomodulatory therapy), or the presence of an autoimmune disease with a known association with bronchiectasis.
5. Traction bronchiectasis – this pathology is related to fibrotic lung change and unlikely to be clinically related to idiopathic bronchiectasis

Patients, in whom a distant history of infection was identified and with a potential diagnosis of infection related bronchiectasis were included in this study. After exclusions 101 patients were identified for inclusion in the study. Patient characteristics and reasons for exclusion are detailed in section 2.2 (page 31).

2.1.5. Controls

Blood was obtained from 115 anonymised healthy control subjects for candidate gene profiling. Control subjects were recruited from a cohort of students without underlying health problems attending DIT. Control subjects for this portion of the study were not age matched, with control subjects being younger having a mean age of 28. Samples from control subjects were aliquoted and stored at -80°C until analysis.

2.1.6. Chart review

Charts from patients in whom study inclusion and exclusion criteria had been met, were interrogated for clinical data in retrospective fashion. Original pulmonary function test results were obtained from the Beaumont Hospital Pulmonary Function Laboratory. Data was collected on a standard case report form (Table 2.2). Collected data was pseudoanonymised and entered into a Microsoft Excel spreadsheet.

Table 2.2 Case Report Form – Beaumont Bronchiectasis Cohort**1. Demographic details**Patient Name D.O.B.

Cohort Identification Number

2. Clinical DetailsDate of diagnosis Dates of HRCT scansAge at first symptoms Duration of symptomsSinus symptoms yes / noAsthma yes / noCOPD yes / noSmoker yes / no Pack yearsPneumovax given yes / no / unclear

Oral steroids

Co morbidities

3. Infections

Sputum volume

Number of hospitalisations with LRTI (5 years)

Frequency of infections

Cultured organisms (list all)

Prophylactic / cyclic antibiotics yes / no**4. Pulmonary parameters (most recent available)**FEV1 FVC FEV1/FVC DLCO**5. Radiology**

Unilateral / Bilateral

No. of lobes involved

Severity

6. Medications

Drug	Dose

2.1.7 Scoring of HRCT images

HRCT scan scoring was carried out by modification of the method previously published by Ooi *et al* (Ooi *et al.*, 2002). Scoring was carried out by one of two radiologists. A small sample (n=6) of CT scans were scored by both radiologists to ensure comparability of results. Differences between independent CT scores were not significant. CT scans were scored in random order and radiologists were unaware of clinical parameters. Each lobe, including the lingula was assigned a score based on an assessment of the percentage of lobar involvement and the severity of bronchial wall thickening. Bronchial wall thickening is calculated as the thickness of the bronchial wall relative to the external diameter of the bronchi (Ooi *et al.*, 2002). The bronchiectasis score and thickening score were combined to give a sum bronchiectasis score which was the main indicator of severity. Lobes were also scored for the severity of emphysema. Details of scoring the scoring system are shown in Table 2.3.

Bronchiectasis score	Definition	Thickening score	Definition	Emphysema score	Definition
0	None	0	None	0	None
1	<25%	1	20-50% EDB	1	Mild
2	25-50%	2	>50% EDB	2	Moderate
3	>50%	3	Obliteration	3	Severe

Table 2.3 showing the scoring system for HRCT scans of bronchiectasis patients adapted from Ooi *et al*

2.1.8. Laboratory data

Laboratory data was obtained on all patients in whom informed consent was obtained. A 'gather' was performed on the Beaumont Hospital Laboratory Information System generating result lists on all patients who had the bronchiectasis profile carried out. Consenting patients

were identified, duplicate tests excluded and results entered into the pseudoanonymised Microsoft Excel spreadsheet.

2.1.9. Analysis

Data was presented graphically using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and was analysed statistically using Graph Pad Prism or SPSS (Version 20, IBM, CA, USA). Continuous variables were analysed using one-way ANOVA with Tukey's multiple comparison test allowing post-test group-group comparisons.

2.2 Results

2.2.1 Cohort characteristics: exclusions

181 patients with bronchiectasis were identified from laboratory records of patients attending respiratory clinics in a tertiary hospital. These laboratory records indicated that a bronchiectasis profile, an extensive panel of immune tests, had been ordered on the patients. Informed consent and further clinical details were sought on this patient group and on this basis some patients were excluded. The main reasons for exclusion were informed consent not obtained and no diagnosis of bronchiectasis on chart review (Figure 2.1). The nature of the consent process (by post, with no face-face request) is the reason for the significant number of patients in the 'no consent obtained' group. Other reasons included alternative diagnoses of Allergic Bronchopulmonary Aspergillosis or traction bronchiectasis secondary to pulmonary fibrosis. No patients in this cohort were excluded because of a diagnosis of adult cystic fibrosis. A small number of patients with clinically relevant immune deficiency were excluded. However, isolated

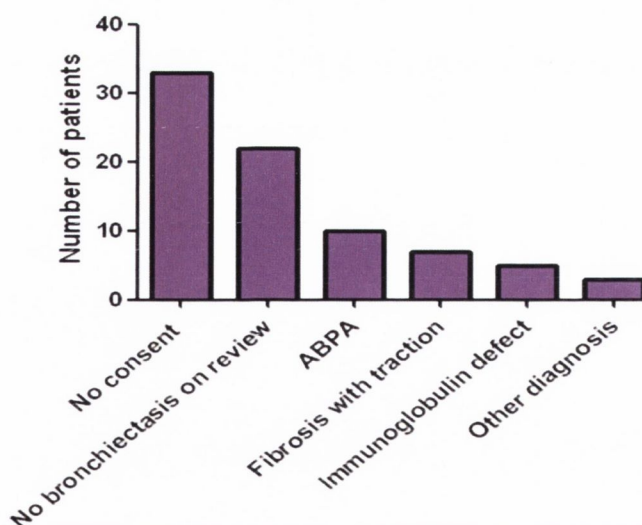


Figure 2.1 showing reasons for active exclusion of patients from the study. Other diagnosis included malignancies and bronchial stricture.

deficiency of IgG3 and IgG4

were not deemed to be significant immune defects in the setting of normal total IgG. Patients

with bronchiectasis related to preceding infection were also included.

2.2.1 Population characteristics of the bronchiectasis cohort

After exclusions 101 Irish Caucasian patients were accepted for further study. 62 (61.3%) of the patients were female and 39 (38.7%) were male giving a male:female ratio of 1:1.6. Most patients were middle aged or older. The mean age was 62 years old and the range was from 22 to 84 years of age. Most patients were in the 50-80 year old age group (Figure 2.2).

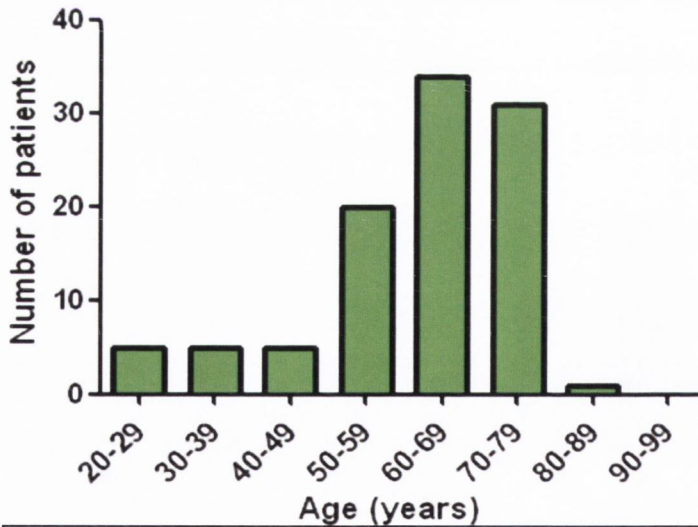


Figure 2.2 showing age profile of patients with bronchiectasis. Bronchiectasis is seen in the older age group. The age of onset of symptoms may suggest a slowly evolving pathology. Alternatively there may be delay in diagnosis.

2.2.3 Symptoms and signs in the bronchiectasis cohort

Examination of the cohort reveals evidence of functional compromise that could affect activities of daily living. Some of this may be related to bronchiectasis. While 95% of patients complained of chronic cough, only 72% described *daily* production of purulent sputum (Table 2.4).

Furthermore, 30% of the patients complained of at least one episode of haemoptysis, although no major episodes were noted. On examination 60% of patients had persistent coarse crepitations on auscultation of the chest, however, the classical sign of finger clubbing was relatively rare (13%) (Table 2.4). 88% of patients had sputum sent for culture and 49% of these grew organisms the most common of which was *Haemophilus influenzae* (15%). Other commonly cultured organisms were *Pseudomonas aeruginosa* (11%) and *Streptococcus pneumoniae* (10%) (Figure 2.3)

Finding	Frequency
Daily Cough	95%
Daily purulent sputum production	72%
Haemoptysis	30%
Crepitations on Auscultation	60%
Finger Clubbing	13%
Other respiratory co-morbidities (Asthma, COPD, rhinosinusitis)	82%
Positive Sputum Culture	43%
Smoking history	58% (14% current, 44% ex-smoker)

Table 2.4 showing the frequency of symptoms, signs and associated findings in the bronchiectasis cohort (n=101).

Breathlessness was graded using the MRC Classification (1-5) and patients were noted to have moderate to moderate-severe debility (Figure 2.4). A history of smoking was a notable feature in this cohort. 58% of patients with bronchiectasis had a history of smoking and of this group 14% continued to smoke at the time of data collection. In those with a history of smoking the mean number of pack-years smoked was 32. This indicates a significant smoking insult.

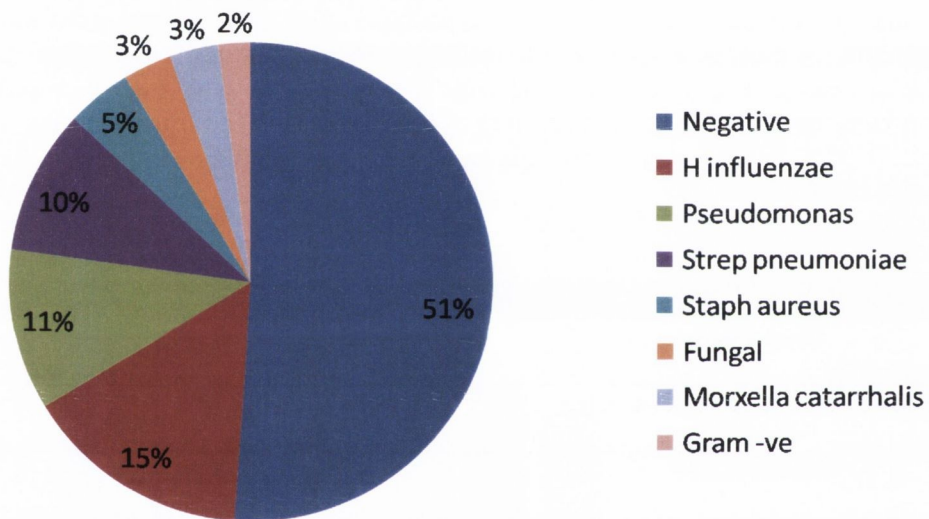


Figure 2.3 showing sputum culture results in patients with bronchiectasis in whom sputum cultures had been sent.

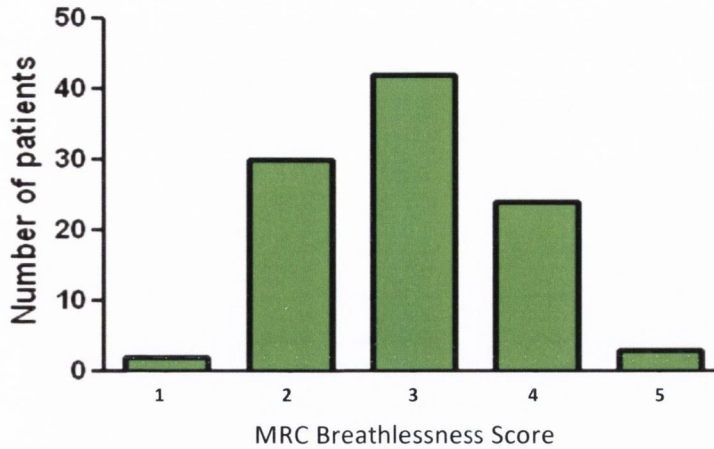


Figure 2.4 showing MRC breathlessness classification in the bronchiectasis cohort. The majority of patients score 3-5 suggesting moderate to severe debility

97% of patients with bronchiectasis reported at least one respiratory tract infection over the previous year. The average number of chest infections reported in the cohort was 4.0 over the previous 12 months. Interrogation of hospital records and charts revealed that 43% of the cohort had been admitted to hospital with a lower respiratory tract infection over the previous 5 years. The mean number of hospital admissions (among those who had been admitted) was 2. These findings are suggestive of significant morbidity within the cohort. These findings may actually underestimate the frequency of hospital admission as patients may have been admitted to other hospitals, data from which was unavailable.

2.2.4 Respiratory co-morbidities in the bronchiectasis cohort

As noted in table 2.4 (page 33), respiratory co-morbidities are prevalent in the bronchiectasis cohort with 82% of the cohort carrying another respiratory system diagnosis. Frequencies of diagnosis of rhinosinusitis, asthma and COPD are shown in figure 2.5. Twenty-four patients had two concomitant diagnoses, while no patients were simultaneously diagnosed with sinus disease, asthma and COPD. Sinus disease may have been under diagnosed in this cohort as it only infrequently formed part of the respiratory assessment.

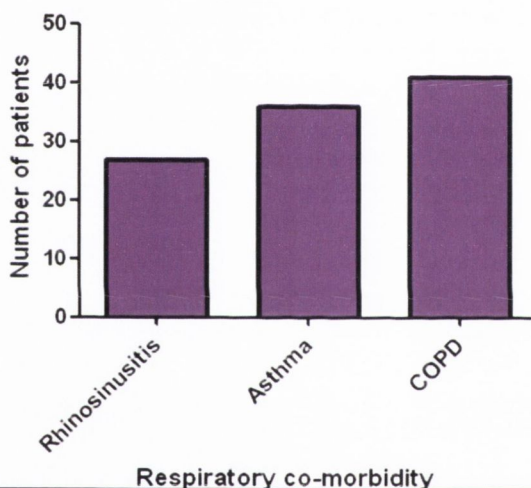


Figure 2.5 showing number of patients in the bronchiectasis cohort with a concomitant diagnosis of other respiratory comorbidities; rhinosinusitis, asthma and COPD.

2.2.5 Relevant medication prescriptions in the bronchiectasis cohort

The vast majority of patients with bronchiectasis were prescribed long term pulmonary medication. Over 80% of patients were prescribed inhaled bronchodilators, usually with a concomitant inhaled corticosteroid (Figure 2.6). Many patients were also on tiotropium, a long-acting anticholinergic inhaler. Leucotriene receptor antagonists such as montelukast

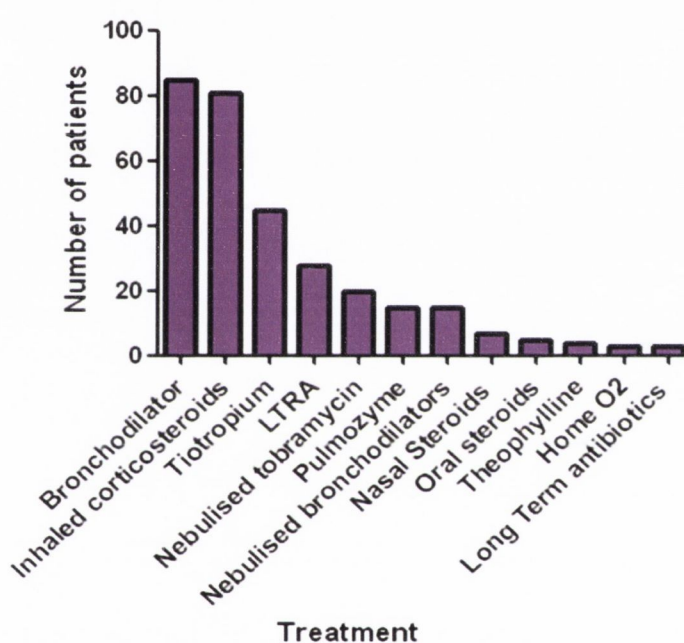


Figure 2.6 showing relevant regular respiratory medications prescribed to 101 patients with bronchiectasis. The vast majority of patients were on inhaled bronchodilators and corticosteroids, with a significant minority on tiotropium. Very few patients were on long-term oral antibiotics. (LTRA=leucotriene receptor antagonists)

were also frequently prescribed. Interestingly, nasal steroids were documented as being prescribed in only 7 patients (6.9%), despite rhinosinusitis being diagnosed in 27 patients (26.5%).

Antimicrobial therapy may be specifically relevant to bronchiectasis, and is used with the aim of reducing the infective burden on patients. 20 (20%) patients were prescribed long term nebulised tobramycin, although 2 (2%) were unable to tolerate this treatment. 15 patients were on pulmozyme. Current data suggests that this drug is not useful in bronchiectasis. Only a very small number of patients were on long term oral antibiotics (Figure 2.6).

2.2.6 Radiological findings in the bronchiectasis cohort

Review of the HRCT scan reports revealed that 75 (74%) patients had bilateral bronchiectasis on HRCT scan. In contrast 26 (26%) had unilateral and more localized disease. The average number of lobes of the lung involved across the cohort was 2.9. In 25 patients bronchiectasis affected a single lobe of the lung only. One patient had two involved lobes on the same side of the mediastinum. In contrast, 23 patients (23%) had diffuse disease affecting all 4 lobes of the lung and the lingula (Figure 2.7). A minority of patients had 3 or 4 lobe disease.

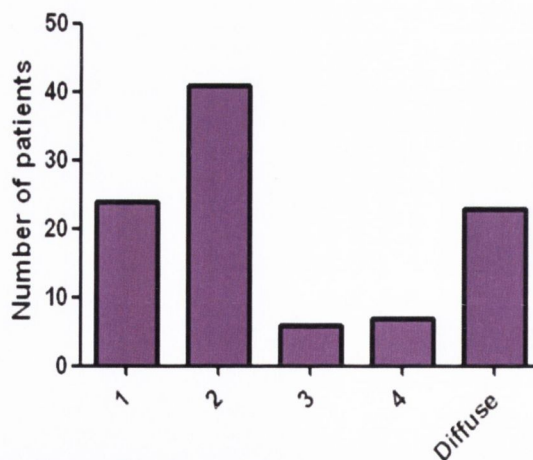


Figure 2.7 showing data on number of involved lobes in the cohort of 101 patients with bronchiectasis. Diffuse disease suggests involvement of all 5 lobes of the lung.

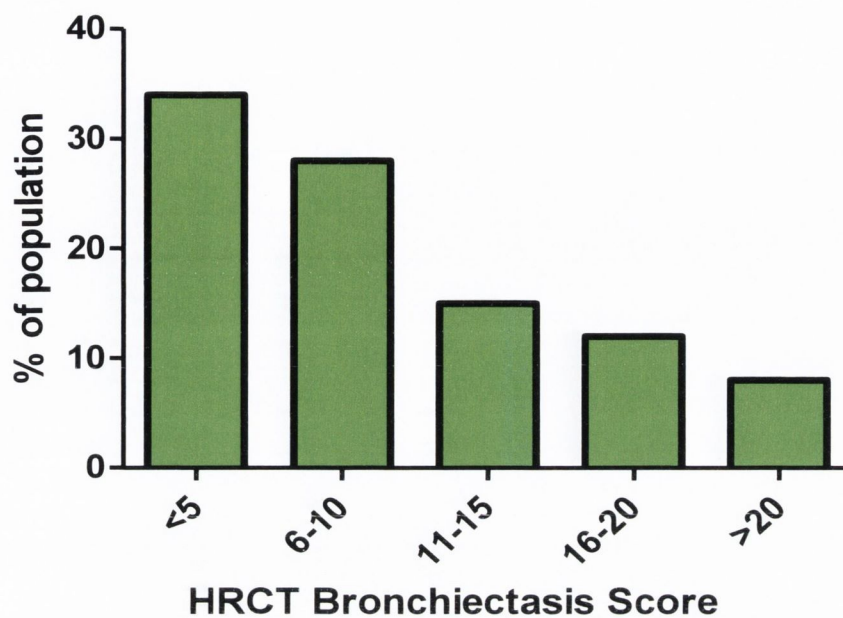


Figure 2.8 showing distribution of bronchiectasis scores in the bronchiectasis cohort. The majority of patients have mild radiological disease. A small proportion are severely affected

The mean bronchiectasis score of this cohort was 9.5. This suggests mild-moderate radiological disease, with approximately 1/3 of patients scoring less than 5. It is clear from these results (Figure 2.7 and 2.8) that there is a considerable sub population with mild disease in one or two lobes.

2.2.7 Lung function tests in the bronchiectasis cohort

Spirometry results were available on all of the 101 patients in the bronchiectasis cohort. The FEV1/FVC ratio which represents the proportion of the forced vital capacity exhaled within the first second was calculated in all patients. The cohort as a whole showed evidence of obstructive lung disease with a mean FEV1/FVC of 62% (Table 2.5, Figure 2.9). Patients with positive sputum cultures had significantly lower FEV1/FVC ratios with a mean of 57% compared with 66% in those with negative sputa ($p < 0.01$ Mann-Whitney). This suggests that those patients with positive sputum cultures have more severe obstructive disease than other bronchiectatic patients.

Parameter	Number of patients	Mean (SD)
FEV1/FVC	101	62.4% (15.0)
DLCO/VA	77	80.8% predicted (20.8)
TLC	51	99.3% predicted (13.8)
RV	51	112.3% predicted (22.8)
FRC	51	108.5% predicted (18.2)

Table 2.5 showing the mean (standard deviation) of a number of parameters from lung function tests in patients with bronchiectasis. Spirometry results were obtained in all patients, however, gas transfer and lung volumes were only obtained in a subgroup.

Mean DLCO corrected for alveolar volume (DLCO/VA) was 81%, marginally reduced on average. This parameter was only available on 77 bronchiectasis patients. Similarly lung volumes were only obtained in some patients (n=51). Mean total lung capacity is close to 100% predicted while both residual volume and functional residual capacity were increased compared to predicted values (Table 2.5).

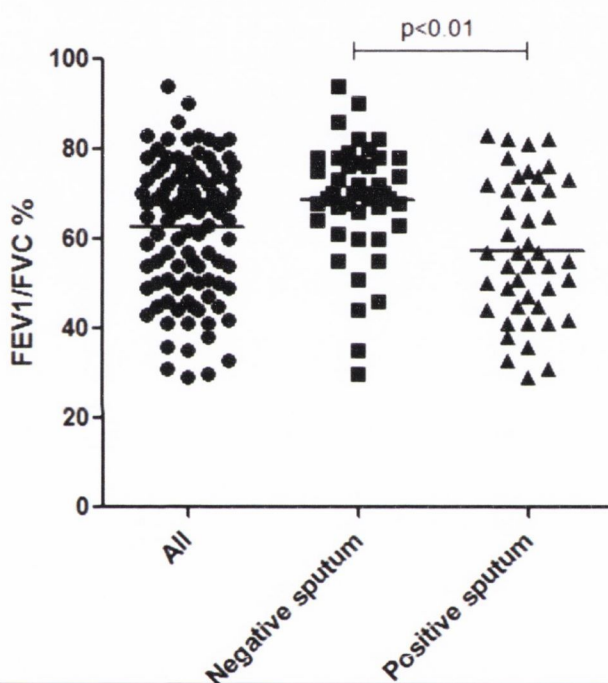


Figure 2.9 showing FEV1/FVC ratios of patients with bronchiectasis. Patients with positive sputum cultures had a lower FEV1/FVC ratio than those with negative cultures ($p < 0.01$).

2.3 Discussion

Bronchiectasis is a heterogeneous disease. The epidemiology of bronchiectasis in developed nations remains poorly studied. This study presents a population of adult idiopathic bronchiectasis gathered from a specialist respiratory clinic in a tertiary adult hospital. This cohort is made up entirely of Caucasian Irish and shares many features of adult bronchiectatic cohorts published in recent years (King et al., 2009b, Kwak et al., 2010, Masters, 2010, O'Donnell, 2008, Pasteur et al., 2000, Sehitogullari et al., 2011, Seitz et al., 2010, Shoemark et al., 2007).

Data from this cohort indicates that adult idiopathic bronchiectasis in Ireland affects individuals in late middle age and affects females slightly more frequently than males (Figure 2.2, page 32). This is in keeping with general findings from a number of different worldwide populations using a variety of recruitment methods. Kwak *et al* in a retrospective CT scan health screening study of 1,409 Korean patients found that bronchiectasis was found more frequently in females than males (male:female, 1:1.45) with a mean age of 55 (Kwak et al., 2010). Weycker *et al* in a retrospective analysis of US health care claims suggested that the prevalence of bronchiectasis increased with age from 4.2/100,000 in 18-35 year olds to 272/100,000 in the over 75s (Weycker et al., 2005). The condition was more common in females in all age groups. Seitz et al in an analysis of US hospital discharge records noted that bronchiectasis associated hospitalisation was most common in females over 60 (Seitz et al., 2010). Other recent studies confirm an increased prevalence of bronchiectasis in late middle age and beyond (Dhar et al., 2010, Finklea et al., 2010). Immunosenescence may be a contributory factor, but this difficult to study area has not been examined in detail in bronchiectasis. The implication of telomere shortening in idiopathic pulmonary fibrosis has lead to an ongoing study examining this phenomenon in non-CF bronchiectasis (<http://public.ukcrn.org.uk>). Some studies have suggested a bimodal disease distribution with subgroups of adult bronchiectasis being divided into those developing initial respiratory symptoms in childhood and an adult onset group (King et al., 2009b). The

relatively low numbers of young patients in our cohort could be explained by low disease prevalence in these age groups, but the possibility of tertiary centre referral bias cannot be easily discounted.

The female excess in modern bronchiectasis cohorts is a consistent finding reproduced in the present study. This observation has echoes of the increased female susceptibility to infectious exacerbations seen in bronchiectasis related to cystic fibrosis (de Boer et al., 2011). The involvement of female sex hormones in cystic fibrosis infective exacerbations is under active consideration. The association of such hormones with adult non-CF bronchiectasis or with specific causative such as non tuberculous mycobacteria remains speculative.

This Irish cohort has striking similarities with data from other bronchiectasis cohorts from the developed world. Despite inherent disease heterogeneity, bronchiectasis in the modern era seems to be mainly a disease of post middle age and affects females more frequently than males.

Symptoms of bronchiectasis can vary widely depending on the severity of the lung lesion. Symptoms range from intermittent expectoration during infective exacerbations to the daily expectoration of large volumes of purulent sputum (Pasteur et al., 2010). Cough was the most frequent symptom in this cohort, present in 95% of patients with daily production of purulent sputum being a frequent accompanying finding (72%) (Table 2.4, page 33). While population screening studies suggest that mild bronchiectasis may be asymptomatic, the majority of cohorts with bronchiectasis have significant symptoms with rates of cough and daily purulent sputum production similar to that seen here (Kwak et al., 2010, Nicotra et al., 1995, Palwatwichai et al.,

2002). Minor haemoptysis was observed in 30% of this cohort, no instances of major haemoptysis were observed. Such symptoms are frequently associated with infection, and while mild, can be the cause of considerable anxiety to patients. Older cohorts noted haemoptysis more frequently, in particular significant (<10mls) haemoptysis in 20% of patients (Warner, 1932, Wynn-Williams, 1953). Whether such studies have relevance in the modern era can be debated.

Auscultatory crackles, detected in 60% of this cohort, are a frequent finding in bronchiectasis. This corresponds well to other studies (Wynn-Williams, 1953, Nicotra et al., 1995). Finger clubbing, long considered a classical sign of bronchiectasis is seen in only 13% of cases in the present cohort. This is infrequent compared to historical data and may represent a shift in disease phenotype in the modern era (Nicotra et al., 1995).

Close to 60% of this cohort had a positive smoking history, the majority of whom were ex-smokers. Bronchiectasis is not considered a classical smoking related disease. However, increasing evidence points to the presence of a distinct subgroup of bronchiectasis related to smoking (King et al., 2009b). Bronchiectasis is frequently identified in patients with COPD although the association is not well characterised and case-control studies are lacking (King et al., 2009b). Furthermore in a large Korean health screening study smoking rates in patients with bronchiectasis compared to those without were not different (Kwak et al., 2010).

In this cohort 88% of patients had sputum results available for analysis. In 13 patients full sputum results were not available, as they had not been sent or were not available at the time of data interrogation (Figure 2.3, page 34). Cross sectional studies of sputum culture show significant variability in the organisms cultured. In the present cohort *Haemophilus influenzae* is a commonly isolated pathological organism, present in 15% of cases. This is a considerably lower

rate of infection/colonisation than many other studies in adult bronchiectasis (Pasteur et al., 2010). *Pseudomonas aeruginosa* was isolated in 11% of cases, while *Streptococcus pneumoniae* accounted for 10% of cases where sputum had been sent for analysis. Figures for culture of these organisms vary considerably within the literature but are broadly comparable with the present cohort. There are several possible reasons for the relatively low rates of *Haemophilus influenzae* infection/colonisation. Patients may have been clear of infecting organisms during a period of clinical stability, sputum was not always routinely collected at clinic visits, and laboratory processing issues all may have contributed to the relatively low rates of *H. Influenzae* detection.

Routine sputum culture was negative in 51% of cases. This is considerably higher than the 'no growth' rates of 23% in a study by Cabello *et al* which used bronchoalveolar lavage (BAL) as a collection method. BAL is not routinely advocated, although induced sputum using nebulised hypertonic saline may increase microbiological yields (Angrill et al., 2002). An increase in positive culture rates may also be obtained by sending samples on three consecutive days. No cases of mycobacterial infection were observed in this study (Figure 2.3, page 34). The prevalence of atypical mycobacteria in Ireland is similar to worldwide data (Kennedy et al. 2003). This suggests that more stringent strategies testing could identify non tuberculous mycobacteria more frequently in Irish cohorts.

Our rates of *Haemophilus influenzae* infection are lower than other published studies in adults. Sputum culture results are essential for the management of bronchiectasis and should be carried out on a routine basis when patients attend for review. Robust clinical and laboratory processes should be put in place for such patients (Angrill et al., 2002, Nicotra et al., 1995).

Respiratory co-morbidities were common in this cohort (Figure 2.5, page 36). Sinusitis and bronchiectasis often coexist (Loebinger et al., 2009). Indeed the vicious cycle of infection and inflammation in chronic rhinosinusitis is strikingly similar to proposed mechanisms for the development of adult idiopathic non cystic fibrosis bronchiectasis (Shoemark et al., 2007). Rhinosinusitis was documented in the medical notes of 27% of patients in the cohort. Only a few publications have addressed this issue. In these studies the prevalence of sinusitis was 45-84% (Guilemany et al., 2011, Guilemany et al., 2009a, Guilemany et al., 2009b). One possible explanation for this low level of sinus disease in our bronchiectasis cohort is failure of recognition of symptoms. Coincident to this is evidence presented in Figure 2.6 (page 37) suggesting that less than 1/3 of those patients diagnosed with rhinosinusitis were being actively managed with nasal corticosteroids. It is possible to infer that there is a failure to treat bronchiectasis and related sinus disease as a single airway disorder, and possibly a failure of documentation of sinus symptoms. Further studies are required to address the issue of sinus disease in bronchiectasis.

Asthma was a common co-morbidity in this cohort (Figure 2.5, page 36). Recent expert opinion has led to the acceptance of asthma as a cause of bronchiectasis in the absence of other aetiologies (Pasteur et al., 2010). Features of mild bronchiectasis are observed in HRCT scans of some asthmatics (Takemura et al., 2004). Paganin *et al* found a significant excess of definite cylindrical and varicose bronchiectasis in patients with non allergic asthma compared with younger atopic asthmatic patients (Takemura et al., 2004). Similar findings have been observed in asthmatics compared with healthy controls (Park et al., 1997). Despite this evidence of bronchiectasis being detected in asthmatics there is little published data on the prevalence of asthma within bronchiectatic cohorts. Our data suggests that asthma is a common association with bronchiectasis observed in 36% of patients in this group.

41% of patients with bronchiectasis had a diagnosis of chronic obstructive pulmonary disease (COPD) (Figure 2.5, page 36). This often preceded the diagnosis of bronchiectasis by several years, with HRCT scanning being performed for clinical investigation of recurrent infection. Many studies associating bronchiectasis and COPD are of poor quality and case control studies are lacking (Pasteur et al., 2010). However, recent evidence suggests that bronchiectasis is seen in over 50% of cases of moderate to severe COPD with the presence of bronchiectasis being associated with sputum pathogen isolation and with increased hospital admission (Martinez-Garcia et al., 2011b). The study by Martinez-Garcia *et al* highlights the importance of the identification of bronchiectasis in COPD patients. It may be that in many such patients it is disease processes relating to bronchiectasis that is driving the phenotype of recurrent infective exacerbations and hospital admissions necessitating more focussed management.

Bronchiectasis is often accompanied by obstructive lung function tests with a reduced ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC)(Pasteur et al., 2010). In keeping with this, the present cohort of adult idiopathic bronchiectatics shows a reduced mean FEV1/FVC ratio at 62.4% (Table 2.5, page 40). This suggests moderate obstruction. This replicates the findings of Pang *et al* that identified mild airflow obstruction (mean FEV1/FVC of 73%) in patients with bronchiectasis (Martinez-Garcia et al., 2011b). More recent work by King *et al* demonstrated moderate airflow obstruction with a mean FEV1/FVC ratio of 65% (King et al., 2010). Interestingly culture of a potentially pathogenic organism was associated with more severe obstructive lung function tests (Figure 2.9, page 41). This is consistent with recent findings on a cohort of patients with COPD and co morbid bronchiectasis (Martinez-Garcia et al., 2011b). The present study adds weight to data suggesting that obstructive disease is the predominant picture in bronchiectasis.

The data sets for other lung function parameters were incomplete. 77 of the patients had gas transfer studies available. Historical data sets suggest that gas transfer values are normal in many patients with bronchiectasis except those with particularly severe obstructive disease. However, King's longitudinal study of gas transfer data noted that DLCO/VA measurements were reduced to 82% predicted after a mean follow up of seven years (King et al., 2010). Reduced gas transfer in bronchiectatics was also noted by Koulouris et al in a smaller study (Koulouris et al., 2003). The present retrospective study concurs with these more recent findings indicating mildly impaired gas transfer with mean values of DLCO/VA being 80.8% predicted. Reduced gas transfer may be taken as evidence of interstitial lung disease. Low values are often seen in disorders where alveolar membranes are thickened such as interstitial fibrosis or where there is alveolar surface area loss as in emphysema (King et al., 2010). In bronchiectasis reductions in gas transfer may be due to repeated interstitial pneumonia with scarring and ultimate destruction of lung parenchyma (King et al., 2010). This suggests that gas transfer impairment may be a marker of more severe bronchiectatic disease.

Approximately half of the bronchiectasis cohort had lung volume data available for analysis. Residual volumes, functional residual capacity and the RV/TLC ratio are often increased in bronchiectasis. Increases in RV and FRC are due to air trapping in dilated and damaged airways. In the present study total lung capacity was normal at 99.3% predicted. Slight increases in residual volume and functional residual capacity were observed at 112.3% and 108.5% predicted. These findings are consistent with other studies suggesting that more severe bronchiectatic lesions are associated with increased residual volume and RV/TLC ratio (King et al., 2010, Stockley et al., 1984, Bahous et al., 1984).

Taken together findings from lung function tests suggest that this cohort is typical of other cohorts of bronchiectasis in terms of respiratory physiology. The incomplete nature of the data set in terms of gas transfer and lung volume data raises the possibility of a sampling bias, with

only more severely affected patients having additional functional tests carried out. This is a clear example of the weakness of retrospective data collection in this study.

HRCT scanning is the current standard for diagnosing and grading radiological severity of bronchiectasis (Pasteur et al., 2010). Bronchiectasis is identified based on characteristic bronchial wall dilatation with the luminal diameter being greater than that of the accompanying pulmonary artery. A further, more inconsistent feature is bronchial wall thickening. Scoring of CT scans is used for research purposes to allow grading of severity of disease. In the majority of studies the severity of bronchiectasis correlates with the degree of airflow obstruction (Ooi et al., 2002, Roberts et al., 2000). The strength of correlation varies between studies and reflects differing patient cohort characteristics, study protocols and scoring methodologies. There is no universally agreed scoring system for bronchiectasis (Pasteur et al., 2010). Indeed many of these scoring systems have been developed from cystic fibrosis studies. No single system has demonstrated superiority in any form of non-CF bronchiectasis. In this study a system developed by Ooi et al, was used with some modifications (Ooi et al., 2002). Bronchiectasis was graded in each lobe (and the lingua) from 1-3 based on both bronchiectasis severity and bronchial dilatation. A maximum sum bronchiectasis score of 36 is possible using this system.

CT scans were available for scoring review on 93 of the 101 patients in the cohort. Reasons for CT scans not being available for review were hard copy scans not available (7 patients) and scans carried out in a different hospital (1 patient). The mean bronchiectasis score for the cohort as a whole was 9.5 with a range 2-29. The mean number of lobes involved was 2.9. Interestingly, 27 patients in the cohort had unilateral bronchiectasis. This more localised phenotype may have a different aetiology, perhaps related to local obstruction during acute infection.

2.4 Conclusions

The Irish bronchiectasis cohort recruited for the present study is similar to many other adult non-CF bronchiectasis cohorts examined in the literature. The age and gender distribution are typical and lung physiology measurements are in keeping with international studies. Radiology scores are slightly lower than the cohort of Ooi et al in which the scoring system was validated (Ooi et al). The relatively low proportion of patients culturing *Haemophilus influenzae* is unusual, but sampling issues may be responsible for this finding. The high prevalence of respiratory co morbidities suggests, in keeping with published data, that asthma and COPD are common diagnoses among patients with bronchiectasis. However, this study detected relatively low levels of sinus and nasal disease. While bronchiectasis is known to be a heterogeneous disease, this Irish cohort is broadly representative of patients with bronchiectasis in modern Western countries.

Chapter 3. CD32A H131R polymorphisms in bronchiectasis

3.1 Introduction

3.1.1 Fc receptors – a possible role in bronchiectasis?

Fc receptors (FcR) are a heterogeneous family of molecules that can bind the Fc portion of immunoglobulins (Daeron, 1997). They exist as membrane bound receptors on the surface of cells of the immune system, or as soluble receptors. They serve as a bridge between the humoral and cellular components of the immune system, conferring antigen specificity on a variety of cells which do not have antigen recognition structures. Specific FcR exist for each antibody class (Daeron, 1997). Fc γ R bind IgG antibodies of the various subclasses, while Fc α R, Fc ϵ R, Fc δ R and Fc μ R bind IgA, IgE, IgD and IgM respectively. Engagement of these receptors with monomeric antibody or immune complexes triggers cellular processes which can result in cell activation or inhibition depending on the specific molecule involved. A second group of FcR which include FcRn and the polymeric receptors for IgA and IgG are involved in immunoglobulin transcytosis and regulation (Daeron, 1997, Freiburger et al., 2010).

3.1.2 Fc γ Receptors

Fc γ receptors bind the Fc portion of IgG and are involved in a variety of important immune processes. Fc γ R can be divided into two categories based on their associated intracytoplasmic signal transduction motif (Daeron, 1997). The majority of these receptors such as CD64 (FcRI), CD32A (Fc γ RIIa) and CD16A (Fc γ RIIIa) possess immunoreceptor tyrosine-based activation motifs (ITAMs) and are involved in cellular activation (Daeron, 1997). CD16B (Fc γ RIIIB) possesses a glycosyl-phosphatidyl inositol link and is weakly activating (Huizinga et al., 1990). The second group consists solely of CD32B (Fc γ RIIB) in humans (Daeron, 1997). CD32B is associated with an immunoreceptor tyrosine-based inhibition motif (ITIM) and is expressed at high levels on human

B cells and at low levels on monocytes, neutrophils and dendritic cells (Su et al., 2007). This receptor acts in a regulatory fashion and inhibits cell responses. These IgG receptors vary in their cell expression and affinity for different immunoglobulin subclasses (Table 3.1). Other Fc receptor classes act as indirect pathogen recognition receptors, binding opsonising immunoglobulin resulting in intracellular signal transduction. Other Fc receptors in particular IgA and IgE receptors have a role in the immune response to bacterial lung pathogens (Urb and Sheppard 2012)

Receptor	Signaling domain	Affinity for Immunoglobulin	Expression
CD64	ITAM	$10^7 - 10^9$ (IgG1>3>4>>2)	Macrophage, monocyte, neutrophil, eosinophil, dendritic cell
CD32A	ITAM	$<10^7$ (IgG3>1,2>>4; HH131) $<10^7$ (IgG1,3>>2,4; RR131)(Shashidharamurthy et al., 2009) H131R polymorphic variation affects binding	Macrophage, monocyte neutrophil, eosinophil, platelet, dendritic cells
CD32B	ITIM	$<10^7$ (IgG3>1>4>2)	High levels – B cells Low levels - macrophage, monocyte, eosinophils, basophils, mast cells, dendritic cells
CD16A	Associates with ITAMs of FcR γ chain or TCR ζ chain (homo- or hetero dimers)	$<2 \times 10^7$ (IgG1,3>>2,4)	NK cell, macrophage, monocyte, mast cell, eosinophil
CD16B	GPI-link May associate with CR3, CD32A or lipid rafts	$<10^7$ (IgG1,3>>2,4)	Neutrophil, eosinophil

Table 3.1 Fc receptor signalling, affinity and expression (after Ravetch *et al* (Ravetch and Bolland, 2001))

3.1.3 Immunoglobulin binding to Fc γ Receptors

The Fc γ R are glycoprotein receptors. They possess a ligand binding α subunit consisting of extracellular immunoglobulin domains. CD64 possesses three immunoglobulin binding domains resulting in a higher affinity for IgG (Ravetch and Bolland, 2001). This allows binding to monomeric IgG. CD32A and CD16A and B possess two immunoglobulin domains and have lower binding affinities. These receptors bind IgG in immune complexes. Cells express multiple low affinity $\gamma\gamma$ R α s, which together will bind opsonised pathogen with high avidity (Ravetch and Bolland, 2001).

These extracellular ligand binding domains interact with IgG molecules at an area near the N terminal portion of the CH2 domain (Ramsland et al., 2011). The interaction of IgG molecules with Fc γ R is facilitated by a conserved glycosylation site near the N terminal. Rearrangements of antigen-IgG-receptor complexes form dimeric activation complexes resulting in the activation of pro-inflammatory signalling cascades (Ramsland et al., 2011). Heterogeneity in glycans observed naturally or the presence of additional sugar moieties as found in intravenous immunoglobulin preparations can impact on binding affinities and hence the cellular outcome of receptor signalling (Forthal et al., 2010).

3.1.4 Function of cell surface Fc γ Receptors

Engagement of activatory Fc γ R by antibody triggers a plethora of important functions. Cross linking of the extracellular domains by immunoglobulin results in tyrosine phosphorylation of the ITAM by src kinases (Daeron, 1997, Ravetch and Bolland, 2001). This initiates an intracellular kinase cascade ultimately leading to the activation of phospholipase C – γ (PLC γ) and the generation of inositol triphosphate (IP3) and diacylglycerol (DAG). This leads to protein kinase C

activation and sustained calcium release, stimulating cell functions (Cox and Greenberg, 2001). These activities can vary depending on the nature of the stimulatory signal and on cell type and include the triggering of phagocytosis, degranulation and antibody dependent cellular cytotoxicity. The efficiency of functionality is receptor dependent. Variations in receptor efficiency may be mediated by differences in affinities for downstream signalling molecules and enhanced receptor clustering (Huang et al., 2004, Ramsland et al., 2011). The transcriptional activation of cytokine and other pro-inflammatory genes results in the propagation of inflammatory pathways. Engagement of certain receptors may yield specific functions. For example, engagement of CD32A on neutrophils is of relative importance for the initiation of the oxidative burst (Dai et al., 2009). A second important function of FcγR is internalisation and clearance of immune complexes (Ravetch and Bolland, 2001). This facilitates antigen clearance, but also in dendritic cells, antigen presentation.

In many cases cells bearing activating FcγR also have the ITIM associated inhibitory FcγR CD32B on the cell surface (Ravetch and Bolland, 2001). This pairing of ITAM / ITIM receptors is now recognised as a common tool used by the immune system to regulate responses (Billadeau and Leibson, 2002). Signalling via CD32B downregulates responses triggered by activating FcγR (Ravetch and Bolland, 2001). CD32B can cross link with ITAM bearing receptor molecules, including the B cell receptor on B cells or activating FcγR. This results in tyrosine phosphorylation of the ITIM and the recruitment of the inhibitory signalling phosphatase SHIP (Ravetch and Bolland, 2001). SHIP hydrolyses PIP3 and inhibits ITAM dependent calcium mobilisation. This downregulates a variety of calcium dependent cellular processes, including ADCC, degranulation, phagocytosis and cytokine release. In B cells, CD32B inhibits B cell receptor ITAM triggered cell proliferation and also generates a proapoptotic signal when CD32B molecules homo-aggregate (Brauweiler and Cambier, 2003). Immunoglobulin engagement with CD32B is an important regulatory tool for the immune system. The balance between cell surface expression of

activating and inhibitory IgG receptors may be key in the maintenance of tolerance (Boruchov et al., 2005).

3.1.5 Binding affinities of cell surface Fcγ Receptors

FcγRs exhibit differing binding affinities for different immunoglobulin subclasses (Table 3.1, page 53). CD64 has a binding affinity one to two orders of magnitude greater than other FcγRs.

Binding affinity may be affected not only by the glycosylation of the immunoglobulin ligand but also by the presence of a number of polymorphisms of the FCGR genes (van Sorge et al., 2003).

Three polymorphisms have been described that impact binding affinities.

A common polymorphism in CD32A is caused by an arginine to histidine amino acid substitution at position 131 (van Sorge et al., 2003). This single base change occurs at a site critical for IgG binding. CD32A H131 (histidine) binds human IgG3, IgG1 and IgG2. It is the only receptor that efficiently binds IgG2. The R131 (arginine) variant binds human IgG3 and IgG1 but has a much lower affinity for IgG2. Cells from heterozygote (HR131) individuals bear both H and R receptor variants (van Sorge et al., 2003).

A second potentially important single nucleotide polymorphism occurs at nucleotide 559 in FCGR3A which codes for CD16A (van Sorge et al., 2003). This results in either a valine or phenylalanine at position 158. The CD16A F158 variant exhibits a differentially higher binding affinity for IgG3.

Finally, two haplotypes of five nucleotides in the FCGR3B gene encode two isoforms; CD16B-NA1 and CD16B NA2 (van Sorge et al., 2003). The CD16B NA1 variant binds its main ligands IgG1 and IgG3 with even higher affinity.

Given the absence of a CD32A molecule in the mouse extrapolation of murine models of FcγR function to humans is difficult (Ravetch and Bolland, 2001). However, studies in mice indicate the importance of the ratio of activatory FcγR binding affinity to inhibitory FcγR binding affinity (Smith and Clatworthy, 2010). This, termed the A/I ratio is a measure, of the efficacy of an IgG subclass in mediating an effector response. These studies suggest that IgG1 is the subclass with the lowest A/I ratio while IgG2 has the highest (Smith and Clatworthy, 2010). This indicates that the activity of the IgG2 subclass is least regulated by the ITIM bearing CD32B, suggesting that IgG2 may have a relatively enhanced role in phagocyte activation. In humans polymorphisms which impact IgG binding to activating Fc receptors may also influence this A/I ratio.

3.1.6 Regulation of expression of FcγR

Emerging evidence demonstrates that the level of expression of FcγRs is influenced by cell activation status and exposure to cytokines. This provides another method of influencing the A/I ratio and altering the threshold for IgG induced activation of FcγR bearing cells (Smith and Clatworthy, 2010). Human monocytes increase expression of CD64 and CD32A under the influence of the prototypic Type I helper T cell (Th1) cytokine interferon γ (Pricop et al., 2001). In contrast IL-4 a type 2 helper T cell derived cytokine upregulates the inhibitory receptor CD32B, while downregulating activating receptors (Pricop et al., 2001). Thus, under the influence of Th1 cytokines, macrophages upregulate activating receptors, reducing the cellular activation threshold (Pricop et al., 2001). Further IgG mediated activation of these receptors has the potential to drive Th1 and Th17 differentiation, modulating the immune response. In contrast CD32B mediated IgG ligation under the influence of Th2 cytokines may inhibit macrophage function and reduce macrophage propagation of immune responses (Pricop et al., 2001).

Hence the ability of a cell to respond to IgG may be influenced by the subclass of IgG and its affinity for a given receptor, by the presence of gene polymorphisms, or by extrinsic factors such as local cytokines. A low activation threshold (high A/I ratio) may be of benefit in infection, but may also propagate sepsis or bystander tissue damage. Excessive activation may also result in impaired tolerance and autoimmunity.

3.1.7 CD32A H131R polymorphisms and infection

CD32A is an attractive candidate gene in the examination of infection susceptibility. In the setting of bronchiectasis the relative phagocytic deficiency of the RR131 variant is an obvious potential risk factor for disease. Alternately, an over-exuberant immune response elicited by immunoglobulin engagement with HH131 receptors on the surface of effector cells of the immune system gives us an opposing hypothesis of excess cellular activation and inflammation. The identification of common allelic variants with likely functional consequences has led to an examination of the association of CD32A with a number of infectious diseases.

Several studies have examined the possible involvement of CD32 H131R (Pricop et al., 2001) variants in infection with encapsulated bacteria. A number of investigators have identified a possible relationship between CD32A RR131 and infection with *Streptococcus pneumoniae* (Yuan et al., 2008, Yuan et al., 2003, Yee et al., 1997, Yee et al., 2000, Sanders et al., 1995). The RR131 variant has been identified in excess in cohorts of adults with pneumococcal pneumonia and with invasive pneumococcal disease. However, others did not observe this effect (Moens et al., 2006). A reduction in the prevalence of CD32A H131 homozygotes in children with upper airway *S. pneumoniae* infections has also been observed (Sanders et al., 1995). Yuan *et al* noted a marked reduction in the numbers of patients heterozygous for CD32A H131R in a study of children with invasive pneumococcal disease in comparison with controls (Yuan et al., 2003).

This observation, replicated in a later study, raises the possibility of a protective optimum afforded by a heterozygote state (Yuan et al., 2008). CD32A H131R heterozygosity may offer ideal levels of immune function and inflammation in the setting of infection with encapsulated bacteria. The possible protective impact of heterozygosity in terms of the induction of ideal levels of infection induced inflammation has been observed in polymorphisms in *mal*, a signal transduction molecule important in innate immunity (Khor et al., 2007).

An examination of patients with community acquired pneumonia observed that CD32A RR131 was associated with more severe disease and with a longer hospital stay (Endeman et al., 2009). The majority of patients with severe disease had invasive pneumococcal infection, however, a marked excess of the RR131 allotype (Odds Ratio 3.03) was also noted in the small group of patients with community acquired pneumonia caused by *Haemophilus influenzae*. CD32A RR131 has also been identified as a risk factor in children presenting with recurrent respiratory tract infections (Bossuyt et al., 2007, Ozturk et al., 2006). CD32A allelic status was identified as one of several risk factors for infection, described as 'partial' immune defects. 56% of study subjects presenting with recurrent respiratory infection had at least two immune defects (Bossuyt et al., 2007). This suggests that the CD32A polymorphism may interact with other subtle immune abnormalities giving rise to a functional immune deficiency state.

Pseudomonas aeruginosa is an important pathogen in patients with preexisting lung disease. It is coated by an alginate capsule. Chronic infection with pseudomonas, common in cystic fibrosis and also encountered in other causes of bronchiectasis, is associated with elevated levels of IgG2-antipseudomonas antibodies (Hodson et al., 1985). The presence of the CD32A R allele has been identified as a risk factor for infection with pseudomonas in the setting of cystic fibrosis (De Rose et al., 2005). Encapsulated bacteria also cause disease outside the respiratory tract.

Neisseria meningitidis infection has been associated with CD32A RR131 (Domingo et al., 2002, Fijen et al., 2000, van der Pol et al., 2001, Bredius et al., 1994, Platonov et al., 1998). In particular

an association with fulminant meningococcal septicaemia has been suggested (Bredius et al., 1994, Platonov et al., 1998). Importantly, the immune response to meningococcus includes complement and multiple IgG subclasses. Polymorphisms in other IgG receptors may play an interacting role as observed in complement sufficient and deficient cohorts (Fijen et al., 2000, van der Pol et al., 2001). These studies suggest that the combination of CD32A RR131 and CD16b NA2/NA2 gives rise to the lowest capacity for phagocytosis of IgG opsonised meningococci resulting in disease susceptibility.

Conflicting data exists on a possible role for CD32 polymorphisms in HIV. Brouwer *et al* observed a two fold increase in vertical transmission of HIV to HH131 infants (Brouwer et al., 2004). The authors hypothesized that virus-IgG immune complexes facilitated HIV infection of target cells. Forthal *et al* noted a more rapid reduction in CD4 count in HIV infected adults with the RR131 CD32A variant (Forthal et al., 2007). The same study observed an increased susceptibility to infection with *pneumocystis jirovecii* in HH patients. A mechanistic explanation for these observations was not readily apparent. CD32A allelic status has been examined on other viral infections with the RR131 variant being associated with a more severe disease course in SARS-Cov (Severe Acute Respiratory Syndrome associated Coronavirus) infection (Yuan et al., 2005).

The potential clinical relevance of polymorphisms in CD32A to disease risk in the developing world is being explored. While several studies associate the HH131 variant with more severe malarial infection, more recent data is conflicting (Cooke et al., 2003, Omi et al., 2002, Ouma et al., 2006, Schuldt et al., 2010, Shi et al., 2001, Sinha et al., 2008). The association of the RR131 variant with more severe disease complications is postulated to relate to the differential binding affinity of CD32A R131 to CRP. Schuldt *et al* suggest that the gain-of-function binding of CD32A R131 to CRP coated erythrocytes may lead to severe malarial anaemia (Schuldt et al., 2010). The HH131 genotype has also been associated with dengue fever and dengue haemorrhagic fever, while the RR131 variant has a protective role (Garcia et al., 2010). In contrast HH131 is

protective against severe manifestations of the parasitic infection onchocerciasis (Ali et al., 2007).

As discussed above, ample evidence implicates the CD32A RR131 polymorphism in the pathogenesis of infectious disease. In contrast, there is some limited evidence to suggest that the H131 variant may play a role in the severity of periodontitis (Dimou et al., 2010, Yamamoto et al., 2004). Periodontitis, an infectious disease of the supporting tissues of the teeth, is thought to result from an inflammatory response to the accumulation of bacteria in the gingival crevice. One hypothesis suggests that the H131 receptors predispose to periodontitis by eliciting an enhanced inflammatory response. *In vivo* studies have noted H131R polymorphism dependent changes in IL-1 secretion from monocytes (Yamamoto et al., 2007). Despite this, evidence from population studies is lacking – perhaps pointing to a weak association only. More robust evidence exists for an association between CD16B polymorphisms, although this too remains controversial (Dimou et al., 2010).

3.1.8 CD32A H131R polymorphisms in atherosclerosis and cardiovascular disease

Atherosclerosis is a chronic inflammatory disease (Ross, 1999). The build up of atherosclerotic plaques in vital blood vessels leads to coronary artery disease and stroke, leading causes of death. Immune processes, based on a background of genetic susceptibility are thought to play a role in the pathogenesis of atherosclerosis. CD32A is a potential candidate gene in atherosclerosis due not only to its immunoglobulin binding properties, but also its importance as an effector receptor for CRP (Zuniga et al., 2003, Rodriguez et al., 2004). Small elevations in CRP may be a risk factor for cardiovascular disease (Ridker et al., 1998). Studies in cardiovascular disease and atherosclerosis have yielded conflicting results. Raaz *et al* observed an association

between the RR131 variant and acute coronary syndrome as a first presentation of coronary artery disease (Raaz et al., 2009). No association with coronary artery disease was noted. The authors postulate that enhanced RR131 binding of CRP could be a risk factor for plaque instability resulting in an increase in potentially catastrophic first presentations (Raaz et al., 2009). Contrasting data from a Finnish group associated CD32 HH131 with more advanced early atherosclerosis in non smokers. An alternate hypothesis of increased inflammation due to enhanced HH131 binding of IgG2 opsonised oxidized LDL was proposed (Sampi et al., 2009). This conflicting data is further confounded by a report examining two large independent German cohorts which found no association with the risk of coronary heart disease (Karakas et al., 2009). Again these conflicting reports may indicate a weak association with these pathological effects. Larger studies may be required to tease such effects out.

3.1.9 CD32A H131R polymorphisms in other lung diseases

Allelic variation in CD32A has also been identified in lung diseases in which a direct infectious aetiology is not readily apparent. Gulen *et al* reported an association with the RR131 genotype and both asthma and allergic rhinitis in Turkish children (Gulen et al., 2007). However, others did not find a similar association while studying a more general group of patients with atopic disease, which included children with food allergy (Pawlik et al., 2004). Immune complexes have been detected in the serum and lungs of patients with idiopathic pulmonary fibrosis (IPF). Based on the possible impact of variations in local inflammation, CD32A allelic status was investigated in patients with IPF (Bournazos et al., 2010). While no difference was identified in genotype distribution between patient and control groups, disease severity and progression was associated with the H131 allele. The authors speculate that HH131 bearing cells amplify inflammatory responses leading to increased immune cell infiltration and production of

fibrogenic molecules (Bournazos et al., 2010). Validation of these results in other cohorts is necessary.

3.1.10 CD32A H131R allelic variation and autoimmune disease

FcγR including CD32A have been studied extensively in autoimmune diseases (Chai et al., 2012, Dijstelbloem et al., 2000, Dijstelbloem et al., 1999, Karassa et al., 2003, Karassa et al., 2002, Karassa et al., 2004, Nikseresht et al., 2006, Tanaka et al., 2005, van de Velde et al., 2006, Wolff et al., 2007). A number of potential pathogenic mechanisms have been proposed. These include alterations in CD32A mediated internalisation of immune complexes by dendritic cells, polymorphism dependent alterations in the efficiency of clearance of immune complexes by reticuloendothelial macrophages and alteration in the magnitude of the inflammatory response (Willcocks et al., 2009). Direct mediation of the pathogenic effects of autoantibodies by CD32A may also be important (Carcao et al., 2003). The duplicitous function of CD32A, with the enhanced role of the R131 variant in CRP binding is a further consideration for the pathogenesis of autoimmune disease. The use of animal models in the study of CD32A and its role in autoimmunity has also been hampered by the absence of CD32A in the mouse.

A body of evidence exists for an association of CD32A polymorphisms and systemic lupus erythematosus (SLE), although individual studies have yielded conflicting results, perhaps confounded by sample size and ethnic variations (Dijstelbloem et al., 2000, Manger et al., 1998, Sato et al., 2001, Villarreal et al., 2001). More recently large meta-analyses have demonstrated an association between SLE and RR131 homozygosity for CD32A (Karassa et al., 2003, Karassa et al., 2002, Yuan et al., 2009). Evidence also exists for an association with antiphospholipid syndrome but not for lupus nephritis (Karassa et al., 2003, Karassa et al., 2002). Further support is given by the results of a genome wide association scan in 720 women with SLE (Harley et al.,

2008). In this study the CD32A H131R polymorphism is associated with SLE with a highly significant p value. In SLE immune complex deposition can result in inflammatory complications in a number of organs (Crispin et al., 2010). The association of CD32A RR131 is consistent with the proposed failure of clearance of immune complexes and apoptotic bodies as an important factor in the pathogenesis of SLE (Sullivan et al., 2003).

Variation at CD32A has also been studied in the vasculitides (Aksu et al., 2008, Biezeveld et al., 2007, Dijstelbloem et al., 1999, Tse et al., 1999). Carriage of *Staphylococcus aureus* has been identified as a possible relapse risk factor in cases of ANCA associated vasculitis (Popa et al., 2002). An IgG2 antibody response is elicited by this microbe, providing the rationale for investigation of CD32A polymorphic status in vasculitic conditions. Indeed one study has demonstrated an association with RR131 receptors and relapse (Dijstelbloem et al., 1999). A UK study reproduced this finding, even though it was only powered to detect quite a large effect size (Tse et al., 1999, Willcocks et al., 2010). The RR131 variant has also been associated with the autoantibody mediated disorders ITP and myasthenia gravis (van der Pol et al., 2003, Williams et al., 1998). In contrast Guillain-Barre syndrome, an acute inflammatory polyneuropathy, often associated with an antecedent bacterial or viral infection was associated in with HH131 genotype in one small study (van der Pol et al., 2000). Evidence exists for HH131 and RR131 CD32A polymorphisms in various autoimmune diseases. There is little doubt that FCGR2A is a mechanistically attractive candidate gene in these disorders. Further work in clearly defined clinical populations with adequate sample size is required to validate many of these often underpowered studies. In addition, the precise pathogenic mechanism operating in these disorders is often unclear, making interpretation of putative associations with CD32A polymorphisms more problematic.

3.1.11 Aims of the chapter

1. To examine whether subtle changes in implied immune function caused by variation in CD32A increase susceptibility to bronchiectasis.
2. To examine whether variation at CD32A could result in susceptibility to a more severe form of bronchiectasis.

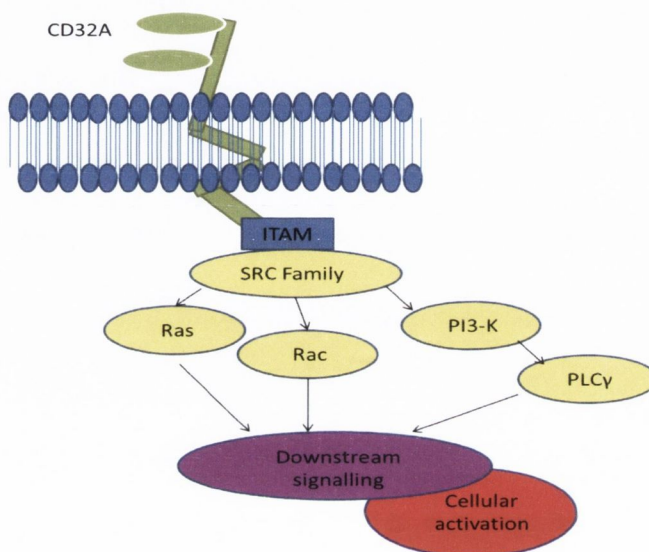


Figure 3.0 showing CD32A downstream signalling pathways activated following receptor crosslinking and ITAM stimulation

3.2. Materials and methods

3.2.1 CD32A genotyping

DNA was extracted from EDTA samples using standard methods (Qiagen Autopure LS, Qiagen, Crawley, UK). CD32A genotyping was performed using allele specific PCR as previously described by Flesch *et al.* (Flesch *et al.*, 1998) Briefly, 25µl PCRs containing 2.5µl (apx. 100ng) of genomic DNA, 2.5ul of 10X PCR buffer containing 15mM MgCL₂, 0.5µl dNTP mix and 0.5U of Taq polymerase (All PCR reaction ingredients from Sigma-Aldrich, Dorset, UK). 0.5µM of H131 and R131 specific sense primers were used in paired PCR reactions. 0.5µM of a common antisense primer from an area of the downstream intron was also used (Table 3.2)(MWG Operon, Munich, Germany). This resulted in a 253 bp fragment (Figure 3.1). A human growth hormone (HGH) internal control was used resulting in a 439 bp fragment.

Primer	5' primer sequence 3'
H131 sense	ATCCCAGAAATTCTCCA
R131 sense	ATCCCAGAAATTCTCCCG
Antisense	CAA TTTTGCTGCTATGGG
HGH I	CAGTGCCTTCCCAACCATTCCCTTA
HGH II	ATCCA CT CACGGATTTCTGTTGTGTTTC

Table 3.2 showing primers used for CD32A allele specific PCR

Hot start PCR was performed as previously described (Mastercycler gradient, Eppendorf, Cambridge UK) (Flesch *et al.*, 1998). Temperature and magnesium gradients were performed and indicated that optimum conditions did not differ from those identified in the original publication (Flesch *et al.*, 1998). The PCR products were identified using a 1.5% agarose gel and stained with ethidium bromide (Sigma-Aldrich, Dorset, UK).

This PCR was demonstrated to be reproducible by repeat of 14 samples on three separate runs. No variation was noted. Specificity was established by commercial sequencing of H131 and R131 PCR products (MWG Operon, Munich, Germany)

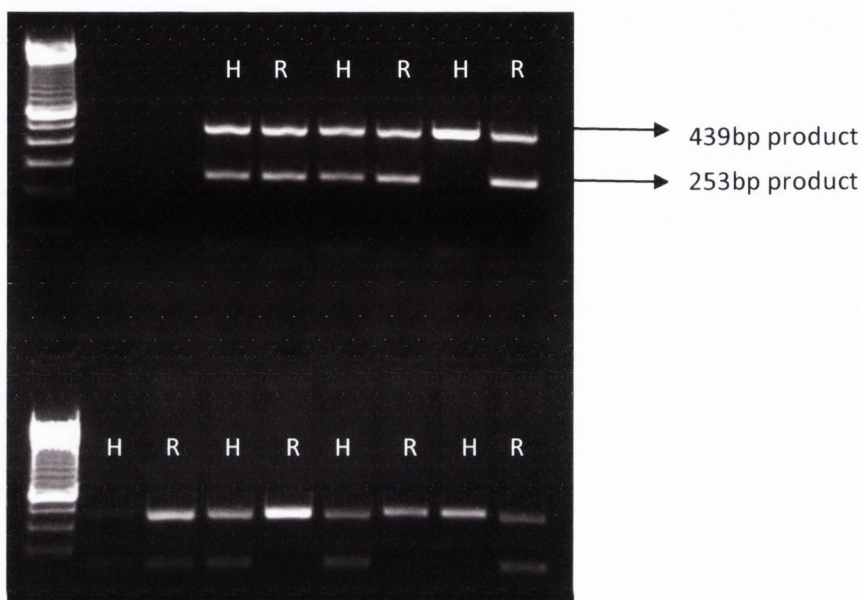


Figure 3.1 showing typical gel used for identification of PCR products. PCRs were run in pairs. (Top row Lanes 1+2 no DNA, Lanes 3+4 CD32A heterozygote, Lanes 5+6 CD32A heterozygote, Lanes 7+8 CD32A RR131. Bottom row Lanes 1+2 CD32A heterozygote, Lanes 3+4 CD32A HH131, Lanes 5+6 CD32A HH131, Lanes 7+8 CD32A RR131)

3.2.2 Analysis

Data was presented using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and was analysed using Graph Pad Prism or SPSS (Version 20, IBM, CA, USA). Continuous variables were analysed using ANOVA/Tukey's comparison, U test or Kruskal-Wallis testing. Comparison of cases and controls was made using χ^2 analysis. χ^2 analysis / Fishers exact test was also used to examine indices of severity in genotypic groups.

3.3. Results

3.3.1 CD32A H131R polymorphisms in healthy Caucasian Irish controls

An unmatched control group of 115 healthy Irish individuals was recruited for genetic comparison. The mean age of this anonymised cohort was 29.6 years and the male to female ratio was 1: 1.3. Genotyping was carried out by allele-specific PCR. Results demonstrated that variation at position 131 of Fc gamma RIIA (CD32A) was common in the healthy Irish population. In the control cohort heterozygotes account for 58% (n=67) of the population while H131 and R131 variants make up 20% (23) and 22% (25) respectively. Comparison with published control cohorts demonstrated no significant difference from our population (as evaluated by χ^2 -testing) (Table 3.3). In particular, no significant difference was observed with a historical group of Irish Caucasian controls recruited by Williams *et al* (Williams *et al.*, 1998). This indicates that our control group conforms to expected patterns and is very likely to be truly representative of a healthy Irish population.

Country	Study Size	HH %	HR %	RR %	p value	Author
UK	259	28%	52%	20%	0.507	Botto <i>et al</i> (Botto <i>et al.</i> , 1996)
United States	149	26%	53%	21%	0.702	Edberg <i>et al</i> (Edberg <i>et al.</i> , 1997)
Russia	107	28%	54%	18%	0.439	Platonov <i>et al</i> (Platonov <i>et al.</i> , 1998)
Canada	95	21%	52%	22%	0.979	Denomme <i>et al</i> (Denomme <i>et al.</i> , 1997)
Finland	93	27%	59%	14%	0.221	Joutsu <i>et al</i> (Joutsu <i>et al.</i> , 1998)
UK	66	18%	58%	24%	0.866	Smyth <i>et al</i> (Smyth <i>et al.</i> , 1997)
Ireland	61	25%	57%	18%	0.617	Williams <i>et al</i> (Williams <i>et al.</i> , 1998)
Current Study	115	20%	58%	22%		

Table 3.3 showing CD32A H131R variation across a range of healthy control populations. χ^2 analysis of polymorphism frequencies in comparison with the current study demonstrated no significant differences (p values as shown).

3.3.2 CD32A H131R polymorphisms in patients with bronchiectasis

CD32A H131R polymorphism status was assessed in the cohort of 101 patients with idiopathic bronchiectasis. Observed genotype frequencies for bronchiectasis patients and healthy controls showed no significant deviation from those expected under Hardy-Weinberg equilibrium.

44 patients (43.5%) of the bronchiectasis patient group were RR131 homozygotes (RR), 43 (42.5%) were HR131 heterozygotes (HR) and 14% were HH131 homozygotes (HH). Figure 3.2 shows a comparison of CD32A genotypic variation in patients and controls. There is a

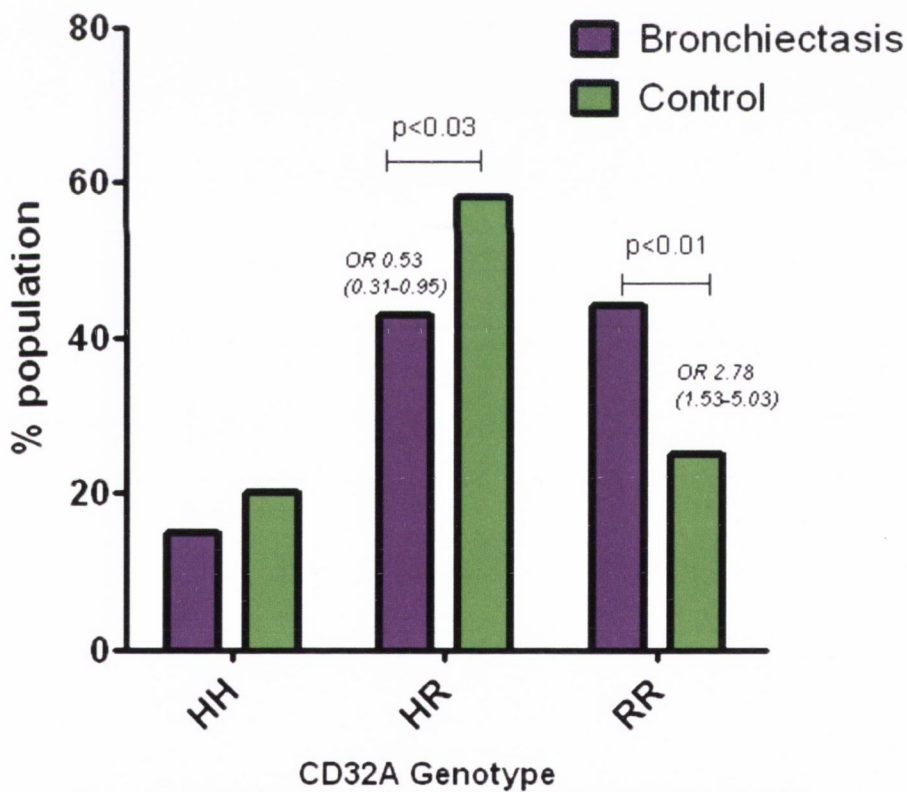


Figure 3.2 showing the distribution of CD32A H131R polymorphisms among 101 bronchiectasis patients and 115 controls (χ^2 analysis, $\alpha=0.05$)

significant excess of CD32A RR131 variants in the bronchiectasis population when compared to healthy controls ($p < 0.01$, OR 2.78, 95% CI 1.53-5.03). The patient cohort also had a reduced frequency of CD32A 131 heterozygotes ($p < 0.03$, OR 0.53, 95% CI 0.31-0.95). No significant difference in the proportion of HH131 variants in disease and control groups was observed. These results suggest that the CD32A RR131 (RR) variant is associated with bronchiectasis in this cohort. There is also a suggestion from these results that the CD32A HR131 heterozygous variant may have a protective effect against bronchiectasis, as this variant is significantly under-represented in bronchiectasis when compared with controls. We have therefore demonstrated for the first time that a gene involved in phagocytic function has a significant association with idiopathic bronchiectasis.

To further determine the relevance of these findings the clinical data was interrogated and differences in the expression of bronchiectasis between the various CD32A variants were sought.

3.3.3 Association of RR131 polymorphism with infection severity

To determine if variation in CD32A H131R had an impact on the presence of symptomatic infection in bronchiectasis, we examined the relationship of different polymorphisms to a variety of markers indicative of clinical disease severity. Infection manifesting as daily purulent cough, recurrent infective exacerbations and hospital admissions have a significant impact on quality of life in patients with bronchiectasis.(Courtney et al., 2008)

CD32A genotype	Reported production of daily purulent sputum		<i>p value</i>	Reported exacerbations/year		<i>p value</i>	Hospital Admissions over last 5 years		<i>p value</i>
	No	Yes		0-3	4+		0	1+	
HH	9	5	<0.001	8	6	<0.001	10	4	<0.001
HR	27	16		32	11		33	10	
RR	8	36		15	29		14	30	

Table 3.4 showing clinical severity parameters of patients with bronchiectasis of known CD32A genotype. (p values based on chi-squared analysis, level of significance =0.05)

Cough was the most common symptom in the cohort of patients with bronchiectasis. However, not all patients complained of persistent and specifically daily production of purulent sputum – in some patients sputum was non purulent, in others sputum production intermittent. RR131 variant patients were significantly more likely to produce purulent sputum ($p < 0.001$) (Table 3.4). In contrast heterozygote bronchiectatics were less likely to produce purulent sputum on a daily basis. No significant difference was observed with HH131 variants.

The number of exacerbations/year based on documentations of patient reports in the notes was also evaluated (Table 3.4). Patients with the RR131 genotype were more likely to complain of four or more infective exacerbations in a given year ($p<0.001$). Again in the H131R subgroup an opposing effect is observed with the majority suffering 3 or fewer lower respiratory tract exacerbations ($p<0.001$). No difference was observed between the small numbers of HH131 homozygotes.

Over the 5 years preceding data collection thirty CD32A RR131 bronchiectatic patients had at least one hospital admission. In contrast only 10 of the heterozygote group were admitted to hospital. These firm findings support the previous data and suggest that patients with the R131 genotype are more likely to suffer a severe infective exacerbation requiring hospital admission ($p<0.001$). H131R variant patients are protected against this likelihood ($p<0.001$).

We have shown that CD32A RR131 is significantly associated with bronchiectasis. Furthermore, these results suggest that variation in CD32A can affect the natural history of bronchiectatic disease. The clear and statistically significant association of the RR131 variant with a more prominent infection history suggests a disease modifying effect.

Patient complaints of dyspnoea were evaluated using the MRC score for functional breathlessness (Table 3.5). Class 1 – 3 represents mild to moderate breathlessness, while 4 and 5 represents more severe symptoms.

CD32A genotype	MRC score for breathlessness		<i>p</i> value
	0-3	4-5	
HH	10	4	0.10
HR	36	7	
RR	28	16	

Significantly fewer bronchiectasis patients that are CD32A heterozygotes fall into the more severe categories of breathlessness . There was a trend towards significance in the R131 category but this did not reach the critical level .

Table 3.5 showing distribution of MRC classification scores across different CD32A genotypes. (p values from chi-squared analysis)

Analysis of sputum microbiology results available on 88 patients that had sputum samples taken reveals further interesting results (Table 3.6).

CD32A genotype	Sputum culture		<i>p</i> value	OR (95%CI)
	Positive	Negative		
HH	7	3	<0.001	2.46 (0.6-10.2)
HR	11	28		0.17 (0.06-0.44)
RR	27	12		3.88 (1.6-9.5)

Table 3.6 showing sputum culture status from 88 patients of the original 101 bronchiectatics. Note: 4 patients had positive cultures for two different organisms.

Results illustrated in Table 3.6 indicate that patients with the RR131 CD32A genotype are more likely to have a positive sputum culture (all organisms) ($p < 0.001$, OR 3.88) than negative. The consistent pattern of clinical protection observed with the heterozygote state is repeated here with H131R being associated with negative sputum culture.

Further examination of results in terms of the three main cultured organisms, *H. influenzae*, *Strep pneumoniae* and *Pseudomonas aeruginosa* reveals that patients with the RR131 homozygous variant genotype has an increased risk of haemophilus or streptococcus culture positivity (Table 3.7). No increased risk of *Pseudomonas* infection/colonization was observed in this sample. Heterozygotes were less likely to culture the encapsulated *Streptococcus* and *Haemophilus* organisms. No significant genotype dependent variation was seen in relation to

Pseudomonas. HH131 homozygosity did not significantly impact on the risk of culture positivity with any of these organisms, although numbers were small.

CD32A	<i>Strep pneumoniae</i>		<i>p value</i>	<i>Haemophilus influenzae</i>		<i>p value</i>	<i>Pseudomonas aeruginosa</i>		<i>p value</i>
	Yes	No		Yes	No		Yes	No	
HH	1	9	<0.05	1	9	<0.001	3	7	0.08
HR	1	38		1	38		2	37	
RR	8	31		12	27		4	35	

Table 3.7 showing numbers of sputum cultures positive for the most commonly encountered organisms in bronchiectasis among the different CD32A genotypes. RR131 variants have an increased risk of culturing *H. influenzae* or *Strep pneumoniae*. (Fisher's Exact Test)

Thus, the CD32A RR131 homozygosity appears to be associated with a striking predisposition to infection with encapsulated bacteria.

3.3.4 The prevalence of respiratory co-morbidities among the bronchiectasis patient cohort is not associated with variation in CD32A

Respiratory co-morbidities are common in this group of bronchiectasis patients (Table 3.8).

Patient data was interrogated to determine if the prevalence of rhinosinusitis, asthma and COPD varied among the different CD32A genotypic subgroups. No significant impact of genotypic variation on the prevalence of respiratory co-morbidities was noted, although there was a trend towards fewer cases of rhinosinusitis in the heterozygote group ($p < 0.07$) (Table 3.8). In particular, none of the genotypic groups showed evidence of an increased risk of COPD, a typical respiratory disease in this age group. This may suggest that the pathogenesis of these co morbid diseases is different from that of bronchiectasis.

CD32A genotype	Rhinosinusitis		<i>p value</i>	Asthma		<i>p value</i>	COPD		<i>p value</i>
	Yes	No		Yes	No		Yes	No	
HH	4	7	0.45	5	9	1.00	4	10	0.39
HR	7	35	0.07	17	23	0.52	18	25	0.84
RR	13	34	1.00	14	26	0.67	19	25	0.69

Table 3.8 showing numbers of individuals with bronchiectasis that also carry a diagnosis of a comorbid respiratory disease in each CD32A variant subgroup. (*p* values from Fisher's Exact Test)

3.3.5 Nebulised antibiotics are more likely to be prescribed to RR131

variant patients with bronchiectasis.

Analysis of prescription patterns of relevant medications in the CD32A subgroups was carried out. No difference in frequency of prescription of bronchodilators, inhaled corticosteroids or leucotriene receptor antagonists was noted (Table 3.9)

CD32A genotype	Inhaled corticosteroids			Leucotriene receptor antagonists			Bronchodilators		
	Yes	No	<i>p value</i>	Yes	No	<i>p value</i>	Yes	No	<i>p value</i>
HH	10	4	0.49	4	10	1.00	11	3	0.16
HR	33	10	0.46	12	30	1.00	33	9	0.42
RR	38	6	0.23	12	32	1.00	42	3	0.06

Table 3.9 showing use of inhaled corticosteroids and bronchodilators along with leucotriene receptor antagonists among the different CD32A genotypic groups. No significant differences are noted (Fisher's Exact Test $\alpha=0.05$).

Additionally in terms of nebulised therapy, no difference in the prescription of pulmozyme is noted. However, nebulised tobramycin was more likely to be prescribed to the RR131 variant group ($P=0.03$). Furthermore, this inhaled antibiotic was less likely to be used in the heterozygote group ($p=0.03$) (Table 3.10).

CD32A genotype	Nebulised pulmozyme		<i>p value</i>	Nebulised tobramycin		<i>p value</i>
	Yes	No		Yes	No	
HH	3	11	0.43	3	11	1.00
HR	4	39	0.26	4	39	0.03
RR	8	36	0.41	14	30	0.03

Table 3.10 showing prescription patterns of the nebulised medications pulmozyme and tobramycin among the different CD32A genotypic groups. Nebulised tobramycin was more likely to be prescribed to R131 variants and less likely to be prescribed to heterozygotes. (Fisher's Exact Test, $\alpha=0.05$)

These results provide further re-enforcement of the clinical findings. The use of nebulised antibiotics, which is significantly higher in the RR group, is likely to correlate with the level of infective morbidity and/or the severity of lung disease.

3.3.6 HR131 variants with bronchiectasis have lung function tests

consistent with less severe obstruction

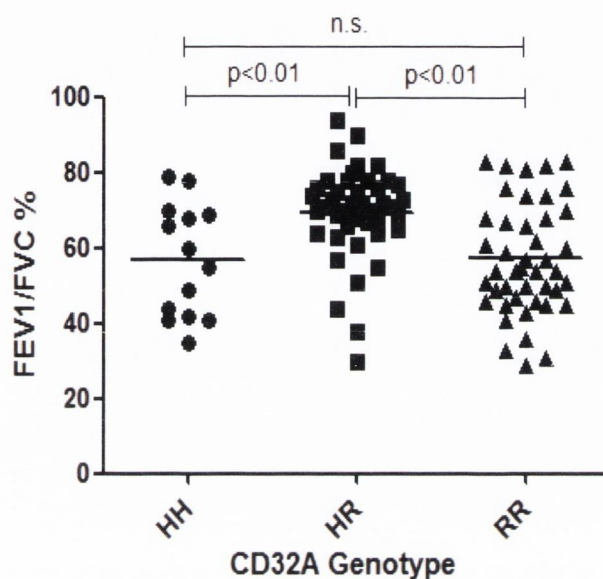


Figure 3.3 showing FEV1/FVC ratios for bronchiectasis patients of known CD32A H131R genotype. Heterozygotes have better FEV1/FVC ratios than other groups (n=101).

Retrospective analysis of lung function tests was carried out and the population divided into different CD32A genotypic groups. Surprisingly, the FEV1/FVC ratios of patients with CD32A heterozygosity were significantly higher with a mean of 70% compared with means of 57% for both the HH131 and RR131 variant groups (Figure 3.3). This suggests that the heterozygote cohort has less significant obstructive disease than the other variants.

Other lung function tests were not available on all patients given the retrospective nature of data collection. DLCO/VA measurements were available on 87 of the 101 bronchiectatic patients (Figure 3.4). RR131 variants had lower levels of this parameter than either HH131 carriers or HR131 heterozygotes, suggesting the presence of interstitial disease accompanying bronchiectasis in this subgroup ($p < 0.001$). The difference between RR131 variants and HH131

patients did not reach the critical level for significance ($p < 0.07$). The numbers in the HH131 group were however, small.

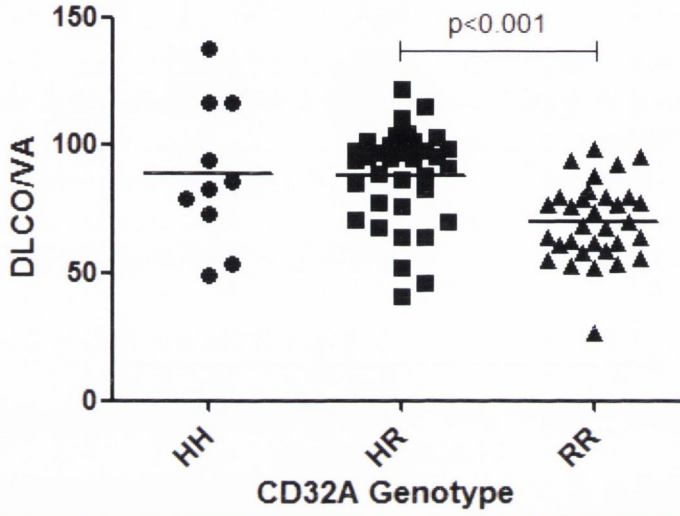


Figure 3.4 showing DLCO/VA ratios for patients with bronchiectasis (n=87). RR131 variants have significantly lower values than heterozygotes ($p < 0.001$ Mann-Whitney)

3.3.7 RR131 variation is associated with significantly higher bronchiectasis scores than other CD32A variants

Full review of HRCT scan results was available on a subgroup of 93 of the 101 patients with bronchiectasis. Full review of CT scan results was not obtained in 8 patients because scans had been carried out in an external hospital or scans were not obtainable. High resolution CT scan images from patients with bronchiectasis were reviewed by radiologists using a scoring system devised by Ooi *et al* (Ooi *et al.*, 2002). Interobserver variability was assessed and showed obtained showed no significant difference.

The mean bronchiectasis score for the RR131 subgroup of patients was 11.6 (range 4-29) (Figure 3.5). This was significantly higher than the score for heterozygote (HR 131) patients (mean 6.7, range 2-18). HH131 variant scores were not significantly different from either heterozygotes or RR131 variants (mean 10.25, range 3-24). These results suggest that RR131 variants have worse structural lung disease than heterozygotes ($p < 0.001$). They also suggest that HR131 patients have milder disease than either HH or RR although the patient numbers in the HR group are quite small.

HRCT records were also assessed for the severity of any concomitant emphysema. Results indicated no significant difference between the CD32A genotypic groups in terms of the severity of emphysema (Figure 3.6)

Intriguingly when scores are examined according to genotype and smoking status there is evidence that smoking has an impact in the CD32A RR131 group alone. Patients in this genetic variant that had a strong smoking history were more likely to have worse bronchiectasis scores. Importantly the prevalence of smoking did not differ significantly between the genotypic groups. This is of interest as there has been much recent attention to gene-environment interactions in the modulation of disease. Indeed in the inflammatory joint disease, rheumatoid arthritis, it has

been shown that cigarette smoking combines with genetic predisposition to significantly enhance disease expression (Sugiyama et al., 2010). Our results could be an example of a gene environment interaction of particular importance to the development of bronchiectasis.

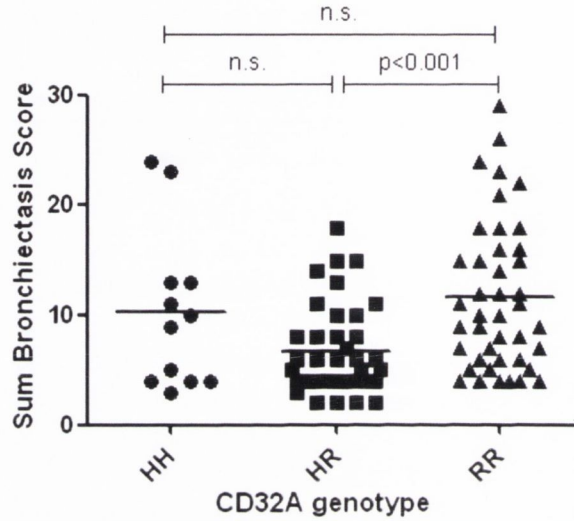


Figure 3.5 showing summed bronchiectasis scores for 93 patients with bronchiectasis. RR131 variants have significantly higher scores than heterozygote variants ($p < 0.001$, Mann-Whitney). HH131 variants are not significantly different from either group but the numbers in this subpopulation are small and the range is wide

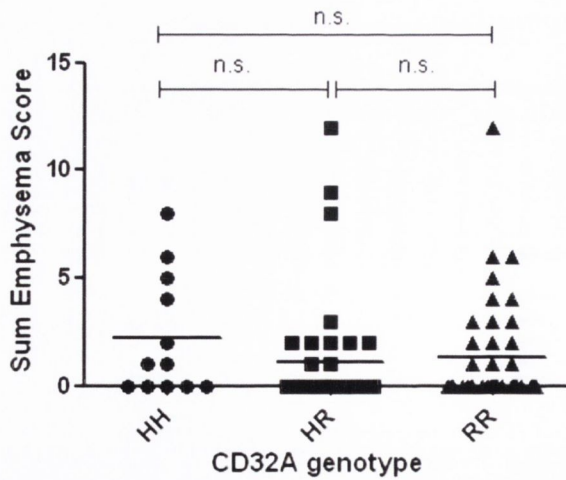


Figure 3.6 showing emphysema scores for 93 patients with bronchiectasis. No significant difference is present between the various CD32A genotypic groups

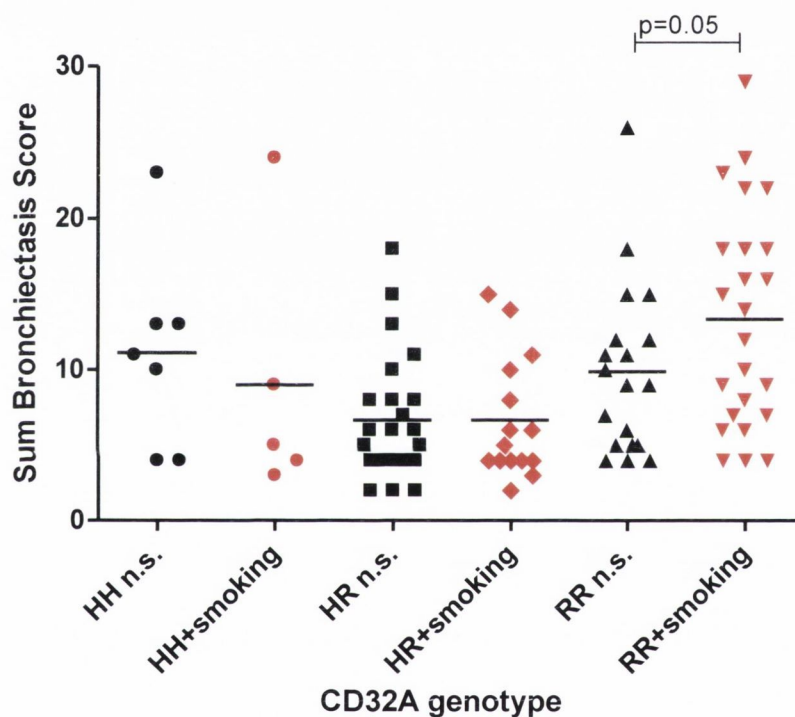


Figure 3.7 showing bronchiectasis scores in CD32A genetic groups divided according to smoking status. Smoking does not have an impact on Bronchiectasis CT scores in HH131 or HR131 groups. However, in the RR131 group smokers have higher bronchiectasis scores that reach borderline significance

3.3.8 Summary of results

These results suggest that CD32A RR131 is associated with bronchiectasis. There is also a suggestion that CD32A HR131 may have a protective effect.

The RR variant of CD32A is associated with increased sputum production, infective exacerbations, hospital admissions and the presence of positive sputum culture, particularly with *Strep. Pneumoniae* and *H. influenzae*. This association with a more severe phenotype is further suggested by an increased rate of prescription of nebulised antibiotics, the presence of impaired lung function testing parameters and the presence of higher HRCT bronchiectasis scores, especially in smokers.

In contrast the HR variant is negatively associated with sputum production, hospital admission, positive sputum culture, impaired lung function tests and HRCT bronchiectasis scores.

In summary CD32A RR131 is associated with bronchiectasis and with more severe disease, while CD32A HR131 might protect against bronchiectasis and be associated with a milder disease state.

3.4 Discussion

CD32A H131R variation was measured in 115 healthy Irish controls and in a comparative population of 101 patients with bronchiectasis. Polymorphic variation in the control population did not differ significantly from other Caucasian populations, including an historical Irish population of healthy individuals (table 3.3, page 68). In the disease cohort, polymorphic variation at CD32A differs considerably from this and other published normal cohorts. The proportion of RR131 variants in the disease population is significantly increased (43.5% vs 20%, $p < 0.01$, Odds Ratio 2.78)(Figure 3.2, page 69). In contrast, a significant reduction in the proportion of heterozygotes is observed (42.5% vs 58%, $p < 0.03$, Odds Ratio 0.53). A non significant reduction in the proportion of HH131 variants in the disease population was also observed (14% vs 20%) (Figure 3.2 page 69). This observation suggests an association of CD32A H131R variation and idiopathic non-CF bronchiectasis in adults in an Irish population.

This study is the first investigation to associate CD32A genotypic variation with idiopathic bronchiectasis. However, CD32A H131R variation has been associated with susceptibility to a number of infective diseases associated with respiratory morbidity. This polymorphism has been studied most extensively in relation to *Streptococcus pneumoniae* infection. Yee *et al* found, in a prospective study, that 50% of patients with invasive pneumococcal pneumonia were CD32A RR131 polymorphic variants (Yee *et al.*, 1997). A subsequent Australian study reported similar findings in invasive pneumococcal disease in children (Yuan *et al.*, 2003). Interestingly, this study noted a reduced frequency of CD32A heterozygosity among patients with invasive pneumococcal disease and postulated a possible protective effect. A subsequent study from the same group published similar findings (Yuan *et al.*, 2008). Examination of community acquired pneumonia patients found that severe sepsis (mainly due to *Strep. pneumoniae* infection) was increased in RR131 adult patients (Endeman *et al.*, 2009). Despite this convincing evidence, some studies have offered conflicting results, including one large, well designed multicentre

Spanish cohort of adult patients which identified an excess of HH131 homozygote variants among patients with pneumococcal bacteraemia (Sole-Violan et al., 2011).

While these conflicting results may be due to the many methodological issues that plague gene association studies or differences between adult and paediatric cohorts, it is also possible that both H131 and R131 can predispose to disease under different circumstances, possibly influenced by other genetic factors or environmental changes (Sole-Violan et al., 2011). The notion that the H131 variant confers disease susceptibility in certain circumstances is supported by associations with cerebral malaria (Omi et al., 2002, Schuldt et al., 2010). Bearing this in mind, a protective effect of CD32A H131R heterozygosity is possible, negating the worst effects of either H or R homozygote variants and striking a happy medium.

The most common pathogen cultured from the sputum of patients with bronchiectasis is *haemophilus influenzae*. Evidence for an association of CD32A RR131 with susceptibility for this pathogen, and other encapsulated organisms is more scant. However, Endeman *et al* identified an association (OR 3.03) of community acquired pneumonia with CD32A RR131 (Endeman et al., 2009). Other evidence suggests that RR131 homozygosity is associated with susceptibility to meningococcal infection (Bredius et al., 1994, Domingo et al., 2002, Fijen et al., 2000, van der Pol et al., 2001). Taken together there is evidence suggesting that CD32A RR131 may be associated with enhanced susceptibility to infection with encapsulated organisms. With respect to pneumococcus and haemophilus, susceptibility to infection could confer an increased risk of recurrent respiratory sepsis, predisposing to bronchiectasis. Studies in these other conditions provide some support for our findings in bronchiectasis.

Our findings in controls indicate that CD32A RR131 variants are common in the healthy population. All of the polymorphic variants of CD32A are compatible with health. The RR131 variant could be considered a subtle or 'partial' immune defect (Bossuyt et al., 2007). This

important idea suggests that humans are by nature susceptible to infection and that multiple partial immune defects along with environmental factors determine an individual's risk of infective disease (Schejbel and Garred, 2007, Bossuyt et al., 2007). In addition, under circumstances where other aspects of the immune system are weak such immune defects may have further importance. A study of children presenting with recurrent respiratory tract infection found that CD32A RR131 did not correlate with disease (Bossuyt et al., 2007). However, they did find that combined partial immune defects including CD32AR R131 were the strongest risk factors for disease. CD32A RR131 is common in the healthy population. It is unlikely that this common polymorphism is acting alone in this disorder.

While this is the first time CD32A polymorphisms have been examined in, and found to be associated with bronchiectasis, other gene associations have been described. Disseminated idiopathic bronchiectasis has been described as a Cystic fibrosis related disorder (Bombieri et al., 2011). The diagnosis of bronchiectasis as a CF related disorder depends on identifying CFTR polymorphisms along with sweat test abnormalities and abnormal nasal potential difference (Bombieri et al., 2011). Bienvenue and colleagues identified a relationship between CFTR sequence variation and consequent protein dysfunction (manifested by abnormal nasal electrophysiology) was associated with CF like disease characterised by susceptibility to *Pseudomonas* and *Staphylococcal* infection, despite normal sweat tests (Bienvenu et al., 2010). However, as Boucher points out in a related editorial, many bronchiectatic patients had nasal potential differences that fell within the reference range (Boucher, 2010). This raises the question on the impact of modifier genes in such patients (Boucher, 2010). How important a role CFTR gene variation plays in bronchiectasis remains the subject of controversy (King et al., 2004, Divac et al., 2005, Bombieri et al., 1998, Casals et al., 2004). Other ion channel mutations may play an additive role with CFTR abnormalities (Fajac et al., 2008, Fajac et al., 2009). Findings of increased CFTR variation in patients with rheumatoid arthritis related bronchiectasis, support

the idea that other disease modifying genes may supply additional hits to the immune system resulting in an end point of bronchiectasis (Puechal et al., 2011).

Whilst CFTR variation is the most extensively studied genetic association with bronchiectasis, other genes have been investigated. Boyton *et al* have identified a number of associated defects (Boyton et al., 2008, Boyton et al., 2006b). They examined polymorphisms in the receptor-ligand pair HLA-C and KIR2D, important in NK cell function. An association with HLA-Cw*03 was identified as more than doubling the bronchiectatic risk. This raised the possibility of NK cell dysfunction and possible excessive activation in bronchiectasis (Boyton et al., 2006b). The same group identified an increased risk of bronchiectasis associated with the presence of the HLA-DR1/DQ5 haplotype (Boyton et al., 2008). They hypothesized that this haplotype could operate as a specific pathogen susceptibility factor or possibly by enhancing self reactivity. These intriguing observations were accompanied by negative association studies of TLR2 and 4 polymorphisms and variations in IFN- γ CXCR1 genes (Boyton et al., 2006a, Reynolds et al., 2007). Other small studies identifying gene associations with MMP-9 and TAP have proved controversial in their findings (Stankovic et al., 2009, Dogru et al., 2007).

These findings point to a possible role for both innate and adaptive cellular immunity in the propagation of bronchiectasis. Despite progress made with these candidate gene studies, clear experimental demonstration of mechanisms of disease are at times lacking. Additionally, CFTR variation excepted, an impact on disease severity has not been clearly demonstrated. Given the likely multifactorial pathogenesis of idiopathic bronchiectasis it is possible that CD32A R131 could combine with other modifier genes to cause disease.

Having identified a possible association of CD32A RR131 variants with bronchiectasis the disease population was then examined for possible markers of disease severity. These findings were based on retrospective analysis of data from clinical charts. Data gathered suggested that

patients with the R131 genotype were more likely to produce purulent sputum on a daily basis ($p<0.001$), were more likely to have four or more infective exacerbations per year ($p<0.001$) and were more likely to have been admitted to hospital over the preceding 5 years ($p<0.001$) (Table 3.4, page 71). An opposing effect was observed in CD32A heterozygotes, who were significantly less likely to have these findings.

This observation is important on a number of levels. Sputum purulence has been shown to be a useful clinical marker in both COPD and non-CF bronchiectasis (Miravittles et al., 2010, Murray et al., 2009). Murray *et al* demonstrated that patient reports of sputum purulence largely matched the observations of medical staff, and, that sputum purulence was associated with a number of factors including increased likelihood of bacterial colonisation and reduced FEV1 (Murray et al., 2009). Evidence also suggests that sputum colour correlates with markers of airway inflammation including elastase and myeloperoxidase (Stockley et al., 2001). The simple clinical observation of patients with CD32A R131 having increased sputum purulence suggests a worse, more inflammatory phenotype.

Infection frequency has been shown to have a clear impact on patient quality of life in bronchiectasis (Wilson et al., 1997a). Furthermore recurrent infective exacerbations are thought to foster further lung damage and disease progression, while the main treatment goal is to reduce the frequency of superinfection (Cole, 1986, Cole, 1991, Pasteur et al., 2010). CD32A R131 variants appear to have more frequent infection and therefore could progress more rapidly. The increased infection frequency will also have associated healthcare costs. The cost issue is even more pronounced when the increased risk of hospitalisation is considered. Seitz *et al* identified an increasing burden of bronchiectasis related hospitalisation in the US and pointed out the need for identification of risk factors (Seitz et al., 2010). Our novel observation of the

association of CD32A RR131 with bronchiectasis related hospital admissions does not explain this increasing burden, but may help identify those at risk of serious morbidity.

The possible protective effect of CD32A heterozygosity is also remarkable. Heterozygotes were significantly less likely to produce purulent sputum on a daily basis ($p < 0.01$), had fewer infective exacerbations ($p < 0.001$) and related admissions ($p < 0.001$). There was no significant difference in these parameters among the small number of HH131 variants, although a trend towards fewer hospital admissions was noted. CD32A heterozygotes were also less likely ($p < 0.04$) to have grade 3-4 breathlessness scores. This differential between RR131 and H131R states is interesting.

Sputum culture results were available on 88 patients and positive in 45. RR131 variants were significantly more likely to have a positive sputum culture ($p < 0.01$, OR 3.88) (Table 3.6, page 74). Furthermore the risk of infection with *Haemophilus influenzae* or *Streptococcus pneumoniae* was significantly higher in the RR131 subgroup ($p < 0.001$ and $p < 0.05$ respectively). RR131 variants were not more likely to demonstrate infection or colonisation by pseudomonas (Table 3.7, page 75). Again an opposing effect is seen with the heterozygotes in terms of a reduced overall likelihood of positive sputum culture ($p < 0.001$) and reduced infection with *Haemophilus* ($p < 0.05$) and *streptococcus* ($p < 0.05$) in particular (Tables 3.6 and 3.7, page 74-75).

Positive sputum culture is common in chronic lung disease (King et al., 2007). These results suggest that RR131 variant patients are more likely to be colonised, in particular with *haemophilus* and *pneumococcus*. Evidence from other sources suggests that subjects colonised with potential pathogens have more severe disease than those with negative sputum cultures (King et al., 2007). Furthermore, there is limited evidence to support the idea that positive sputum culture with *Haemophilus influenzae* gives rise to a worse bronchiectasis score (Wilson et al., 1997b). In the light of this evidence the microbiological data supports the hypothesis that

CD32A RR131 subjects have more severe disease, while heterozygotes are protected against the more severe manifestations of disease. Despite this the majority of evidence suggests that *Pseudomonas aeruginosa* is associated with lower quality of life scores, more rapid disease progression and more severe manifestations of disease than other colonising organisms (Martinez-Garcia et al., 2007, King et al., 2007, Wilson et al., 1997b). There is no difference in the prevalence of *Pseudomonas* positive sputum cultures among the various CD32A genotypic groups. This result suggests that CD32A genotypic variation does not play a role in the risk of colonisation with *pseudomonas* among patients with bronchiectasis. However, the numbers of patients colonised with *pseudomonas* was small in each group. In addition, *Pseudomonas* colonisation may represent the end point of a continuum of progressive bronchiectasis (King et al., 2007). Longitudinal studies would be needed to determine if CD32A genotype played a role in progression towards a clinical end point of *pseudomonas* infection.

Examination of the frequency of the respiratory co morbidities allergic rhinitis, asthma and COPD among the various genotypic groups showed no significant differences (Table 3.8, page 76). Keistinen *et al* suggest that bronchiectasis related mortality is increased by the presence of asthma or COPD (Keistinen et al., 1997). Despite this evidence of the presence of co morbid respiratory disease as a marker of severity, the conspicuous absence of genotype dependent differences suggests that the differences in severity indices observed in this cohort are specific to bronchiectasis. The absence of genotype dependent differences in the frequency of rhinosinusitis could be viewed as unusual given that the pathogenic mechanisms underlying the two diseases may be similar. However, the small numbers of patients diagnosed with rhinosinusitis and lack of focus on these symptoms during the routine clinical follow up of the cohort may make this data unreliable. Investigation of a role for CD32A genotypic variation in rhinosinusitis related to bronchiectasis is a potential area for future observational research.

The management of non-CF bronchiectasis has often translated treatment pathways directly from cystic fibrosis (Pasteur et al., 2010). The recent British Thoracic Society guideline on non-CF bronchiectasis highlights the importance of developing treatment goals specific to bronchiectasis and critically evaluating the risk benefit of long-term medications in the condition. Patients in this cohort were being managed before the publication of these clear guidelines. No genotype dependent differences in the prescription of inhaled bronchodilators, inhaled corticosteroids or leucotriene receptor antagonists were noted (Table 3.9, page 77). No genotype dependent differences in the prescription of the recombinant human DNase Pulmozyme were noted. This treatment breaks down DNA released from neutrophils in the infected lung reducing the viscosity of secretions. Despite evidence of efficacy in CF, a large multicentre trial of Pulmozyme in non-CF bronchiectasis demonstrated its association with a reduction in lung function and increase in exacerbation frequency (Fuchs et al., 1994, O'Donnell et al., 1998). No significant excess of pulmozyme prescription was noted in the CD32A RR131 group. However, there was an increase in the proportion of patients being prescribed tobramycin in the CD32A RR131 group ($p<0.03$) with a reduction in tobramycin use in the heterozygote group ($p<0.03$) (Table 3.10, page 78). Nebulised tobramycin is normally reserved for cases in which exacerbations are frequent (more often than 3 per year) or associated with significant morbidity (Rubin, 2008). Its evidence based effects in non-CF bronchiectasis are thus far minimal. It seems intuitive that the reason for more tobramycin being prescribed in the CD32A RR131 group is an attempt to respond to the increased exacerbation frequency in this group. Similarly the converse in heterozygotes is true. It seems that this pattern of drug prescription supports the data that suggests CD32A RR131 gives rise to a worse disease phenotype in bronchiectasis, while CD32A H131R is protective. However, heterogeneity in prescribing habits, in the absence (at the time of recruitment) of clear management guidelines, means that it is difficult to draw clear conclusions from this data.

Lung function testing in bronchiectasis frequently shows obstructive lung disease (Cherniack and Carton, 1966, Bahous et al., 1984, King et al., 2010). Data presented here suggests a surprising genotype dependent pattern. Figure 3.3 (page 79) indicates that heterozygotes have less severe obstructive lung disease than other groups. Both RR and HR CD32A variants are more severely affected with a mean FEV1/FVC of 57%. Therefore the HR131 variant may have a less severe phenotype. However, caution should be used when extrapolating FEV1/FVC ratios to clinical state as they may correlate only weakly with symptoms (Martinez-Garcia et al., 2005). Despite this recent studies have indicated that a reduced FEV1 is associated with an increased risk of mortality (Finklea et al., 2010). These findings do point to a clear difference between HR131 and RR131 in terms of disease severity.

Examination of DLCO/VA scores in the subgroup in which data was available showed different results (Figure 3.4, page 80). This group of tests indicated a significantly lower DLCO/VA in RR131 patients compared with other CD32A variants. DLCO/VA is the transfer coefficient and is representative of the diffusion capacity when corrected for alveolar volume. While DLCO/VA is often normal in bronchiectasis, compromise of this parameter has been associated with increased mortality (Bahous et al., 1984, Loebinger et al., 2009). Reduced DLCO is associated with more severe disease, and a spread of inflammation from the airways to the lung parenchyma affecting gas transfer (King et al., 2010). Concomitant emphysema could also influence this factor however, a genotype dependent difference in emphysema scores was not observed in this study. DLCO/VA may suggest more extensive inflammatory disease in the lungs of patients with the RR131 CD32A variant. It is interesting to speculate whether such findings could have an association with mortality in these patients. Not all of the patients in the cohort had gas transfer studies carried out. Therefore selection bias may be an issue. A prospective study would be required to confirm these findings.

These lung function test results provide further support for CD32A variation as a risk factor for more severe bronchiectatic disease. The relationship of RR131 to a reduction in transfer factor may indicate more severe inflammatory disease in the lung.

High resolution CT scan data provides a further index of clinical severity (figures 3.5 and 3.6, page 82). Examination of available HRCT films in our cohort did not provide any evidence that the severity of emphysema was affected by CD32A genotype (Figure 3.6). Examination of bronchiectasis scores suggested that as a whole this Irish cohort had mild radiological disease. This is in contrast to the numerous indices of severity already examined, and may suggest an as yet unexplained disconnect between clinical and radiological findings. However, in keeping with the previously presented clinical data, RR131 CD32A variants had higher HRCT scores than heterozygotes ($p < 0.001$), although in absolute terms the difference in mean scores is small (RR 11.6 vs HR 6.7). Studies of HRCT scores and bronchiectasis associate more severe radiological disease with a greater degree of airflow obstruction (Pasteur et al., 2010). Other studies have indicated that CT findings are associated with other parameters including sputum volume (Ooi et al., 2002). These findings again support the suggestion that CD32A RR131 is a disease modifying gene in bronchiectasis. Examination of bronchiectasis scores and their relationship to smoking status in each of the genotypes provides another tantalizing finding (Figure 3.7, page 83). This suggests that smokers in the CD32A RR131 group have higher CT scores than non smokers from the same group. This finding is not replicated in the other CD32A variants. This result must be interpreted with caution due to the small sub-group numbers and borderline statistical significance. Despite this, it is interesting to speculate about the influence of smoking in the bronchiectasis population described here. The putative immunosuppressant effect of smoking on the lung immune system, perhaps acting via nicotinic receptors may be a key area for future examination (de Jonge and Ulloa 2007). While smoking may be a confounding variable, it is also

possible that it represents an environmental factor that could have an interaction with the subtle immune deficiency associated with the CD32A RR131 genotype. This might increase the risk of clinical disease. Further studies will be necessary to tease out this interesting observation.

3.5 Conclusions

Examination of CD32A H131R variation in bronchiectasis has produced some striking findings. CD32A RR131 is increased in this Irish cohort of patients with bronchiectasis, suggesting an association with this otherwise unexplained disease process. In contrast the heterozygous CD32 HR131 appears to be under represented. These interesting findings are strengthened by subsequent clinical data. The clinical data associates CD32A RR131 with a series of markers of severity including purulent sputum production, exacerbation frequency and hospital admission. In addition RR131 is associated with more frequent positive sputum culture particularly with encapsulated bacteria. These indices are supported by the increased frequency of prescription of inhaled antibiotics to the CD32A RR131 group. Respiratory physiological data also suggests that CD32A RR131 patients are more severely affected. This patient group also has more severe radiological disease. In contrast to this CD32A HR131 heterozygotes seem to be spared the worst manifestations of bronchiectasis. In particular the heterozygote state is associated with less obstructive lung disease than other genotypes.

This provides good evidence that CD32A may be a disease modifying gene in bronchiectasis. The association with a number of different clinical parameters strengthens this finding and supports the need for further study in the area. A final observation of a possible gene-environment interaction between CD32A RR131 and smoking may be important. This may partially explain

why some people with the RR131 variant may develop disease despite the compatibility of this polymorphism with perfect health. These findings are worthy of further investigation.

The investigation of disease modifying genes in bronchiectasis is an area of ongoing research. However, new investigations into the role of pulmonary bacteria in health and disease have the potential to offer further insights into the pathogenesis of bronchiectasis. Studies have indicated variation in the microbiome associated with clinical parameters in both COPD and cystic fibrosis (Han et al 2012). It is possible that a complex interaction between the lung microbiome and immune system genotypic variation is playing a key role in bronchiectasis.

In summary, these results give important insights into the pathophysiology of bronchiectasis. In addition they highlight subgroups of patients with different disease phenotypes. These previously undetermined genetic predispositions may allow the more aggressive targeting of patient subpopulations at risk of more severe disease.

Chapter 4. Functional impact of CD32A polymorphic variation on neutrophil phagocytosis

4.1 Introduction

4.1.1 History and epidemiology

Streptococcus pneumoniae has long been recognised as an important human pathogen.

Denounced by Osler, usurping Bunyan's earlier reference to tuberculosis, as 'the captain of all the men of death', the pneumococcus remains over a century later a major cause of mortality and morbidity (Watson et al., 1993). Pneumococci cause disease in a variety of sites, ranging from relatively benign acute otitis media and bronchitis to pneumonia, meningitis and septicaemia. *S. Pneumoniae* results in 1-2 million deaths per year worldwide with the elderly and very young being particularly at risk (Hausdorff et al., 2005). In addition to the mortality costs are the considerable financial implications of minor infections. Acute otitis media caused by *S. pneumoniae* is responsible for almost 2 million emergency department visits in the U.S. usually resulting in antibiotic prescription. The emergence of effective vaccination strategies is rapidly changing the epidemiology of *S. pneumoniae*, especially in relation to invasive disease (Hausdorff et al., 2005). Despite these advances, pneumococci continue to adapt. Increasing knowledge of *S. Pneumoniae* and its interaction with the human immune system is necessary to identify new therapeutic strategies to control this formidable disease causing agent.

Streptococcus pneumoniae is of importance in bronchiectasis, although other bacteria, including *Haemophilus influenzae* and *Pseudomonas aeruginosa*, are found more frequently in adults (Table 1.1, page 3). The data on which such figures are based is taken from patients with established bronchiectasis. Whether the pneumococcus, a widely identified colonising agent, plays a role earlier in the pathogenesis remains unknown (Grimwood, 2011).

4.1.2 *Streptococcus pneumoniae*; the organism and its survival strategies

Streptococcus pneumoniae is a gram positive encapsulate bacteria. There are currently 93 immunologically distinct serotypes identified by the chemically distinct composition of the polysaccharide capsule, dictated by the order and type of monosaccharide units and side branches (Gladstone et al., 2011, Bentley et al., 2006). Pneumococcus is often found as a commensal in the upper respiratory tract. A single serotype may colonise adults and children for weeks and months respectively, normally without any adverse consequences (Bentley et al., 2006). These serotypes such as 6B, 19F and 23F successfully evade local defence and allow long term transmission within humans in the setting of low pathogenicity (Hausdorff et al., 2005). In contrast, other serotypes such as serotype 14 and serotype 9V can produce invasive disease relying on rapid-person to person transmission in the acute setting (Hausdorff et al., 2005).

The young represent a major reservoir for the organism with 20-60% of infants and children acting as carriers (Bentley et al., 2006). In contrast, colonisation rates in healthy adults may be less than 10% (Bogaert et al., 2004). Certain pneumococci exhibit a non invasive phenotype (Sulikowska et al., 2004). These bacteria use immune evasion strategies to facilitate persistence in the nasopharynx. Transmission is perpetuated by direct contact with respiratory secretions. Colonisation phenotypes exhibit low pathogenicity, but long lasting transmissibility allows persistence in the population (Kronenberg et al., 2006). The polysaccharide serotype is an important predictor of the propensity of a pneumococcus to cause invasive disease. In the case of immune compromise, non invasive phenotypes become more pathogenic (Kadioglu et al., 2008).

A different strategy is used by invasive disease causing phenotypes to facilitate survival (Kadioglu et al., 2008). These phenotypes induce disease rapidly resulting in person-to-person spread by

coughing. Recent acquisition of a new invasive serotype is an important risk factor for the development of invasive pneumococcal disease (Gray et al., 1980).

4.1.3 Virulence factors of *Streptococcus pneumoniae*

The polysaccharide capsule is of paramount importance as a virulence determinant for pneumococci. The capsule impairs mechanical clearance and restricts autolysis (Nelson et al., 2007, Bogaert et al., 2004). It reduces phagocytosis by steric hindrance of the interactions between complement and Fcγ receptors and their respective opsonins. Although other virulence factors probably contribute to invasive pneumococcal disease, unequivocal evidence exists for the importance of the pneumococcal capsule (Kadioglu et al., 2008).

Pneumolysin is another virulence factor expressed by invasive pneumococcal serotypes. In sufficient quantities pneumolysin is lytic to cells of the immune system. It also has numerous other inflammatory properties, including the production of cytokines, T cell activation and, the inhibition of phagocyte respiratory burst (Kadioglu et al., 2008). Numerous other putative virulence factors may also contribute to invasive pneumococcal disease, including pneumococcal surface proteins A and C, neuraminidase and IgA protease.

4.1.4 The immune response to *Streptococcus pneumoniae*

The immune response to pneumococci consists of innate immune responses and antibody mediated adaptive immune responses and, more recently identified, Th17 mediated responses. Innate immunity represents the earliest non-specific step in host defence. The innate immune system relies on the recognition of pathogen associated molecular patterns by pattern

recognition receptors on lung epithelial and immune cells. Toll-like receptors (TLR) have been shown to recognise pneumococcal surface molecules (Dessing et al., 2009, Malley et al., 2003, Yoshimura et al., 1999). TLR2 recognises cell wall lipoteichoic acid and peptidoglycan (Yoshimura et al., 1999). TLR4, which recognises lipopolysaccharide, is also important in the recognition of pneumolysin, although mouse models suggest that TLR3 may also play a role (Dessing et al., 2009, Malley et al., 2003). TLR9 has also been implicated in pneumococcal pathogenesis, recognising DNA from autolytic bacteria (Albiger et al., 2007). Pattern recognition receptor signalling converges on the NF κ B and interferon regulatory families of transcription factors (Hajishengallis and Lambris, 2011). Signal transduction results in the production of proinflammatory cytokines and activation of airway macrophages and the recruitment of neutrophils (Mizgerd, 2008). In addition to cell surface pattern recognition molecules, circulating molecules such as CRP and MBL bind to pneumococcal PAMPs acting as early acute phase opsonins. However, the contribution of MBL to pneumococcal phagocytosis remains controversial (Brouwer et al., 2008, Chudwin et al., 1985).

4.1.5 Alveolar macrophages in the immune response to pneumococci

Alveolar macrophages are the predominant macrophage population in the lung. These cells are the resident phagocytes of the lung and are capable of opsonin independent phagocytosis using a number of scavenger receptors (Palecanda and Kobzik, 2001). However, opsonisation with complement, circulating pentraxins or immunoglobulin is necessary for efficient phagocytosis and pneumococcal eradication (Gordon et al., 2000). In contrast to other organisms, alveolar macrophage apoptosis plays an important role in the control of pneumococcal infection (Marriott and Dockrell, 2007). Pneumococcal phagocytosis and killing is associated with increased alveolar macrophage apoptosis (Ali et al., 2003). Inhibition of apoptosis reduces

expression of nitric oxide and inhibits intracellular bacterial killing (Ali et al., 2003, Marriott et al., 2004). In this way alveolar macrophage apoptosis inhibits invasive pneumococcal disease (Srivastava et al., 2005, Dockrell et al., 2003). While macrophage apoptosis enhances the killing of pneumococci, the phagocytosis of apoptotic cells by alveolar macrophages results in down regulation of pro-inflammatory cytokines (Marriott et al., 2006). This inhibits the recruitment of neutrophils and appears to be an important regulatory step, promoting the resolution of inflammation when the pathogen is cleared. Murine models demonstrate the importance of alveolar macrophages in low dose pneumococcal infection models (Dockrell et al., 2003). Furthermore, when the defences of resident alveolar macrophages are overcome, neutrophil recruitment is required to clear infection (Dockrell et al., 2003). Hence, the alveolar macrophage may be an important cell in infection containment with a function at the transition point between asymptomatic colonisation and disease causing infection.

4.1.6 Neutrophils in the immune response to pneumococci

The recruitment of inflammatory neutrophils and macrophages to the seat of infection and the secretion of inflammatory products can result in alveolar consolidation. In this setting, typical of lobar pneumonia, air spaces are filled with immune cells and inflammatory exudates, facilitating the phagocytosis of bacteria (Balamayooran et al., 2010). Recruitment of neutrophils, by macrophage derived chemokines and cytokines and the chemotactic properties of pneumococcal factors such as N-formyl peptides, is important for the clearance of *Streptococcus pneumoniae* infection (Gauthier et al., 2007, Marriott et al., 2008). Neutrophils ingest and kill opsonised pneumococci and other pathogens using NADPH oxidase derived reactive oxygen species (ROS) and granule associated proteases such as elastase (Reeves et al., 2002). Murine models suggest that ROS are not necessary for pneumococcal killing but do contribute to the

activation and survival of neutrophils in the lung (Marriott et al., 2008). Furthermore, the generation of ROS downregulates neutrophil recruitment to the lung acting as a break on the inflammatory influx (Marriott et al., 2008). This is a potentially important regulatory step as neutrophil infiltration and activation within the lung can cause tissue damage (Matute-Bello et al., 1997, Herbold et al., 2010). The impact of neutrophil influx whether beneficial or detrimental appears to depend on the virulence of the pneumococcal serotype involved (Garvy and Harmsen, 1996, Marks et al., 2007).

4.1.7 Adaptive immune response to pneumococcus – the role of antibodies

Antibodies directed against the pneumococcal capsular polysaccharide have traditionally been viewed as critical to the immune response in naturally exposed patients. Antibodies interact with the pneumococcal capsule, opsonising the bacteria and activating complement (Malley, 2010). The IgG2 subclass has been shown to be particularly important in this regard (Soininen et al., 1999). This suggestion is supported by several lines of evidence, including the efficacy of treatment of invasive pneumococcal disease with serotype specific antibody containing serum, the generation of effective protection by polysaccharide vaccination, and the increased risk of disease in hypogammaglobulinaemic patients, and those with IgG2 subclass deficiency (Casadevall and Scharff, 1994).

Serotype specific antipneumococcal antibodies can be elicited by mucosal carriage, otitis media and by invasive disease (Malley, 2010, Soininen et al., 2002). Antipneumococcal antibody responses generated by the administration of vaccines containing polysaccharide capsules of different serotypes are protective against invasive pneumococcal disease (Jackson and Janoff, 2008). Antibodies directed against other cell wall components may also play a role in the

development of immunity in non-immunised persons (Lipsitch et al., 2005). Levels of antipneumococcal antibodies that offer protection against invasive pneumococcal disease and nasopharyngeal carriage have been loosely defined, although much debate exists about how generally applicable such levels are (Paris and Sorensen, 2007). The importance of the humoral immune response has been emphasised over recent years, however, new data suggests Th17 cells may also be important.

4.1.8 Th17 cells and the immune response to pneumococci.

More recently pro-inflammatory Th17 cells have been identified as playing a possible role in the immune response to pneumococci. These cells produce a family of cytokines such as IL-17 and IL-22 that favour neutrophil recruitment and antimicrobial responses. Conditions with absent or defective Th17 cells such as hyper-IgE syndrome result in susceptibility to a variety of infections including *Streptococcus Pneumoniae*. Recent *in vitro* data suggests that human monocytes exposed to pneumococci induces a mixed Th1 and Th17 response from co-cultured lymphocytes (Olliver et al., 2011). Despite this further studies have suggested that it is antibody mediated mechanisms that determine protection against fulminant pneumonia, at least in mouse models (Cohen et al., 2011). Limited evidence also raises the possibility that vigorous Th17 responses may be detrimental to outcome in pneumococcal disease (Cohen et al., 2011). Further studies to determine the role of Th17 induced responses are required.

4.1.9 Phagocytosis

Phagocytosis is the process of endocytosis of solid particles by the cell membrane to form an internal phagosome, facilitating intracellular killing mechanisms. Phagocytosis is necessary for the clearance of infection and the prevention of chronic lung injury. The process of phagocytosis has been placed at centre stage in the battle of the immune system against foreign particles since its identification by Metchnikov in the late 19th century (Underhill and Ozinsky, 2002). Since then significant progress has been made in the understanding of this key immune mechanism (Underhill and Ozinsky, 2002). Phagocytosis is a complex process, potentially involving multiple receptors that vary depending on pathogen. The process of phagocytosis is also coupled to the generation of inflammatory mediators and cytokines that may affect the efficiency of the phagocytic process (Underhill and Ozinsky, 2002).

Phagocytosis is initiated by contact of microbial cell surface moieties or labeling opsonins with phagocyte surface molecules (Underhill and Ozinsky, 2002). Receptors involved include Fc receptors, complement receptors and a variety of receptors for integrins and scavenger particles. CD32A and CD16 are the constitutively expressed IgG receptors on neutrophils (Rivas-Fuentes et al., 2010). These receptors initiate phagocytosis by engaging the Fc portions of opsonising IgG bound to target particles (Daeron, 1997). Evidence suggests that CD32A is by a considerable magnitude the most efficient receptor at inducing phagocytosis (Rivas-Fuentes et al., 2010). In contrast, CD64 which is not constitutively expressed but is inducible on neutrophils appears incapable of mediating phagocytosis in isolation (Rivas-Fuentes et al., 2010).

Complement receptors are also important in phagocytosis. Complement can opsonise target particles through antibody dependent and independent mechanisms. A variety of complement receptors on the phagocyte cell surface can recognise, bind and internalise the opsonised particle. CR1 is capable of binding a variety of opsonins including C1q, C4b, C3b and MBL (Ghiran

et al., 2000). CR1 engagement can enhance phagocytosis occurring via Fc receptors, an example of cross talk between innate and adaptive immune systems. CR2 (CD21) is not known to be a phagocytic receptor. CR3 and CR4 are heterodimeric integrins capable of recognising iC3b (Kohl, 2006). Complement receptor mediated phagocytosis is enhanced by exposure of the phagocytic cell to activating cytokines and microbial products (Wright and Griffin, 1985).

While complement and Fc receptors can mediate phagocytosis in isolation they can also act synergistically (Huang et al., 2011). Blockade of CR3 reduces Fc receptor mediated phagocytosis (Brown et al., 1988). Similarly stimulation of CD32A but not CD64 enhances CR3 mediated phagocytosis (Huang et al., 2011). Thus simultaneous engagement of Fc and complement receptors increases phagocytosis and can compensate for low level opsonisation (Huang et al., 2011). Other receptors such as scavenger receptors SR-A and MARCO can also facilitate target binding often without significant internalisation (Underhill and Ozinsky, 2002). Engagement of the target particle or pathogen with the phagocytic cell is the initiating step in the process of phagocytosis.

Target binding to the phagocyte triggers rearrangement of the cell cytoskeleton (Joshi et al., 2006). Engagement of complement receptors facilitates engulfment without membrane extension. In contrast, when Fc receptors are involved cytoskeletal rearrangement allows extension of the plasma membrane and particulate engulfment (Joshi et al., 2006).

Internalisation of the target particle is accompanied by induction of killing and inflammatory pathways. Fc receptor engagement is critical for efficient activation of inflammatory responses (Underhill and Ozinsky, 2002).

The destruction of internalised pathogens by monocytes, macrophages and especially neutrophils, is in part due to the generation of the oxidative burst. Signalling cascades through Fc receptors generate a series of kinases which activate the multienzyme NADPH oxidase

complex (Underhill and Ozinsky, 2002). This enzyme complex produces toxic reactive oxygen metabolites such as O_2 , H_2O_2 and $HOCl$ (Nauseef, 2007). These toxic molecules, in association with enhancements in antimicrobial enzyme activity at low pH, contribute to phagosomal pathogen death. While CR3 engagement is sufficient for internalisation it does not lead to the production of reactive oxygen species. In contrast Fc receptors are important in this regard (Dai et al., 2009, Fossati et al., 2002). In particular CD32A is important in both neutrophils and monocytes in the generation of oxidative burst (Dai et al., 2009, Fossati et al., 2002). Other Fc receptors utilise different signalling pathways and have a more pronounced role in the generation of inflammatory mediators (Dai et al., 2009).

4.1.10 Measurement of phagocytosis

Methods to measure phagocytosis have been developed over many years and have helped elucidate defects in phagocytic function. Experimental assessment of phagocytosis requires appreciation of the interaction between phagocytes, pathogens or targets and opsonising serum factors (Lehmann et al., 2000). A number of techniques have been used. The basis of each assay is the incubation of the phagocytic cell with a pre-opsonised target followed by measurement of target ingestion or target removal from medium (Hampton and Winterbourn, 1999).

Early techniques used to assess phagocytosis have largely been superseded by flow cytometry. Methods used previously, included visualisation by direct and electron microscopy, bacterial viability assays, and chemiluminescence assays (Lehmann et al., 2000). Direct visualisation methods demonstrate particle engulfment, while chemiluminescence assays indicate the generation of post phagocytosis oxidative burst (Welch, 1980, Lehmann et al., 2000). Finally, viability assays allow estimation of the removal of bacteria by the entire phagocytic process.

Microscopy measured target uptake by direct visualisation but was limited by resolution issues. Electron microscopy and confocal microscopy have enhanced resolution but are cumbersome techniques for high throughput experimentation (Hampton and Winterbourn, 1999). Chemiluminescence with luminol offered a less subjective method of measuring phagocytosis (Gardner et al., 1982). Microbiological assays utilise viable colony counting after bacterial incubation with phagocytes and allow concurrent assessment of bacterial viability. ³H-thymidine incorporation into viable bacteria has also been used as a combined phagocyte and killing assay (Roberts and Ford, 1982).

Flow cytometry has become popular for the measurement of phagocytic parameters. It offers a versatile platform for the high throughput assessment of phagocytosis and related processes including oxidative burst. A variety of targets have been used including bacteria, zymosan, fungi and latex or polystyrene beads (Lehmann et al., 2000).

In the simplest terms, flow cytometric protocols involve the incubation of targets with an opsonising mix of complement and serum, followed by exposure of opsonised targets to phagocytosing cells (Lehmann et al., 2000). Phagocytosis is stopped by rapid temperature reduction, cells are then fixed and analysed on a flow cytometer. Cells which have phagocytosed fluorescent targets are often easily identified. Lehman *et al* in their review of opsonophagocytic assay techniques draw attention to a number of important technical issues which can have an impact on results (Lehmann et al., 2000). These include isolation media, mixing during incubations and opsonising agents and ratios. The importance of the target:cell ratio is also highlighted. A low target:cell ratio reduces the rate of coincidence (the simultaneous appearance of one or more cells with a free target in the laser beam) to negligible levels (Lehmann et al., 2000). In addition, a low target:cell ratio favours opsonin dependent phagocytosis, as opposed to non specific opsonin independent phagocytosis.

4.1.11 Measurement of differential Fc receptor function using phagocytosis assays

Opsonophagocytic assays have been used to explore the importance of Fc receptor polymorphisms. Research in this area has highlighted the importance of standardization of assay conditions. Sanders *et al* highlighted the importance of CD32A H131R variation in the phagocytosis of a killed encapsulated strain of group B streptococci (GBSIII)(Sanders et al., 1995). Using purified IgG2 anti-GBSIII antibodies as an opsonin they demonstrated that isolated HH131 neutrophils had a higher phagocytic capacity than RR131 neutrophils. Phagocytosis could be blocked by the addition of monoclonal antibodies against CD32A. The phagocytosis model in this study used an approximate 1:1 cell:target ratio and was, notably, found to be complement independent.

Several studies have examined phagocytosis of different *Streptococcus pneumoniae* serotypes under a variety of assay conditions. A chemiluminescence approach was used to demonstrate the importance of antibody and complement to the neutrophil phagocytosis of serotype 14 pneumococci (Gardner et al., 1982). Results indicated that chemiluminescence activity was proportional to antibody concentration. Furthermore this assay system highlighted the importance of complement in pneumococcal phagocytosis, with heat inactivation of serum resulting in markedly reduced phagocytosis (Gardner et al., 1982). In contrast, and again using serotype 14 pneumococci, Rodriguez *et al* found phagocytosis by isolated neutrophils to be complement independent (Rodriguez et al., 1999). These experiments used very different assay conditions including a 1:30 cell:target ratio and a pre-phagocytosis cold incubation to allow bacterial attachment to bacterial targets (Rodriguez et al., 1999). However, using H131 and R131 transfected cell lines, this study confirmed a superiority of H131 in terms of phagocytosis

(Rodriguez et al., 1999). The importance of H131R variation at CD32A was emphasized by data using serotype 23F pneumococci as targets (Jansen et al., 1999). This study also estimated that an unfavourable Fc receptor genotype could require the production of four times as much antibody to produce similar levels of phagocytosis (Jansen et al., 1999). Information from these assays suggests that under certain different experimental conditions CD32A H131R variation produces considerable difference in phagocytosis.

While these studies address variations in the magnitude of phagocytosis following neutrophil stimulation opportunities to address unanswered questions in this area remain. New technologies allow assessment of isolated pneumococcal polysaccharide induced phagocytosis investigating the unknown impact of CD32A variation on this. Extension of these assays has the potential to add to build on previous data by examining CD32A related variation in cell surface and secreted activation markers along with oxidative burst. Such findings may offer unique insights into pathogenic pathways of relevance to bronchiectasis.

Interest in opsonophagocytic assays as a way of measuring functional antibody levels post vaccination has grown. Flow cytometric platforms offer the possibility of generating high throughput assays which can be multiplexed, allowing the simultaneous assessment of several different antibody specificities. A number of strategies have been utilized to remove sources of variability from investigational opsonophagocytic assays. These include the use of CD32A R131 expressing HL-60 neutrophil cell lines and latex or polystyrene beads (Romero-Steiner et al., 1997). These cell lines eliminate the variability, much of which is due to CD32A genotypic variation, seen among human donors. The development of polysaccharide labeled fluorescent beads eliminates variations in bacterial staining and culture techniques (Lehmann et al., 2000). Such beads contain a fluorochrome within the bead polymer and have a similar size to pneumococci. Use of bead targets allows the assessment of anti-polysaccharide antibodies and

their contribution to phagocytosis in isolation, without the involvement of other virulence factor receptors (Martinez et al., 2006).

4.1.12 Aims of this chapter

- To establish flow cytometric assays to measure the phagocytosis of pneumococcal targets from isolated donor neutrophils of different genotypes.
- To determine the impact of CD32A H131R on phagocytic efficiency using this assay
- To determine the effect of CD32A H131R on neutrophil activation following stimulation with pneumococcal targets

4.2 Materials and Methods

4.2.1 Subjects

24 healthy control subjects were selected on the basis of CD32A genotype. Eight subjects from each genotypic group were identified (HH131, HR131, and RR131) for inclusion in the functional analyses. Subjects were run in random order. These individuals were typed for H131R. However, the results of genotyping were coded and therefore these experiments were conducted without the knowledge of CD32A H131R status.

4.2.2 Neutrophil isolation

Neutrophil isolation was carried out using an adaptation of a technique used by Lichtenberger *et al.* (Lichtenberger *et al.*, 1999). Briefly, 40mls of EDTA whole blood was obtained from healthy volunteers and mixed gently (Stuart SRT9 roller mixer, Staffordshire, UK).

- 40 mls of whole blood (in EDTA) was placed in a 50ml Sarstedt tube (Sarstedt, Drinagh, Ireland).
- Plasma was removed after spinning at 210 rcf (Beckman Coulter Allegra X 17R, Beckman Coulter, High Wycombe, UK) for 20 minutes (no brake).
- 10mls of 6% Dextran (6g Dextran in 100mls HBSS, without calcium, magnesium or phenol red, pH 7.0-7.4)(Life Technologies, Paisley, UK) was added to the pelleted blood. This was then made up to 40mls with 0.9% saline. The suspension was placed at a 30° angle for 30 minutes and then placed upright for a further 10 minutes, allowing gentle sedimentation of the red cells.

- The white cell rich upper layer was removed and spun at 210 rcf for 10 minutes with no brake.
- A 90% Percoll solution (Sigma Aldrich, UK) in 10X PBS was initially made up. From this a series of dilutions in 1X PBS were made resulting in a 79%, 68% and 55% solutions. The white cell solution was centrifuged gently at 210 rcf (with no brake), for 10 minutes following which the pellet was resuspended in the 55% Percoll solution.
- A gradient was set up in a 15mls Sarstedt tube (55%, over 68% over 79%). The gradient was placed in a centrifuge and spun at 210 rcf for 20 minutes.
- Following centrifugation the separated neutrophil layer was carefully removed using a 21 gauge needle. These neutrophils were washed (twice in HBSS) and resuspended in HBSS (with calcium and magnesium, without phenol red, pH 7.0-7.4)(Life Technologies, Paisley, UK)

Neutrophils were found to be >99% viable using EB/AO viability staining. There was no appreciable loss of viability over 6 hours of assessment, in contrast to a number of other evaluated methods (Data not shown). Purity was >96% and confirmed on light microscopy and flow cytometry (Figure 4.1). Cell counts were performed by light microscopy and the cell suspension diluted to give a final concentration of 1000 cells/ μ l.

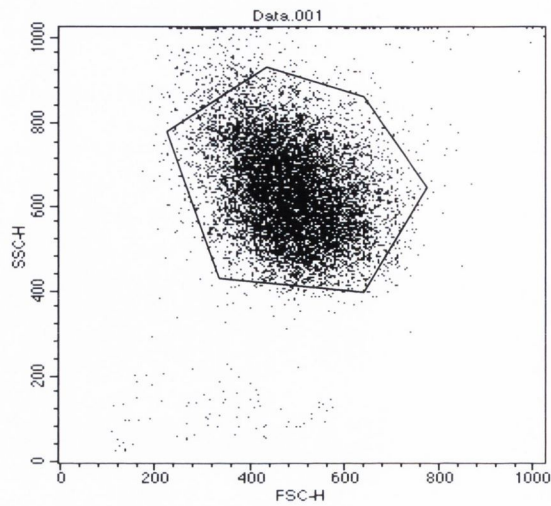


Figure 4.1 showing a pure freshly isolated neutrophil population on flow cytometry. Gating of neutrophils was by forward scatter and side scatter

4.2.3 Bead based phagocytosis assay

A phagocytosis assay using pneumococcal coated polystyrene beads (Flow Apps Ltd, Illinois, USA) was established. This assay was based on the assay developed by Martinez *et al* (Martinez *et al.*, 2006).

4.2.3.1 Opsonophagocytic assay buffer (OPA buffer)

OPA buffer was made up in 20ml aliquots. 0.04g of powdered bovine serum albumin (BSA) was added to 20mls of HBSS with calcium and magnesium but without phenol red, under sterile conditions.

4.2.3.2 Antibodies

Pooled serum was obtained from healthy individuals vaccinated with the standard 23 valent pneumococcal vaccine Pneumovax II who had made an excellent specific antibody response. An excellent response was defined as Pneumococcal IgG > 250 mg/l on functional IgG measurement by ELISA. Serum from 15 such individuals was pooled to make a standard reference sera to be used in all experiments. This reference sera was aliquoted and stored at -80°C until use. Aliquots were sent for serotype specific pneumococcal antibody analysis (Papworth NHS Trust, Cambridge, UK). Results for pooled samples are shown in Table 4.1.

To inactivate complement pathways portion of antibody aliquots were heat inactivated in a 56°C water bath for precisely 30 minutes. Confirmation of the absence of complement function was confirmed by assessment of classical and alternative complement pathways using standard RID plates (Binding Site, Birmingham, UK)

In selected experiments opsonising antibodies were diluted. In these experiments antibody dilution was carried out in opsonophagocytic assay buffer. Volumes were kept constant.

Pneumococcal serotype	Antibody level mg/l
4	8.9
6b	10.2
9v	15.6
14	23.3
18c	5.75
19f	13.9
23f	5.6

Table 4.1 showing serotype specific anti-pneumococcal antibody concentrations in pooled high titre standard reference serum.

4.2.3.3 Complement

6 week old baby rabbit sera was used as a complement source (Pel-Freeze, AK, USA). Lyophilised aliquots were stored at 4°C. They were reconstituted as directed using 1ml of sterile water, aliquoted and stored at -80°C until use.

In selected experiments complement was titrated out. In these experiments dilution was carried out in opsonophagocytic assay buffer.

4.2.3.4 Polysaccharide conjugated polystyrene beads

Polystyrene beads conjugated to different pneumococcal polysaccharides were obtained from FlowApps (FlowAppsLtd, Illinois, USA) (Table 4.2). Seven serotypes were obtained for initial assessment. The beads were of four different emission wavelengths.

Pneumococcal serotype	Colour	Excitation/Emission spectra
14	Yellow	488/530nm
9V	Pink	488/585nm
18C	Pink	488/585nm
6B	Red	488/>600nm
19F	Red	488/>600nm
4	Blue	635/>670nm
23F	Blue	635/>670nm

Table 4.2 showing colours and excitation/emission spectra for different pneumococcal conjugated polysaccharide beads

The concentrated bead solution was diluted in HBSS (with calcium and magnesium) according to manufacturer's instructions to give a final concentration of 10,000 beads/ul. Each opsonophagocytic reaction mix required 20ul of the diluted bead solution amounting to 2×10^5 beads. Fresh diluted bead solutions were made up for each assay with the volumes calculated dependant on the number of experimental wells used.

4.2.3.5 Opsonophagocytic assay (OPA)

The OPA was carried out with 100µl reaction mixes in sterile 96 well round bottomed tissue culture plates (Corning Life Sciences, USA).

- The appropriate number of required wells was calculated. Volumes of all reagents were made up specific to the number of wells required to limit wastage.
- A cell:target ratio of 1:4 was used except where otherwise specified
- Reactions were carried out at 37°C except in cold pre-incubation experiments
- Control wells were laid out as follows
 - A. Cells only/Blank control (50µl cells + 50µl OPA buffer)
 - B. Bead control (50µl cells + 20µl bead mix + 30µl OPA buffer)
 - C. Complement control (50µl cells + 20µl complement +30µl OPA buffer)
 - D. Serum control (50µl cells + 10µl serum +40µl OPA buffer)
 - E. Single colour controls (experiment dependent)
- Experimental wells contained
 - A. 50µl cell suspension of varying CD32A H131R genotype
 - B. 10µl high titre standard or other experiment specific sera
 - C. +/- 20µl complement source
 - D. 20µl bead suspension of varying polysaccharide serotype

- A 100µl reaction mix was used in all wells; in those where reagents were omitted for experimental or control reasons volume was replaced with OPA buffer.
- Initially 20µl bead mix and opsonising serum was mixed in selected wells for 1hr at 37°C with slow agitation on a plate shaker 50rpm (Stuart SSM1, BibbyScientific, Staffordshire UK)
- After antibody opsonisation, in appropriate wells, 20µl of the complement source was added and incubated at 37°C for 20 minutes with slow agitation on a plate shaker
- Finally following pre-opsonisation and complement activation phases, 50µl of cell suspension was added.
- The final OPA mixture was returned to the 37°C incubator and agitated gently on a plate shaker for a further hour (or experimentally appropriate time).
- On completion reaction mixes were removed from the 96 well plate, placed in Falcon tubes and washed twice in ice cold CellFix (BDBiosciences, Oxford, UK)
- Where appropriate multiple supernatants were removed, pooled and spun at 1100rcf in a high speed centrifuge to remove cells and debris and stored at -80°C until required for analysis of secreted activation markers.
- Samples were analysed on a BD FACS Calibur Flow Cytometer (BD Biosciences, Oxford UK)

4.2.3.6 Flow cytometry

- Samples were analysed immediately
- The isolated and pure neutrophil population was gated by simple forward and side scatter (Figure 4.1, page 114). Samples with significant cellular or other contamination were rejected and assays repeated. This happened on three runs only and was due to errors in the centrifugation stage of neutrophil separation.

- Phagocytosis was measured as the percentage of total cells with an increase in fluorescence above the gated negative population (Figure 4.2)
- Suitable compensation strategies were carried out
- Flow cytometry was analysed using Cell Quest software (BD Biosciences, Oxford, UK)

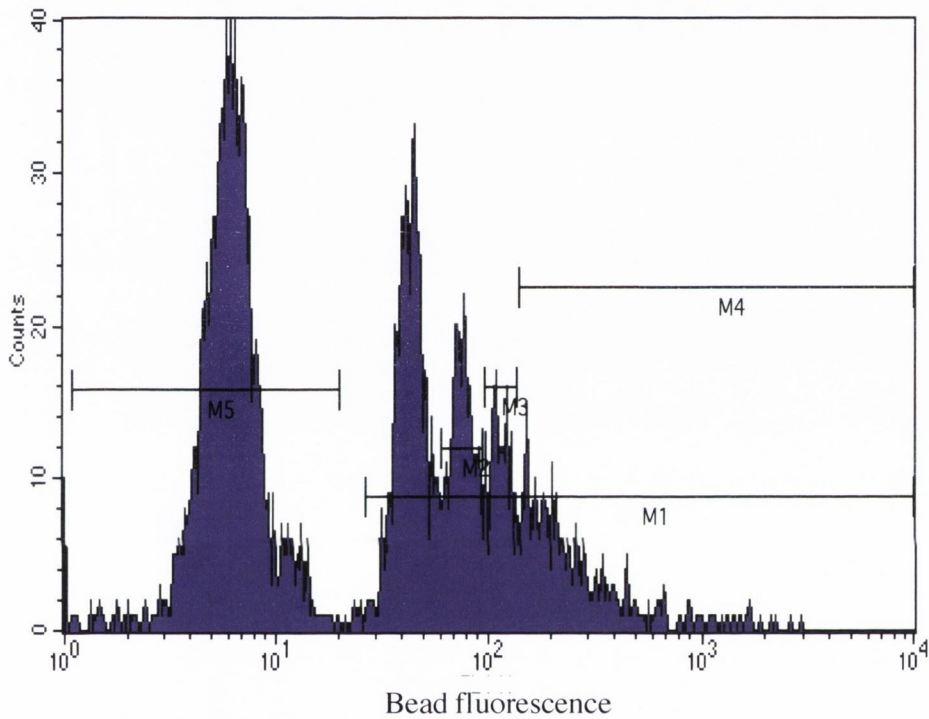


Figure 4.2 showing neutrophil phagocytosis of serotype 14 polysaccharide conjugated polystyrene beads. The M1 bar identifies cells that have phagocytosed beads, while the M5 bar shows cells that have not taken up beads

4.2.4 Inter- and intra-assay variation and time course

A series of experiments were carried out using CD32A heterozygote cells and serotype 9v beads from an individual donor to determine the variability of the basic opsonophagocytic assay. Intra-assay variability was minimal with an average coefficient of variation of 3%. There was more significant inter-assay variability, on assays carried out on different days (Figure 4.3). However, these initial experiments indicated that the assay was robust and reproducible. Analysis of time course indicated that neutrophils phagocytosed bead targets rapidly and there was a gradual increase in the proportion of neutrophils phagocytosing beads up to 2 hours. In most experiments a 1 hour time point was used (Figure 4.4).

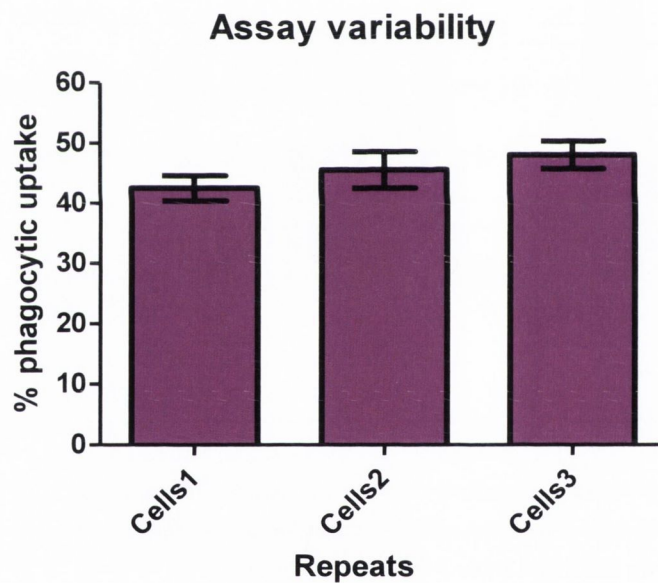


Figure 4.3 showing assay variability over three separate experiments. Low intra-assay variability is demonstrated.

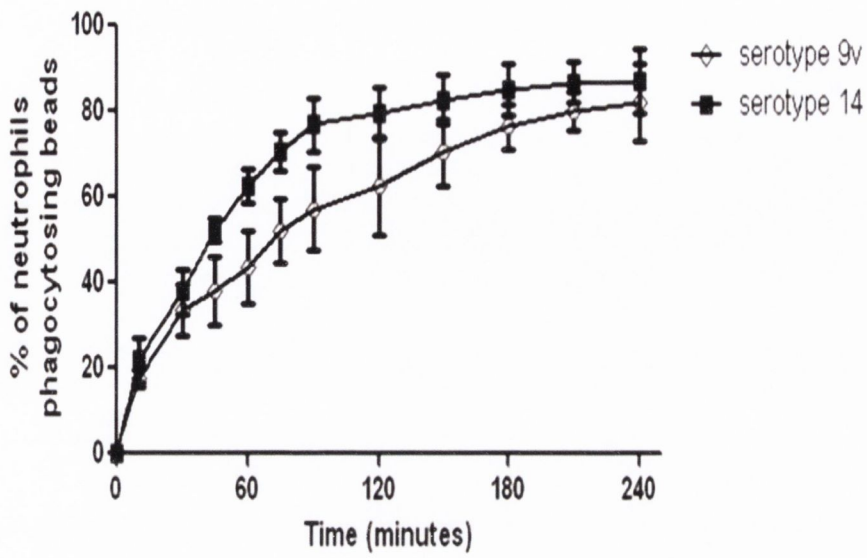


Figure 4.4 showing phagocytosis of serotype 9v and 14 polysaccharide coated polystyrene beads at several time points over 4 hours (n=3). Phagocytosis is initially rapid and tapers off as time progresses.

4.2.5 Assessment of importance of peaks in phagocytosis flow cytometry plots

Phagocytosis plots produced indicated that phagocytosing cells produced a number of immunofluorescence peaks (Figure 4.2, page 119). It was hypothesised that these peaks represented the number of bead targets phagocytosed by neutrophils. This was addressed by measuring the proportion of total number of neutrophils that had phagocytosed 0, 1, 2, 3 or more than 3 targets as counted by two independent observers under light microscopy. Observers counted 100 cells on three separate experiments. This was compared with proportions measured by flow cytometry taken in the corresponding experiment. Results are presented in table 4.3.

Targets phagocytosed	% as measured by light microscopy (mean of two observers)(n=3)	% measured by flow cytometry (n=3)	P value
0	45	49	n.s.
1	24	22	n.s.
2	13	11	n.s.
3	8	7	n.s.
>3	10	11	n.s.

Table 4.3. The proportion of cells phagocytosing different numbers of targets corresponds to proportions observed by light microscopy. Therefore this flow method can serve as a way of measuring efficiency of phagocytosis by indicating the proportion of cells that phagocytose multiple targets. (unpaired t test $\alpha=0.05$)

4.2.6 Confirmation of internalisation of phagocytosed beads

Confocal microscopy was used to determine internalisation of beads during the OPA and to exclude the possibility of beads sticking to the cell surface. Cells were examined after the standard 1hr phagocytosis assay on an Olympus FV1000 (Olympus, Essex, UK) confocal microscope (Figure 4.5) (with thanks to Gavin McManus, TCD). Cell surface adherence of the beads was not observed at this single time point and was not a problem in previous studies using these beads (Martinez et al., 2006).

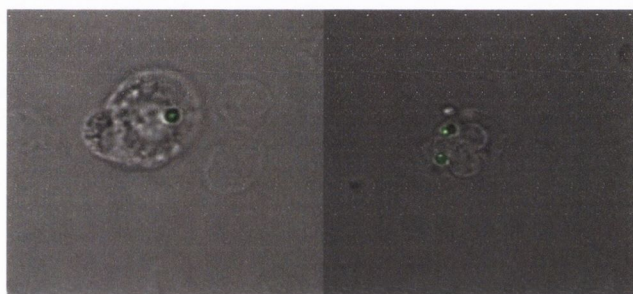


Figure 4.5 showing phagocytosis of serotype 9v beads by neutrophils

4.2.7 Simultaneous measurement of oxidative burst using flow cytometry

A previously described technique was adapted to assess oxidative burst in neutrophils on stimulation with pneumococcal conjugated polystyrene beads (Lehmann et al., 2000). The method is described briefly:

Preparation of hydroethidine (Dihydroethidium)

- Add 1ml of DMSO to 10mg hydroethidine
- Add 100 μ l of the hydroethidine solution to 10mls of sterile deionised water
- The stock solution was aliquoted and stored in the dark at -20 $^{\circ}$ c until use

- 3 μ l of hydroethidine was added to selected wells of the opsonophagocytic assay and mixed gently. All reactions and washes were carried out in the dark.

Oxidative burst flow cytometry OPA

- The opsonophagocytic assay was carried out as previously with a negative control, stained with hydroethidine being included in every run
- Compensation was carried out using single colour bead controls. Samples being used for the measurement of oxidative burst were not used for assessment of bead phagocytosis.
- Samples were analysed by two colour flow cytometry after appropriate compensation
- Cells were considered oxidative burst positive when there was a shift in immunofluorescence above the limit for the corresponding negative control (Figure 4.6)
- The proportion of cells generating an oxidative burst was recorded

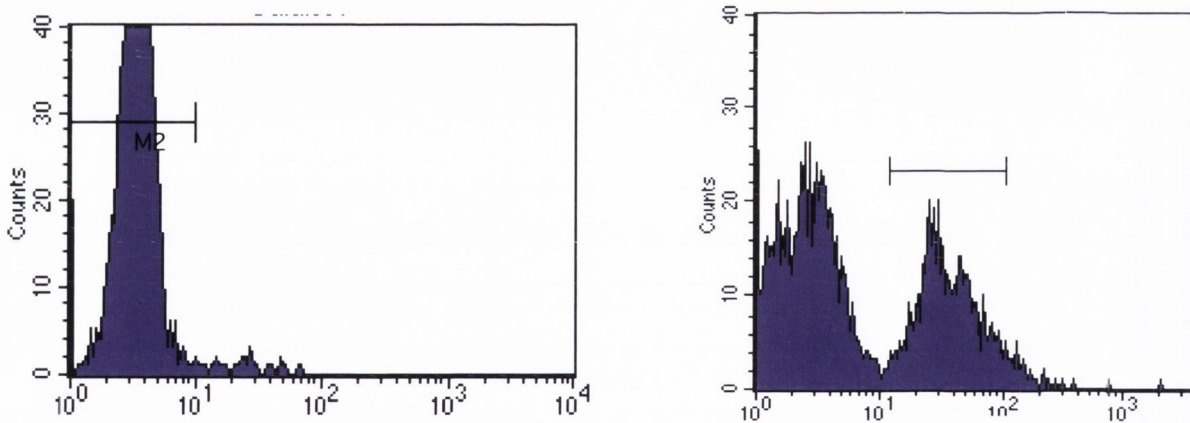


Figure 4.6. Flow cytometric oxidative burst measurement. Stimulation with polysaccharide beads in the right panel causes a right shift with increasing fluorescence caused by the oxidation of hydroethidium

4.2.8 Receptor blockade experiments

A series of experiments to assess the consequences of receptor blockade on phagocytosis were established. In these experiments monoclonal antibodies against CD16 (3G8 BD, Pharmingen, Oxford, UK), CD32 (AT10, Invitrogen, NY, USA), CD64 (10.1, BD Pharmingen, Oxford, UK) and CD11b (ICRF44 BD Pharmingen, Oxford, UK), CD18 (IB4 Merck Biosciences, Darnstadt, Germany) and CD35 (J3D3, Beckman Coulter, High Wycombe, UK) were used to saturate the cell suspension as previously described (Cotter et al., 2005). A peptide Fc receptor blocker (Innovex Biosciences, CA, USA) was also used for preincubation according to manufacturer's instructions.

- 20µl of the chosen antibody was added to the cell suspension
- 20µl of HBSS was added to negative wells to ensure volume consistency
- Preincubation with cells was carried out for 30 minutes at 4°C
- Opsonophagocytic assay and analysis was carried out as described previously

4.2.9 Toll-like receptor antagonist experiments

Experiments were carried out to determine the impact of additional TLR stimulation on phagocytosis. Cells were preincubated with PAM3CSK4; a TLR1/2 agonist, peptidoglycan (PG); a TLR 2 agonist and lipopolysaccharide (LPS); a TLR4 agonist, for 20 minutes (Invivogen, CA, USA). The OPA assay was then carried out as previously described. Several concentrations were used (data not shown) but as no significant impact was observed only maximal concentrations are reported here. Stock solutions were made up according to manufacturer's instructions and used to make final experimental concentrations of 100ng/ml for LPS, 10µg/ml peptidoglycan and PAM3CSK4 1µg/ml, in the 100µl final OPA solution.

4.2.10 Assessment of neutrophil activation by flow cytometry

Neutrophil activation markers were measured in phagocytosing cells by two colour flow cytometry. Anti-CD11b, CD35 and CD62L PE labeled antibodies were obtained from BDBiosciences (BDBiosciences, Oxford, UK) (Table 4.4). Staining was carried out after the OPA assay was completed.

- Selected samples were removed from the 96 well opsonophagocytic assay plate
- Samples were placed in Falcon tubes
- Negative controls were included each run. The negative control contained serum and complement but beads were substituted with OPA buffer.
- 5µl of fluorescent monoclonal antibody was added to chosen samples
- Staining was carried out at room temperature in the dark for 20 minutes
- Samples were washed twice in cold HBSS and fixed with BD Cellfix
- Samples were analysed by flow cytometry after appropriate compensation using negative and single colour controls (BDBiosciences, Oxford, UK)(Figures 4.7-4.9). Appropriate compensation was carried out with each experimental run.
- For analysis purposes experimental results were expressed as the fold change in mean fluorescence intensity when compared with the experimental negative control. This serves as an internal correction for experimental variation.

Antibody	Fluorochrome	Clone	Catalogue no.
Anti-human CD11b	PE	ICRF44	BD, Pharmingen 555388
Anti-human CD35	PE	E11	BD, Pharmingen 559872
Anti-human CD62L	PE	Dreg/56	BD, Pharmingen 555544

Table 4.4 showing antibodies used for the detection of neutrophil activation following stimulation with serotype 14 polysaccharide coated beads

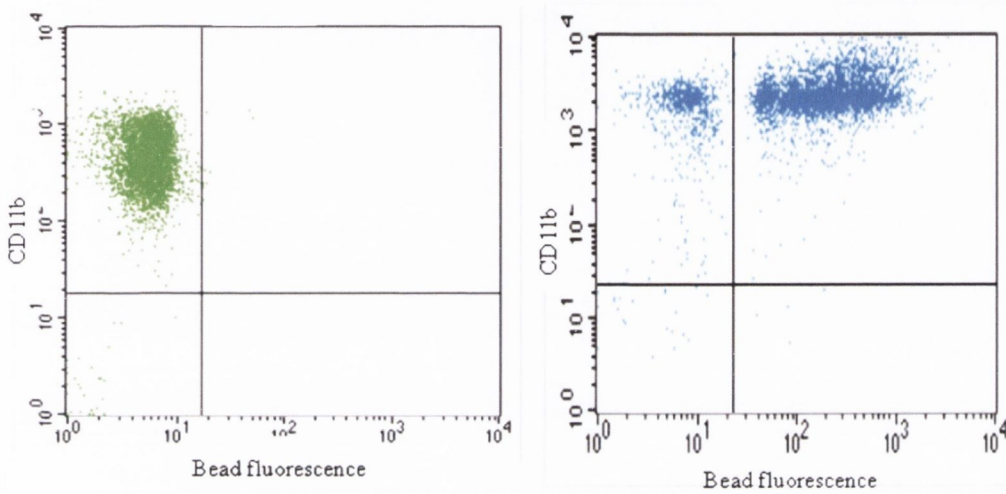


Figure 4.7 showing CD11b staining in control (left) and serotype 14 bead (right) stimulated neutrophils after 1 hour incubation. Expression of CD11b was increased with bead stimulation compared with the negative control. CD11b expression was higher in neutrophils that had phagocytosed beads than in those that had not phagocytosed neutrophils.

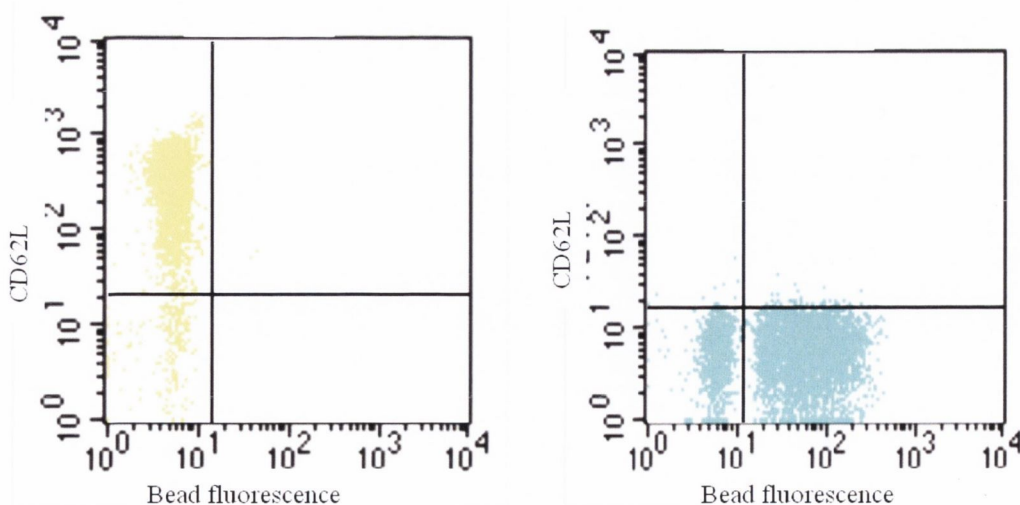


Figure 4.8 showing CD62L staining in control (left) and serotype 14 bead (right) stimulated neutrophils. CD62L expression is completely abrogated in the activated neutrophil sample with no difference observed between phagocytosing and non-phagocytosing cells.

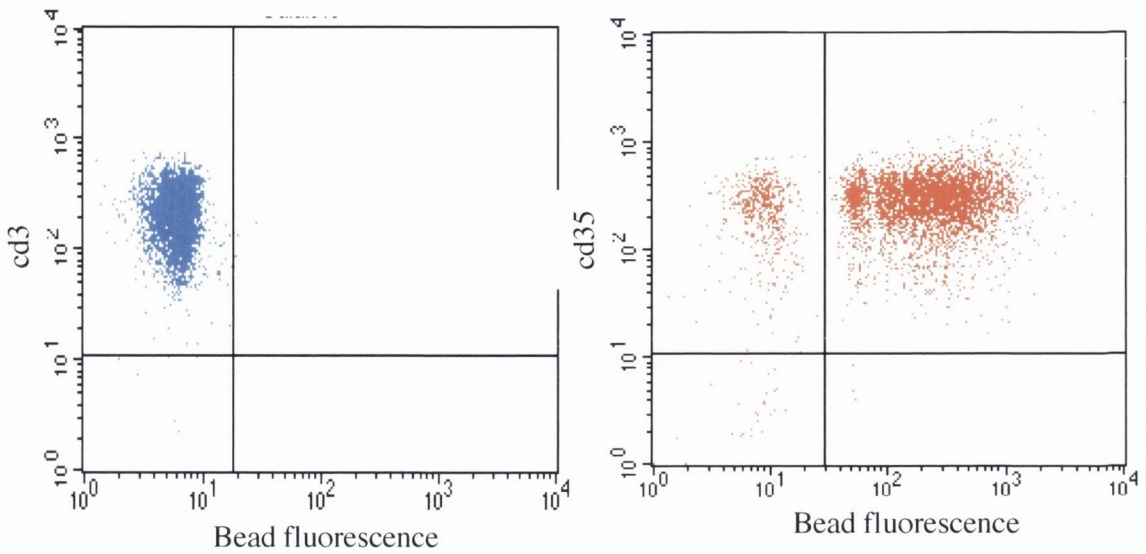


Figure 4.9 showing CD35 staining in unstimulated control and serotype 14 bead stimulated neutrophils after 1 hour incubation. CD35 expression is slightly increased when compared with the negative control.

4.2.11 Assessment of neutrophil activation by ELISA

Aliquoted supernatants from OPA sample mixes were returned to room temperature by slowly thawing. Measurements of elastase (Hycult Biotech, Netherlands), myeloperoxidase, matrix-metalloproteinase 9, CD62L, TNF- α , IL1- β and IL-8 (R&D Systems, UK) were made by ELISA according to manufacturer's instructions.

4.2.12 Bacterial opsonophagocytic assay

To complement information obtained from the bead based phagocytosis assay, a bacterial phagocytosis assay was established. This assay was adapted from that established by Jansen *et al* (Jansen *et al.*, 1998).

Growth, heat killing and staining of bacteria

- Serotype 14 bacteria (kind gift of Dr. B. O Connell, Department of Microbiology, St. James's Hospital, Dublin) were grown overnight on blood agar plates at 36°C in 5% CO₂ atmosphere.
- 3mls of Todd-Hewitt Broth with 0.5% yeast extract and 5% heat inactivated human pooled serum was inoculated with the bacteria (OD₆₆₀ of 0.05-0.08)
- Bacteria were grown to log phase three times
- Bacteria from the third culture was washed with PBS (1100 rcf, 15 minutes 4°C) and resuspended in PBS to an OD₆₆₀ of 1.0
- The bacterial suspension was incubated at 60°C for 1hr
- The suspension of killed bacteria was washed with PBS and resuspended at an OD₆₆₀ of 1.0
- Serotype 14 bacteria were labelled with a 0.5mg/ml solution of fluorescein isothiocyanate (FITC, Sigma Aldrich, UK) in PBS for 1hr at 4°C.
- Labelled bacteria were then washed twice in HBSS (with calcium and magnesium) and resuspended at an OD₆₆₀ of 1.0
- Bacteria were stored in 100ul aliquots containing 10⁸ bacteria approximately
- Aliquots were protected from light and stored at -80°C until use
- Just prior to use, bacterial aliquots were returned to room temperature and made up to 1ml in HBSS (with calcium and magnesium, without phenol red), giving a concentration of 10⁸ cells/ml.

The opsonophagocytosis assay was carried out as described previously with the substitution of 20µl of a 1/10 dilution of serotype 14 bacteria in OPA buffer. Other stages and parameters were kept identical, maintaining the cell:target ratio at 1:4.

Analysis was carried out on the BD FACSCalibur flow cytometer as described previously following appropriate compensation with single colour controls. Compensation controls were included with each experimental run.

Notably the discrete peak pattern observed with the beads was not as prominent with bacteria, probably due to variable stain uptake by the bacteria resulting in an indistinct fluorescence spread (Figure 4.10).

Time course experiments (n=3) carried out with CD32A heterozygote neutrophils suggested that phagocytic capacity over 2 hours was greater with labelled bacteria than with beads (Figure 4.11). Despite this, assays at the 1 hour time point were used. There was greater intra and inter assay variability in the bacterial assay than in the bead assay (Figure 4.12). The intra-assay mean coefficient of variation for the bacterial assay was 11.4%

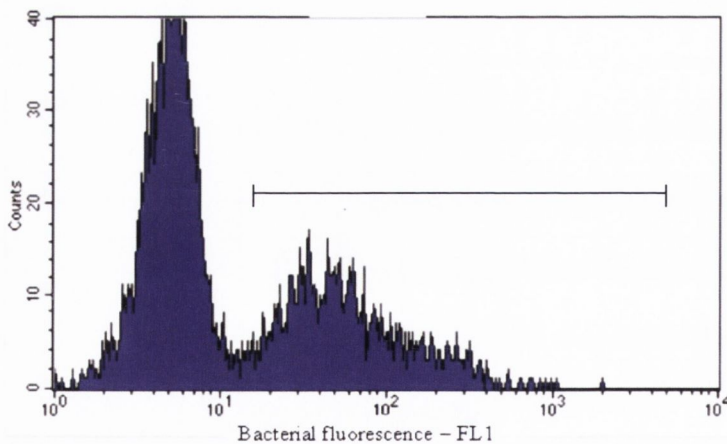


Figure 4.10 showing phagocytosis of FITC labelled heat killed serotype 14 bacteria. The gated area represents bacteria that have been taken up by neutrophils.

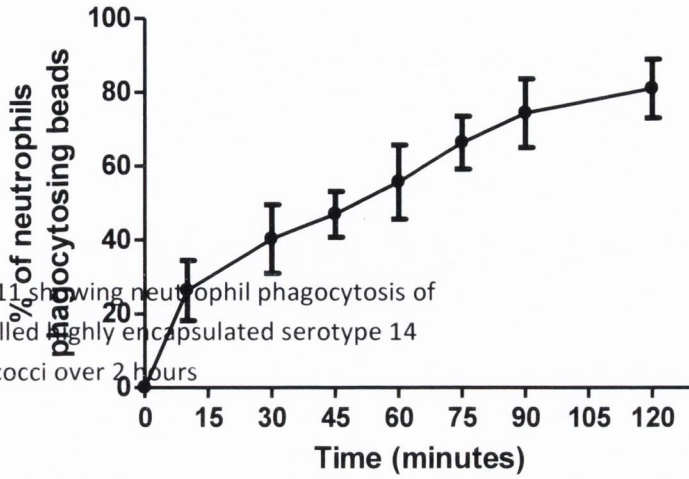


Figure 4.11 showing neutrophil phagocytosis of FITC labelled highly encapsulated serotype 14 pneumococci over 2 hours

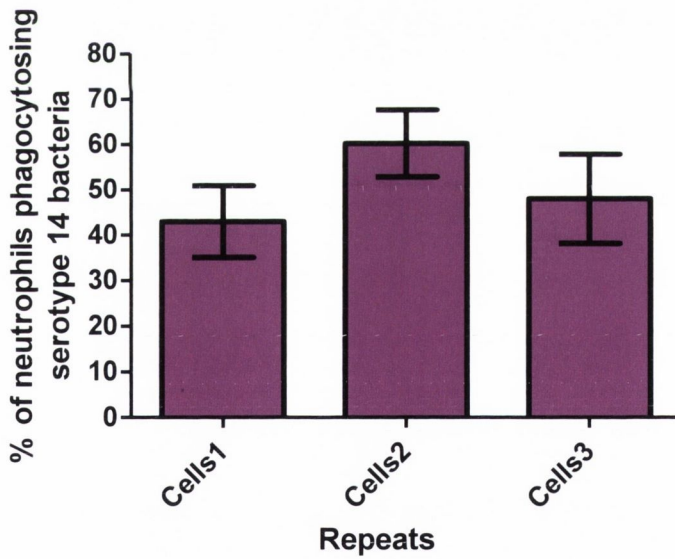


Figure 4.12 showing reproducibility of bacterial phagocytosis assay. This assay showed more intra and inter assay variability than the bead based assay (n=3 experiments).

4.2.13 Analysis

Data was presented using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and was analysed using Graph Pad Prism or SPSS (Version 20, IBM, CA, USA). Standard error bars are presented unless otherwise specified. Continuous variables were analysed using one way ANOVA/Tukey's multiple comparison test. Non parametric data was analysed by U testing or Kruskal-Wallis for multiple comparisons. $\alpha=0.05$ for all comparisons.

4.3 Results

4.3.1 Assessment of the relative importance of components of the opsonising mix for phagocytosis of serotype 9v beads

A series of experiments were carried out to identify the relative importance of opsonising complement and serum for efficient phagocytosis. As expected, cells exposed to polysaccharide coated beads that were not pre-incubated with an opsonising mix phagocytosed small numbers of targets only (Figure 4.13a). When beads were pre-incubated with baby rabbit complement (RC), processed to contain no antibodies, phagocytosis was again negligible. Efficient phagocytosis was observed both when cells were pre-incubated with the pooled high titre serum (PHTS) and when this serum was supplemented with additional rabbit complement (Figure 4.13). Further analysis of the PHTS sample in a complement haemolysis assay indicated residual complement activity. To assess the importance of antibody in isolation a PHTS aliquot was incubated at 56°C for 30 minutes precisely. Abolition of complement function was confirmed by complement haemolysis assay.

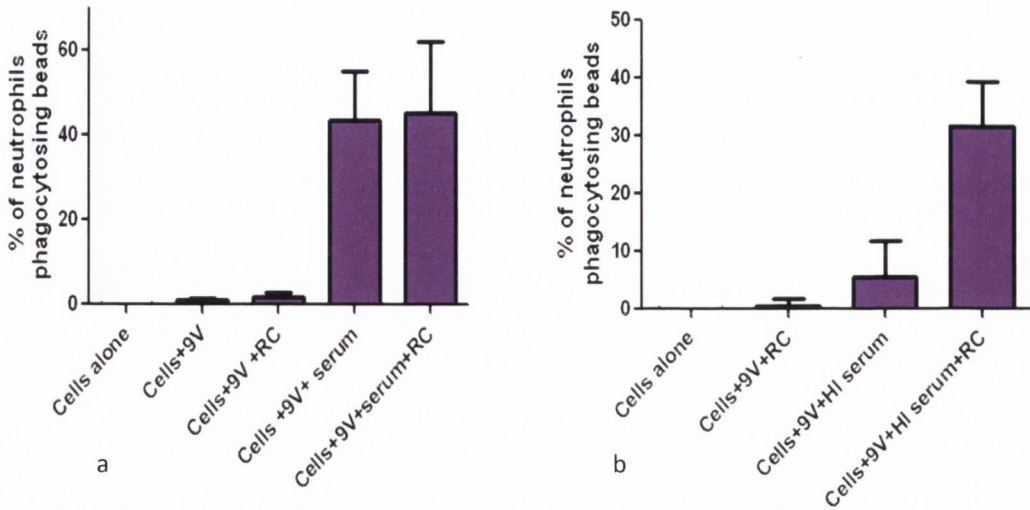


Figure 4.13a showing phagocytosis of serotype 9v coated polysaccharide beads under different opsonising conditions. The serum used is PHTS. Phagocytosis is inefficient in the presence of rabbit complement alone (n=3). Figure 4.13b showing phagocytosis of serotype 9v coated polysaccharide beads with heat inactivated PHTS as an opsonising agent. Phagocytosis is minimal in the presence of complement alone and is present at low levels in the presence of antibody alone (n=3)

When beads were pre-incubated with heat inactivated PHTS some phagocytosis was observed, although this was at reduced levels. When heat inactivated serum was supplemented with baby rabbit complement at a 1:4 dilution, efficient phagocytosis was observed at levels slightly lower than with non heat activated serum (Figure 4.13b).

It can be concluded from this experiment that antibody and complement together are important for efficient phagocytosis of beads in this assay system.

4.3.2 Efficient phagocytosis demands the presence of antibody and complement

To further explore these issues we used a preparation of intravenous immunoglobulin G. Such preparations contain varying levels of antipneumococcal antibodies as part of a polyclonal IgG preparation. IVIG does not contain complement and has absent or very low levels of other antibody isotypes. Using IVIG excludes the possibility of residual complement fragments contributing to phagocytosis via complement receptors. Pre-incubation of serotype 9v beads with IVIG alone results in low level phagocytosis of a magnitude not significantly different to heat inactivated serum (Figure 4.14). Supplementation of IVIG with baby rabbit complement resulted in a marked increase in phagocytosis. To further expand on these issues checkerboard dilution was carried out over a range of dilutions of baby rabbit complement and IVIG pre-incubation mixes (Figure 4.15). These results demonstrate that both antibody

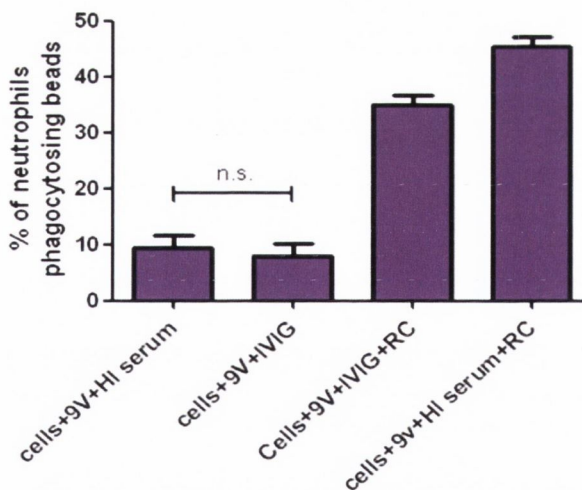


Figure 4.14 showing neutrophil phagocytosis of serotype 9V beads following preincubation with HI PHTS or IVIG +/- baby rabbit complement (RC). Antibody alone, in the form of IVIG or heat inactivated serum produces low but detectable levels of phagocytosis. This is enhanced 3-4 fold by supplementation with 1:4 RC

and complement are necessary for efficient phagocytosis of serotype 9v pneumococcal polysaccharide coated beads. Notably, however, low level phagocytosis is consistently detected in the absence of complement. In contrast, complement alone is not capable of supporting phagocytosis in the absence of antibody (Figure 4.13 a and b, Figure 4.15).

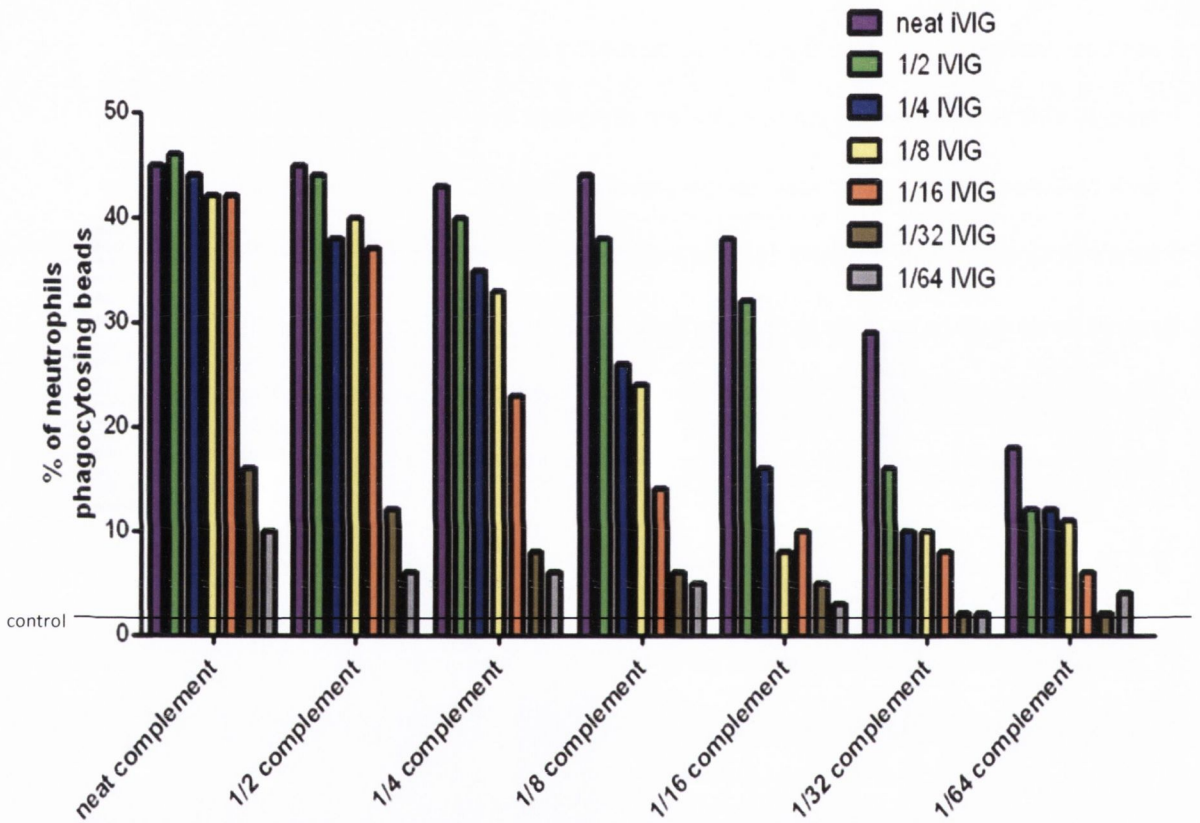


Figure 4.15 showing the percentage of neutrophils phagocytosing serotype 9V beads across a range of dilutions of IVIG and complement in the pre-incubation opsonising mix. Undiluted IVIG can still support measurable phagocytosis at low complement concentrations. The background phagocytosis in control samples is indicated

From Figure 4.15 it may be deduced that a 1:16 dilution of IVIG is able to support phagocytosis and this level of phagocytosis begins to drop off (especially at higher dilutions) as complement is diluted beyond 1:2.

To determine if the findings for serotype 9v coated beads were generalisable to other serotypes phagocytosis was examined across a range of serotypes. The serotypes examined corresponded to those available in the Prevenar conjugate vaccine. Findings regarding different opsonising conditions were broadly in agreement over the different pneumococcal serotypes (Figure 4.16). However, serotypes 14 and 9v in particular, demonstrated reproducible phagocytosis > 10% in the absence of functional complement. This is of importance as the main focus of experimentation is to determine phagocytosis occurring via FcγR in general and CD32A in particular.

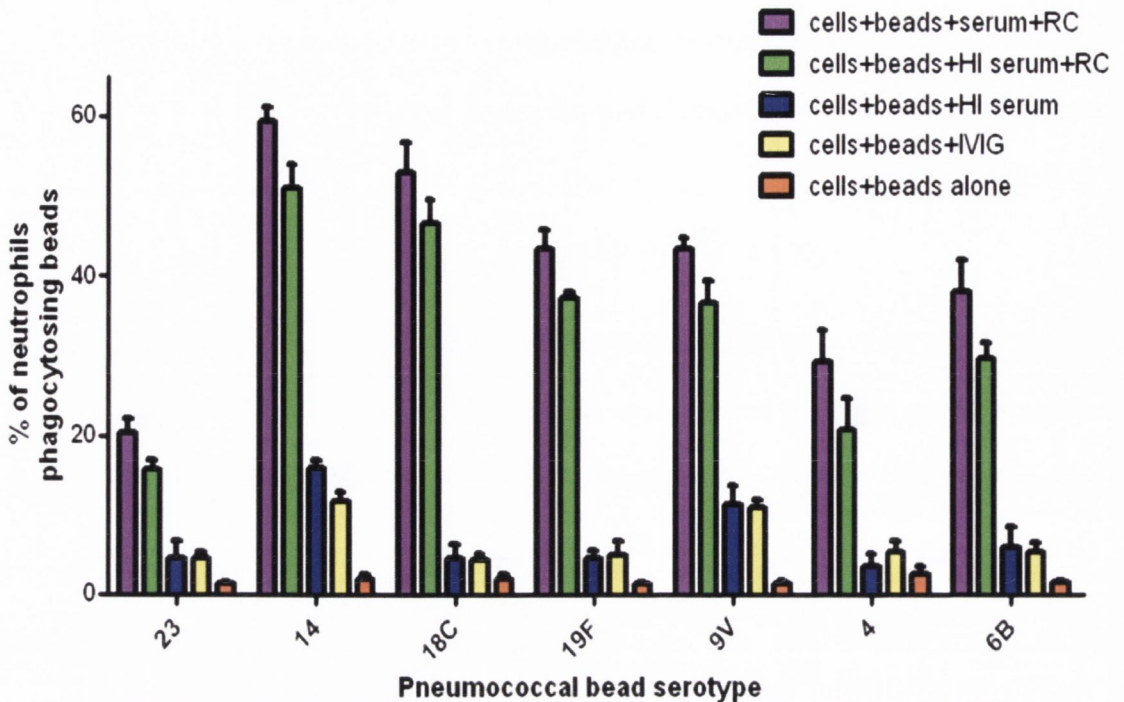


Figure 4.16 showing phagocytosis of a variety of beads coated in different pneumococcal polysaccharides in differing opsonising mixes. Serotype 14 and serotype 9V beads demonstrate the highest phagocytosis in heat inactivated serum at the one hour time point.

Other investigators using serotype 14 bacteria as targets found phagocytosis to be complement independent (Rodriguez et al., 1999). These authors used a 30:1 target to cell ratio and incubated the cells with opsonised beads at 4°C. To determine whether such changes in our experimental conditions could increase complement independent phagocytosis we incubated serotype 9v beads with neutrophils at a series of higher target: cell ratios and at low temperatures. Results showed that in this assay system a reduction in temperature did not facilitate an increase in phagocytosis (Figure 4.17a). Increasing the ratio of bead targets to cells did produce a moderate increase in phagocytosis in heat inactivated serum (Figure 4.17b). However, the observed increase in phagocytosis was not large enough to warrant the quantity of beads that would be required to use a high target:cell ratio for routine assay use.

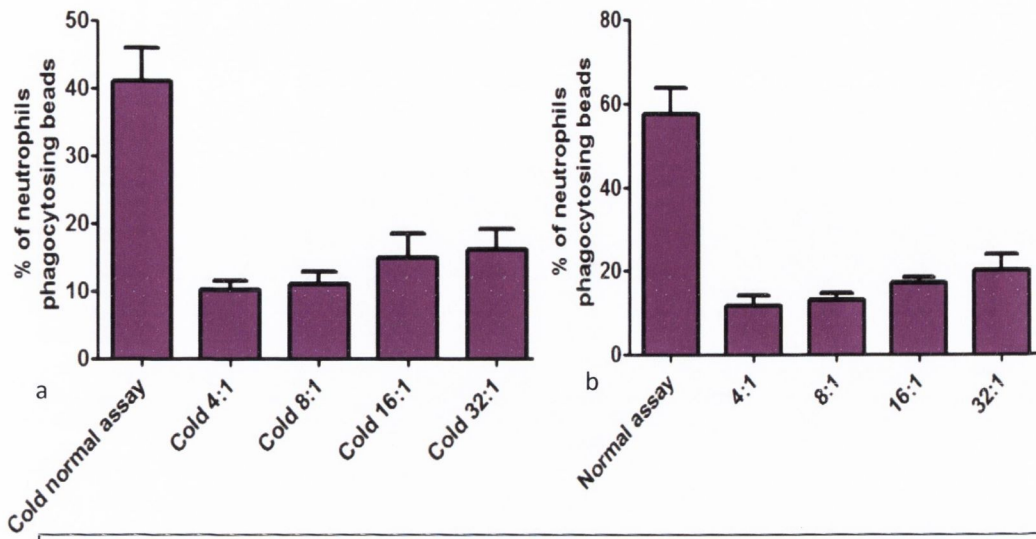


Figure 4.17a showing neutrophil phagocytosis of serotype 14 pneumococcal polysaccharide coated beads at a variety of target:cell ratios with 4°C preincubation in the opsonising mix. The normal assay contains complement while all other assays used HI PHTS. The cold temperature causes a slight reduction in phagocytosis compared to the standard 37°C incubation shown in Figure 4.17b (note different scales).

These experiments suggest that using serotype 14 beads could potentially be used to measure Fc receptor function. The data suggests that a reproducible amount of complement independent phagocytosis is observed, and that phagocytosis in the presence of complement is consistently more efficient.

4.3.3 CD32 plays a partial role in the phagocytosis of serotype 14 pneumococcal polysaccharide coated beads

To determine that CD32a is playing a role in the phagocytosis of pneumococcal polysaccharide coated beads blocking experiments were carried out. Isolated neutrophils were preincubated with monoclonal antibodies directed against the Fc gamma receptors CD16, CD32 and CD64. The opsonophagocytic assay was then carried out as normal, and phagocytosis subsequently assessed (Figure 4.18a). Results from this small experimental series (n=3) suggest that preincubation with Anti-CD32 or a combination of anti-CD16/32/64 monoclonal antibodies reduces the amount of phagocytosis by 25 – 30%.

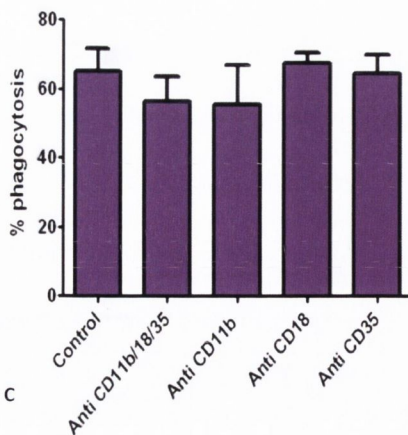
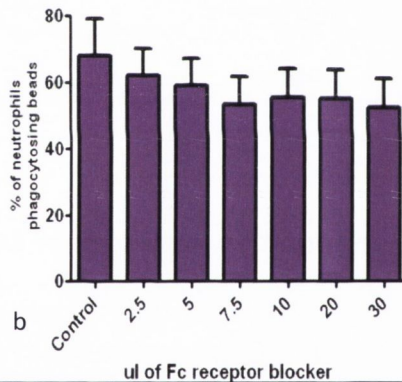
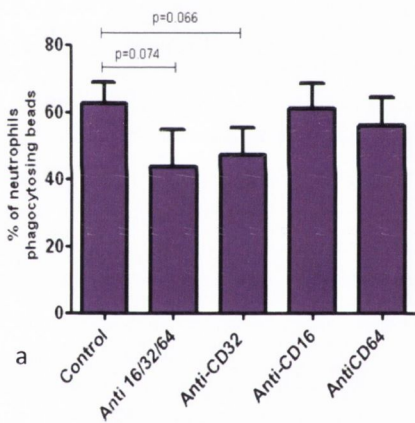


Figure 4.18a showing phagocytosis of serotype 14 beads following neutrophil pre-incubation with a combination of monoclonal antibodies against Fc receptors (n=3). Pre-incubation with anti-CD32 results in a reduction in phagocytosis which approaches statistical significance (p=0.066) as does pre-incubation with an anti-CD16/32/64 mix (p=0.074)(unpaired t test). Figure 4.18b shows neutrophil phagocytosis of serotype 14 pneumococcal polysaccharide beads following pre-incubation with Fc receptor blocker (n=3). The peptide receptor blockade produces a reduction in phagocytosis comparable to anti-CD32 monoclonal antibody. Figure 4.18c shows the result of three experiments using blocking antibodies to complement receptor 1 (CD11b and CD18) and complement receptor 3 (CD35). A small reduction in phagocytosis was observed with antibodies against CD11b and with the antibody mixture. However, results were variable and did not approach statistical significance

This effect did not reach statistical significance in this sample although there was a trend towards significance. In an attempt to reinforce the results of these experiments, neutrophils were preincubated with a peptide Fc receptor blocker (Innovex Biosciences, Richmond Ca, USA) (Figure 4.18b). This produced similar magnitude of blockade but, again, in the small sample (n=3) did not reach statistical significance. In a similar crude fashion blockade of complement receptors with monoclonal antibodies was carried out with assessment of phagocytosis after neutrophil preincubation (Figure 4.18c). Preincubation of neutrophils with anti-CD11b directed against a component of complement receptor 1 produced a small reduction in the magnitude of phagocytosis, however, this did not reach statistical significance. The antibody mix appeared to produce a similar reduction in phagocytosis of serotype 14 beads. The variability in results and the small number of experiments mean it is difficult to draw firm conclusions from this data. However, we can note that blockade of anti-CD32A reduces phagocytosis by 10-20%, this is the same amount of phagocytosis that occurs reproducibly in the absence of complement.

These results suggest that CD32A plays a role in mediating phagocytosis in the presence of complement. However, other factors such as complement receptors, cellular activation or receptor cross-linking are playing an additional role in the presence of complement.

4.3.4 Addition of Toll-like receptor agonists to serotype 14 pneumococcal polysaccharide coated beads does not increase phagocytosis in this model.

The polysaccharide coated beads based used in these assays are clearly different from bacteria. In particular, the beads lack many other virulence factors present in the bacterial surface structure. Experiments were carried out to determine if supplementation of the bead suspension with a selection of relevant toll-like receptor agonists would increase phagocytosis in standard or heat inactivated serum. Agonists used were PAM3CSK4; a TLR1/2 agonist, peptidoglycan (PG); a TLR 2 agonist and lipopolysaccharide (LPS); a TLR4 agonist. No significant effect on the phagocytosis of serotype 14 pneumococcal polysaccharide coated beads was noted with any of the TLR agonists in either the complement sufficient or deficient opsonising mix (Figure 4.19). Furthermore no significant difference was noted with a number of different concentrations of LPS across a range of dilutions of PHTS in the standard assay (not shown).

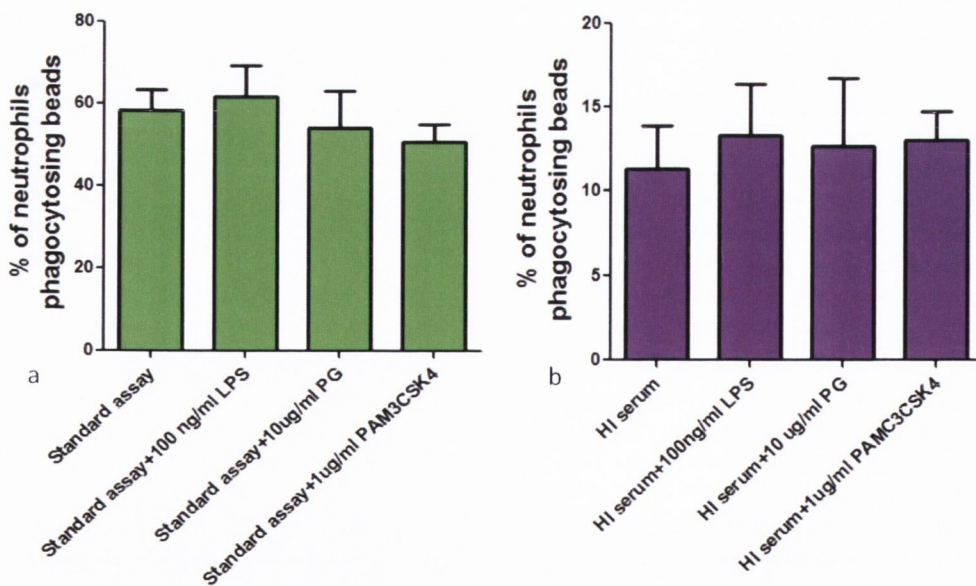


Figure 4.19 showing phagocytosis of serotype 14 beads following co-cubation with a variety of TLR agonists. No significant difference in the magnitude of phagocytosis was observed in the standard assay (Figure 4.19a) or with HI PHTS (Figure 4.19b) (n=3)

4.3.5 Efficient phagocytosis of heat killed highly encapsulated serotype 14 bacteria requires the presence of antibody and complement

To have a more biologically relevant comparison group for the bead based opsonophagocytosis assay, we established a similar experimental system using highly encapsulated heat killed bacteria. We carried out preliminary experiments similar to those in the bead based assay.

Further experiments indicated the dependence of serotype 14 bacterial phagocytosis on the presence of both antibody and complement (Figure 4.20). Minimal bacterial phagocytosis occurs in the presence of complement alone. In complement deplete serum, heat inactivated or purified IVIG, a small amount of reproducible phagocytosis (about 15%) occurs. This phagocytosis is increased by the supplementation of the opsonising mix with a 1:4 dilution of baby rabbit complement.

For practical and experimental purposes it was decided to carry out all further bacterial opsonophagocytic assays using a 1hr time point and with a 1:4 dilution of supplemental baby rabbit complement as standard.

4.3.6 Summary

These results taken as a whole suggest a robust assay for assessing phagocytosis of beads and whole bacteria. Therefore this assay was taken forward to examine possible differences in Fc receptor function in the various CD32A genotypic group.

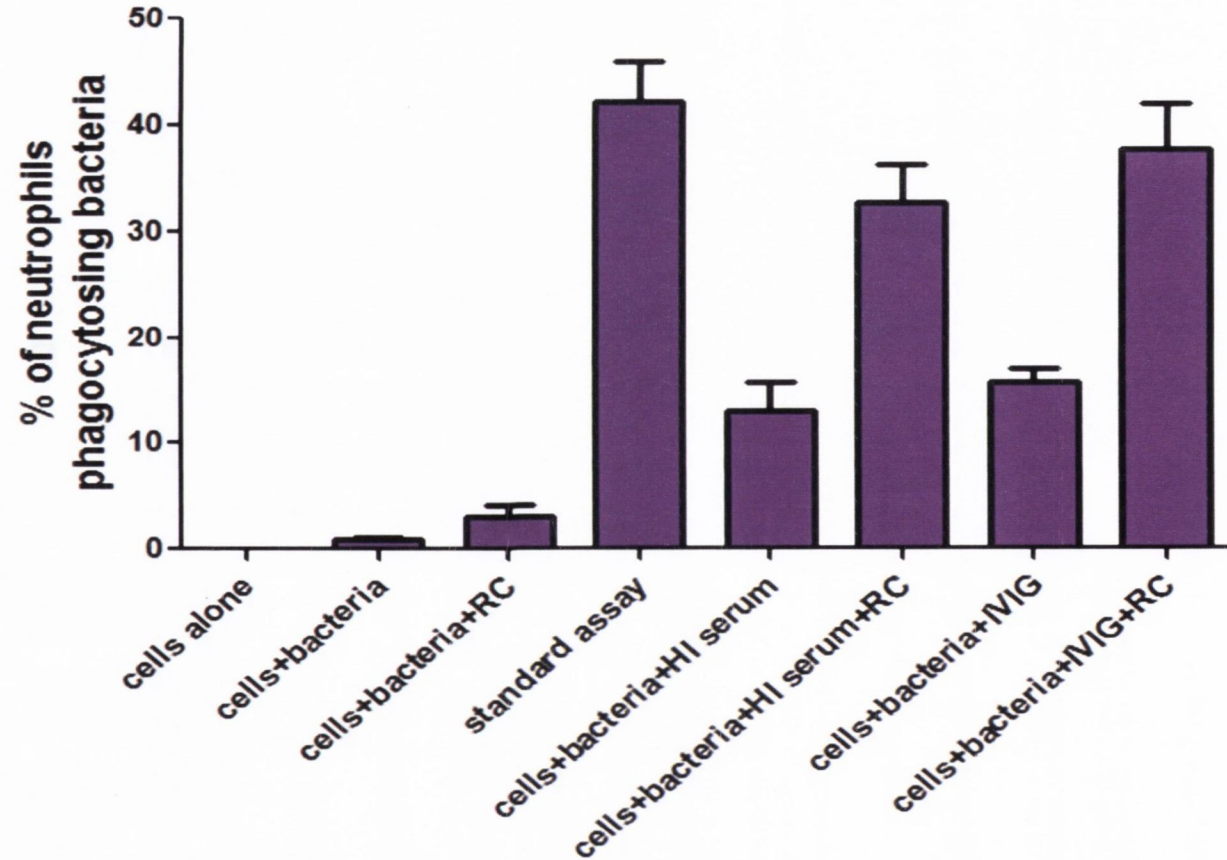


Figure 4.20 showing neutrophil phagocytosis of serotype 14 bacteria under a range of different pre-opsonising mixes (n=3). In a similar fashion to the bead assay, efficient phagocytosis requires antibody and complement. In complement deplete opsonising mixes (HI serum or IVIG) reproducibly measurable low level phagocytosis is observed. Supplementation of a ¼ dilution of rabbit complement in these assays increases phagocytosis.

4.3.7 CD32A genotype influences the magnitude and efficiency of neutrophil phagocytosis of serotype 14 pneumococcal polysaccharide coated beads

To investigate a possible effect on the phagocytosis of serotype 14 beads by variation in CD32A genotype, neutrophils were isolated from healthy individuals of known CD32A genotype. These neutrophils were exposed to serotype 14 bacteria in the standard opsonophagocytic assay and relevant measurements made on flow cytometry. Figure 4.21 shows the proportion of neutrophils of different CD32A genotype that phagocytose serotype 14 beads in neat pooled high titre standard serum (PHTS). A typical overlaid flow plot is shown in Figure 4.22. Significantly less of the CD32A RR131 bearing neutrophils phagocytose serotype 14 beads than neutrophils from heterozygote or H homozygote donors ($p < 0.002$). The CD32A H131R group also exhibited less phagocytosis than the HH131 group ($p < 0.001$).

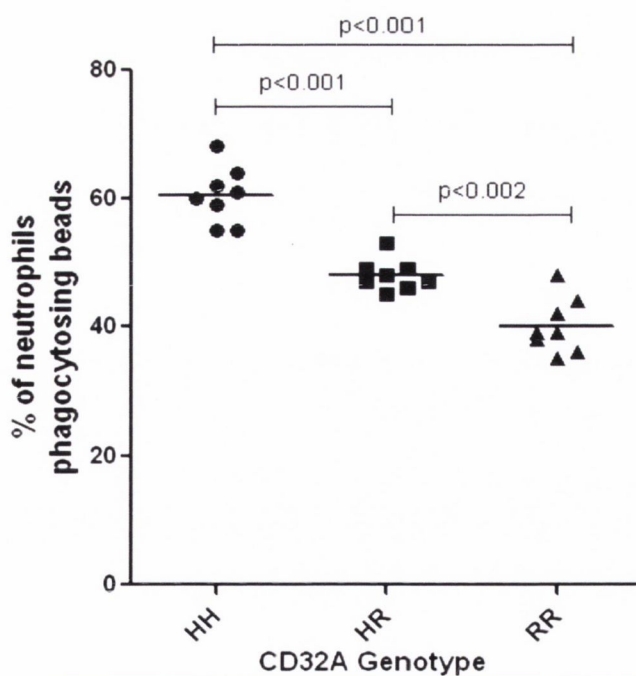


Figure 4.21 showing the percentage of neutrophils of known CD32A genotype that phagocytose serotype 14 beads. RR131 variants phagocytose significantly fewer beads. (ANOVA, Tukey $\alpha = 0.05$)

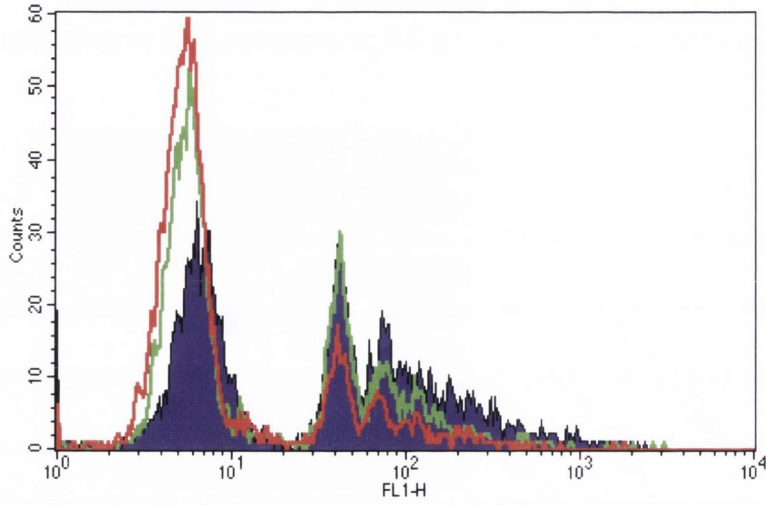


Figure 4.22 showing an overlay flow plot of bead phagocytosis. Solid purple HH131 neutrophils have phagocytosed more beads than HR131 (green line) and RR131 (red line) neutrophils.

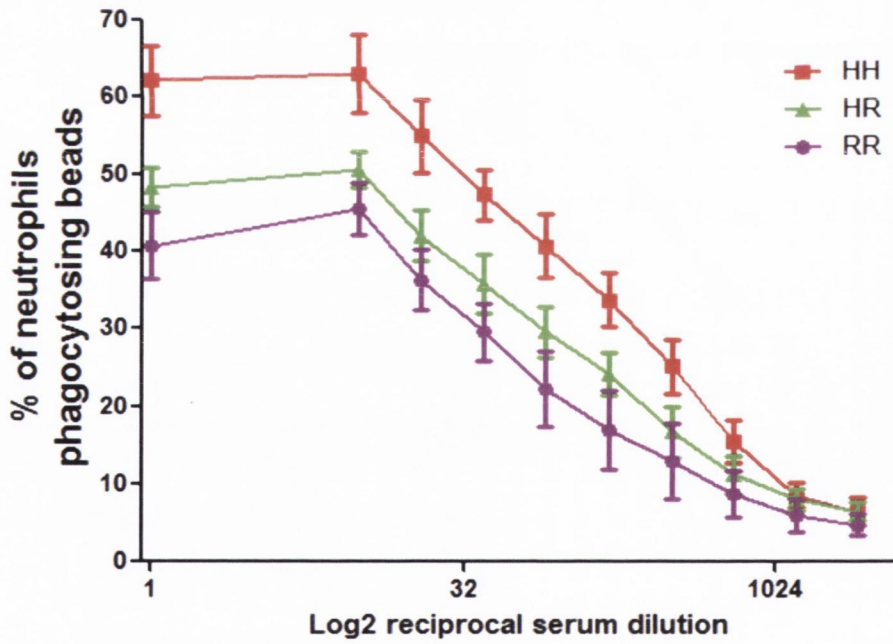


Figure 4.23 showing neutrophil phagocytosis of serotype 14 beads over a range of dilutions of pooled high titre serum.

These genotype specific differences in phagocytosis are maintained across a range of serum dilutions, with curves coinciding as antibody concentrations fall (Figure 4.23). This demonstrates that the phagocytosis of beads is dependent on antibody concentration and at each concentration tested the RR variant shows significantly less phagocytosis. In addition neutrophils have the capacity to phagocytose multiple beads, this may be also be a marker of the efficiency of phagocytosis. Data was interrogated to establish if CD32A RR131 genotype also impacted on the proportion of neutrophils that phagocytose multiple beads. Results indicated that a mean of 48% of CD32A R131 bearing neutrophils ingested two or more beads, significantly less than other genotype variants (Figure 4.24). Again, in this parameter the HH131 bearing cells demonstrated superiority with a mean of 65% phagocytosing multiple beads.

It has therefore been demonstrated that in this assay system that RR131 cells exhibit a reduced phagocytic capacity and lower phagocytic efficiency.

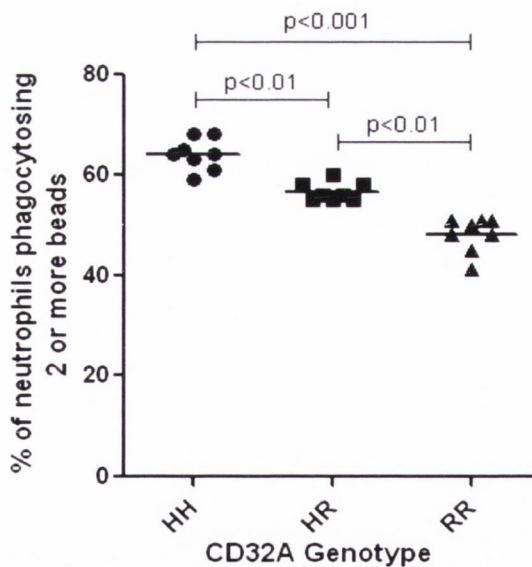


Figure 4.24 showing the percentage of neutrophils of known genotype that phagocytose 2 or more serotype 14 beads.

4.3.8 CD32A genotype influences the neutrophil phagocytosis of highly encapsulated heat killed serotype 14 pneumococci

FITC-labeled highly encapsulated heat killed serotype 14 bacteria were used to assess whether results from the bead based assay would also apply to whole cell pneumococci. In undiluted serum CD32A genotype was found to be an important influencing factor on the proportion of neutrophils phagocytosing pneumococci over 1 hour (Figure 4.25 and 4.26). This assay displayed more variability than the bead based opsonophagocytic assay. Despite this, the percentage of CD32A RR131 bearing neutrophils that phagocytosed pneumococci was significantly less than other genotypes. In contrast to the bead based assay, no significant difference between HH131 and heterozygote groups was observed. However, there was a trend towards significance with heterozygote neutrophils producing intermediate levels of phagocytosis. Larger numbers would be required to confirm this trend.

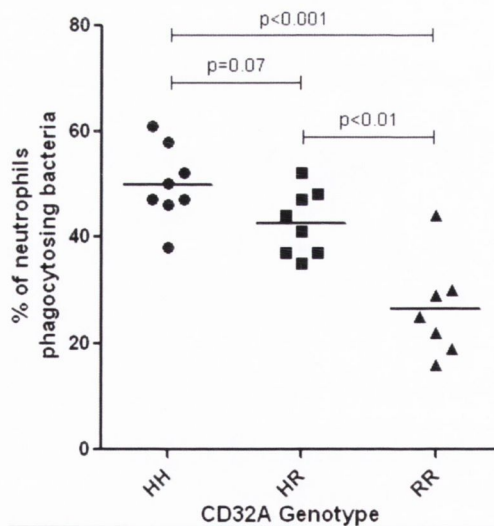


Figure 4.25 showing the percentage of neutrophils that phagocytose serotype 14 pneumococci. Fewer CD32A RR131 variants phagocytose pneumococci than neutrophils bearing other receptors.

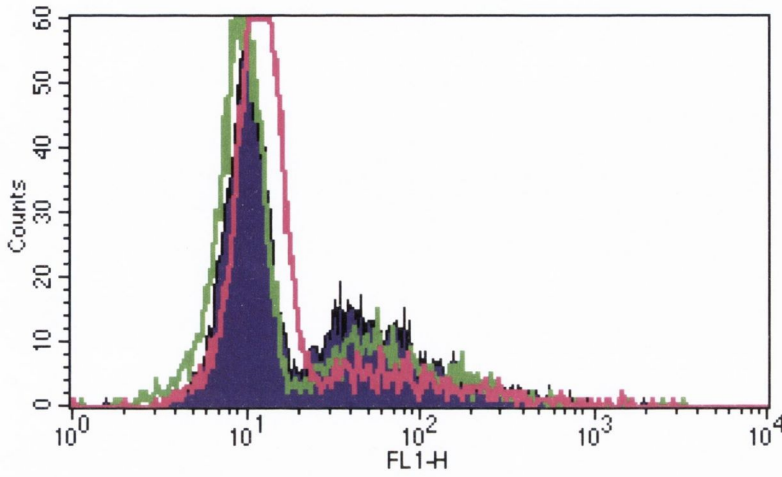


Figure 4.26 typical overlay flow plot showing neutrophil phagocytosis of serotype 14 bacteria. Proportionately more HH131 (solid purple) neutrophils phagocytose bacteria than HR131 (green line) or RR131 (red line)

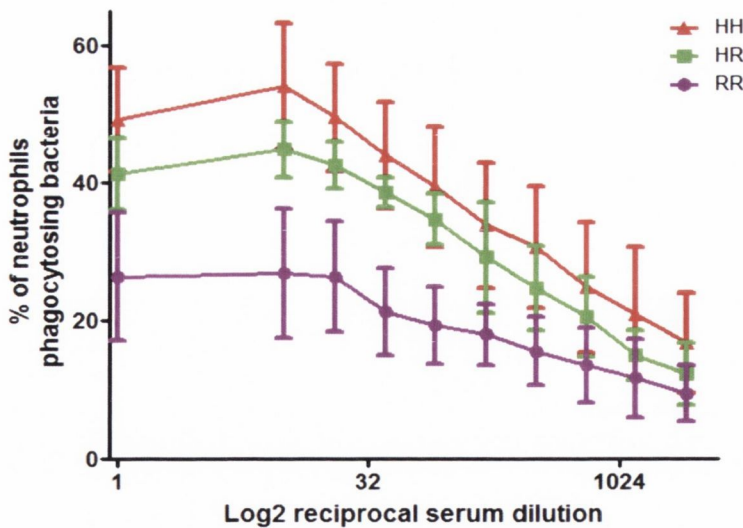


Figure 4.27 showing the percentage of neutrophils of known CD32A genotype phagocytosing serotype 14 bacteria across a range of dilutions of the pooled high titre antipneumococcal serum.

Similarly, across a range of serum dilutions CD32A RR131 neutrophils exhibit reduced phagocytosis (Figure 4.27). There is no significant difference in the proportion of HH131 and heterozygote neutrophils that phagocytose bacteria across a range of dilutions with both demonstrating superiority over neutrophils bearing RR131 variants. At dilute antibody concentrations there is convergence of error bars and results, however, the overall trend is maintained.

These experiments confirm the previous data generated with the bead based phagocytosis assay and clearly demonstrate the significantly reduced phagocytic capacity of the RR variant CD32A Fc receptor. However, as this assay is quite complement dependent we examined whether Fc receptor variation affects phagocytosis in the absence of complement.

4.3.9 CD32A variation affects phagocytosis of serotype 14 beads and bacteria in the absence of complement

Serotype 14 beads and bacteria were pre-incubated with heat inactivated serum to examine if variation in CD32A affects phagocytosis in the absence of functioning complement. In the presence of heat inactivated high titre antipneumococcal serum, RR131 variant neutrophils demonstrated inferior phagocytosis of serotype 14 beads ($p < 0.001$) (Figure 4.28a). Results are similar to previous experiments showing approximately half the number of RR131 bearing neutrophils phagocytosed beads when compared with HH131 bearing cells. Heterozygote neutrophils are intermediate and significantly inferior to HH131 variants ($p < 0.05$). The pattern repeats for the bacterial assay (Figure 4.28b).

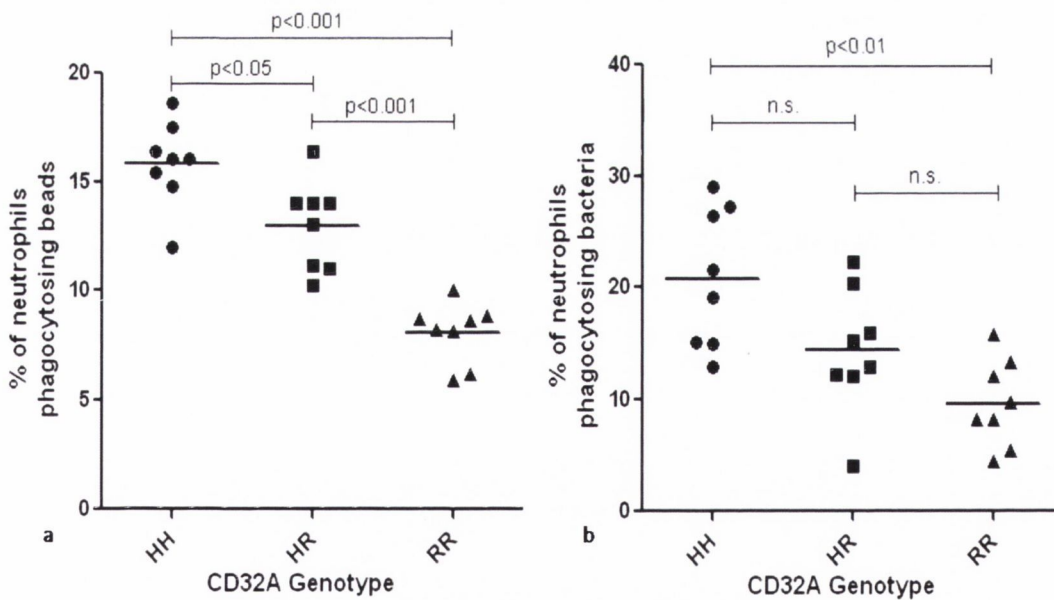


Figure 4.28a showing the percentage of neutrophils that phagocytose serotype 14 beads in heat inactivated serum (no complement). Figure 4.28b showing the percentage of neutrophils that phagocytose serotype 14 bacteria in heat inactivated serum

4.3.10 Assessment of neutrophil activation

CD11b, CD62L and CD35 are cell surface markers of neutrophil activation (Berger et al., 1989, Neufert et al., 2001, van Eeden et al., 1999). Previous studies have demonstrated that stimulation of neutrophils by phagocytic targets results in rapid changes in surface expression of these molecules (Neufert et al., 2001, Mann and Chung, 2006). CD11b, combined with the common $\beta 2$ integrin chain CD18, forms CR3 a receptor for complement and an important leukocyte adhesion molecule (von Andrian et al., 1991). CD11b is found on the cell surface of neutrophils. On activation surface expression is upregulated, following mobilization of cytoplasmic stores (von Andrian et al., 1991). CD35 is another complement receptor CR1, similarly upregulated on neutrophil activation (Berger et al., 1984). In contrast levels of CD62L, L-selectin, fall on neutrophil activation due to surface shedding of the molecule (McEver et al., 1995). Neutrophils of known CD32A genotype were stimulated with serotype 14 beads preopsonised with high titre anti-pneumococcal serum and complement, for 1 hour. Surface expression of these neutrophil activation markers was assessed by flow cytometry.

4.3.11 CD32A genotype does not influence expression of surface markers of neutrophil activation after 1 hour stimulation with serotype 14 beads

Expression of surface markers behaved as expected on stimulation. Cells phagocytosing serotype 14 pneumococcal polysaccharide beads increased expression of CD11b and CD35 when compared with controls containing complement and serum. Figure 4.29 shows the increase in mean fluorescence intensity (MFI) of CD11b on phagocytosing neutrophils of different CD32A genotypes (when incubated with serotype 14 beads) relative to serum/complement controls. No significant difference in the magnitude of CD11b up-regulation was detected. A statistically significant genotype dependent difference in CD35 upregulation was detected (Figure 4.30).

CD32A RR131 bearing phagocytosing neutrophils express significantly more CD35 after bead incubation than HH131 neutrophils ($p < 0.05$). Again heterozygote cells occupy a position of intermediate upregulation, but the differences in the increase in CD35 expression for heterozygote neutrophils did not reach statistical significance when compared to other groups. In contrast to other results, RR131 neutrophils express the highest levels of CD35. However, there is considerable overlap between these groups with respect to CD35 expression.

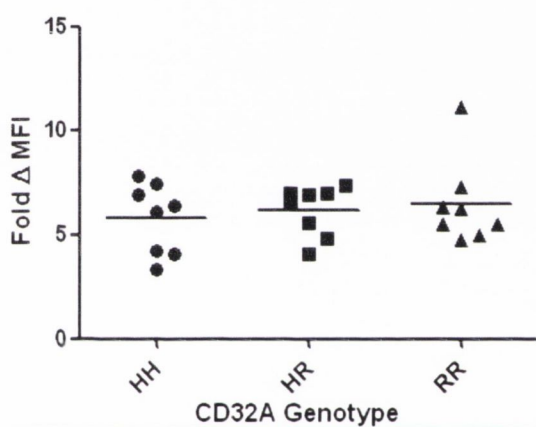


Figure 4.29 showing change in CD11b expression on neutrophils after 1 hour incubation with serotype 14 beads. No significant difference is observed.

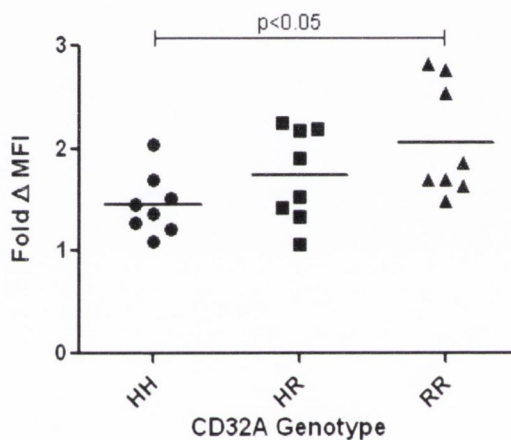


Figure 4.30 showing fold change in CD35 expression in phagocytosing neutrophils of known CD32A genotype after 1 hour incubation with serotype 14 beads.

Preservation of CD62L expression was noted in control experiments, with no genotype dependent differences noted (Figure 4.31). Incubation with beads resulted in CD62L expression falling to essentially undetectable levels as CD62L is shed from the cell surface. No differences associated with the presence of different CD32A genotypic variants were noted (Figure 4.31).

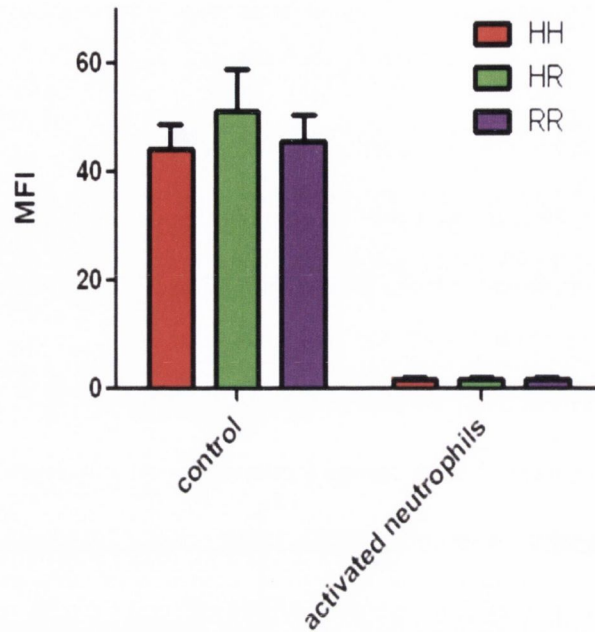


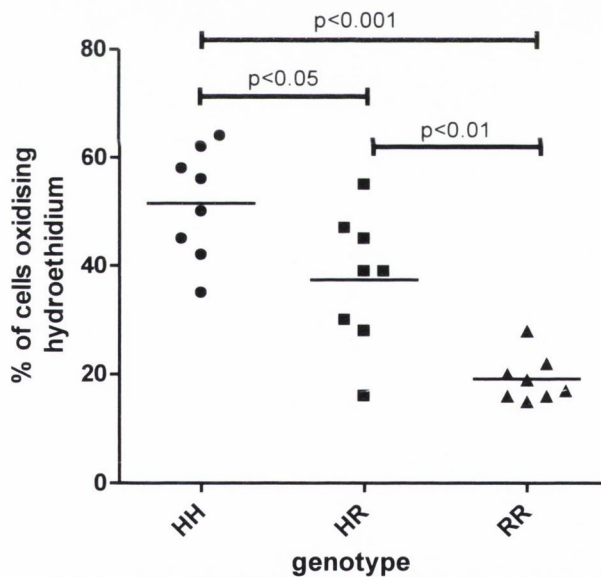
Figure 4.31 showing CD62L (Mean Fluorescence Intensity) expression by neutrophils incubated with serotype 14 beads and control neutrophils. No significant differences are noted between the groups.

In summary, CD32A RR131 neutrophils upregulate CD35 to a greater extent than other genotypic groups. No other genotype dependent differences are noted. Having examined adhesion molecule expression, we then went on to examine oxidative burst generation following bead stimulation.

4.3.12 CD32A genotype influences the magnitude of oxidative burst generated by neutrophils incubated with serotype 14 beads.

Evidence suggests that signalling through CD32A is important in the generation of neutrophil oxidative burst. To determine if CD32A H131R variation influenced the magnitude of oxidative burst reaction, neutrophils of known genotype were incubated with opsonised serotype 14

beads in the presence of hydroethidium. Hydroethidium (DHE) is dehydrogenated to red fluorescent ethidium in the presence of superoxide (Walrand et al., 2003). This results in a right shift in fluorescence measurable by flow cytometry. The proportion of neutrophils generating an oxidative signal is shown in figure 4.32.



These results show that the magnitude of oxidative burst, as measured by dihydroethidium oxidation by superoxide in neutrophils, is considerably smaller in RR131 neutrophils than other variants ($p < 0.01$ vs HR and $P < 0.001$ vs HH cells) at the 1hour time point. HH131 neutrophils produce approximately three times the amount of superoxide as measured by this method. Based on these results, it is apparent that CD32A genotype significantly affects oxidative burst generation after stimulation with serotype 14 polysaccharide coated beads.

4.3.13 CD32A genotype affects the amount of elastase, and myeloperoxidase, released from neutrophils stimulated by serotype 14 pneumococcal polysaccharide coated beads but has no impact on other secreted markers of activation

Activated neutrophils produce cytokines, chemokines and degranulation products. Following neutrophil stimulation pathways which produce and release a variety of neutrophil products are induced (Nauseef, 2007). These products may cause by-stander damage or impact on the recruitment of other inflammatory cells (Nauseef, 2007). Neutrophil elastase is a highly destructive enzyme contained within azurophilic granules (Moraes et al., 2006). On neutrophil activation preformed elastase can be released rapidly into the extracellular space resulting in local tissue damage. The supernatant of neutrophils of known CD32A genotype stimulated with serotype 14 beads for 1hour was collected. Residual cells and beads were removed and neutrophil elastase was measured by ELISA (Figure 4.33). In a manner similar to the previously presented phagocytosis results, RR131 neutrophils produced lower levels of elastase than either HH131 neutrophils ($p<0.01$) or heterozygote H131R neutrophils ($p<0.05$). No significant difference between HH and HR variants was detected. Measurement of myeloperoxidase (MPO) by ELISA was also carried out on reaction supernatants. MPO is also contained within neutrophil azurophilic granules and released into the extracellular environment on neutrophil stimulation (Moraes et al., 2006). RR131 neutrophils gave rise to the lowest levels of MPO (Figure 4.34). Levels were significantly lower than heterozygote variants ($p<0.05$), but showed no significant difference from H131 bearing neutrophils. Levels of MMP-9, a gelatinase of the matrix metalloproteinase (MMP) family were also measured (Figure 4.35a). These enzymes can be involved in tissue destruction and remodeling, and are found in neutrophil specific granules (Kessenbrock et al., 2010). Levels of this enzyme did not differ significantly between CD32A

genotypic variants. Similarly levels of CD62-L or L-selectin which is shed from activated neutrophils did not display relevant differences (Figure 4.35b). This finding complements the results of surface expression of CD62L shown in figure 4.31.

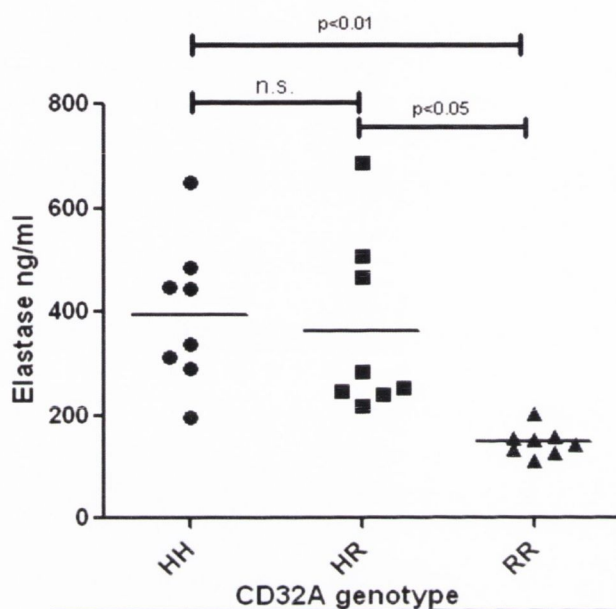


Figure 4.33 showing elastase production by neutrophils of known CD32A genotype after 1 hour incubation with serotype 14 beads. RR131 neutrophils produce low levels of elastase in comparison to other genotypes

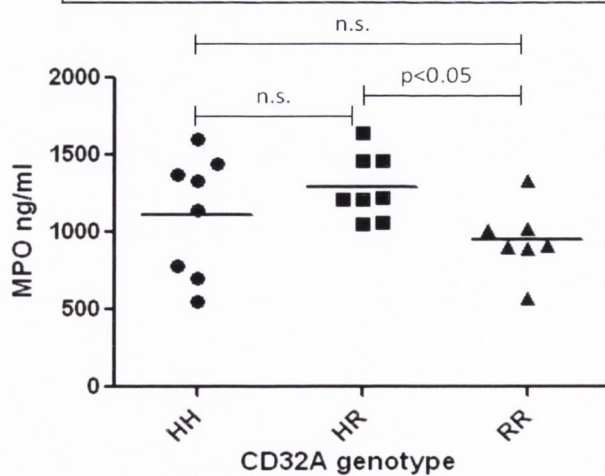


Figure 4.34 showing myeloperoxidase (MPO) production by neutrophils of known CD32A genotype after 1 hour incubation with serotype 14 beads. RR131 neutrophils produce low levels of MPO in comparison with HR neutrophils

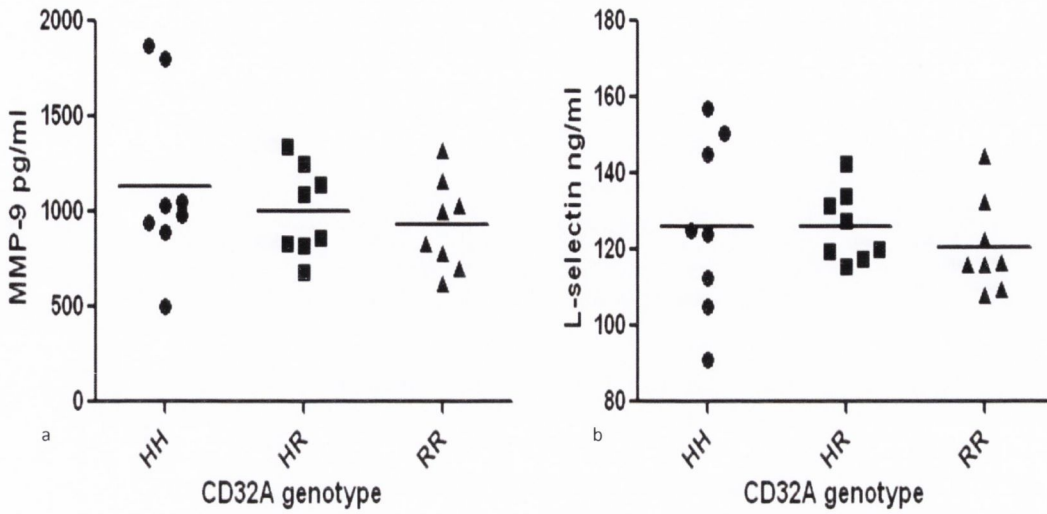


Figure 4.35a showing matrix metalloproteinase 9 and Figure 4.35b showing L-selectin (CD62L) released by neutrophils after stimulation with serotype 14 beads. No significant differences were noted between the various genotypic groups.

These results demonstrate that RR131 variants produce less oxidative burst, less elastase and less myeloperoxidase. This is further evidence of the relative functional inferiority of CD32A RR131.

4.3.14 CD32A genotypic variation has no impact on neutrophil cytokine secretion

Recently, it has become apparent that neutrophils are able to synthesize cytokines and chemokines in response to a wide variety of stimuli (Kasama et al., 2005). We measured levels of the pro-inflammatory cytokines IL-1 β and TNF- α and the CXC chemokine IL-8 in serotype 14 opsonophagocytic assay supernatants at the 1 hour time point by ELISA. Low levels of each cytokine were detected, however, no significant genotype dependent differences were noted (Figure 4.36 a, b and c).

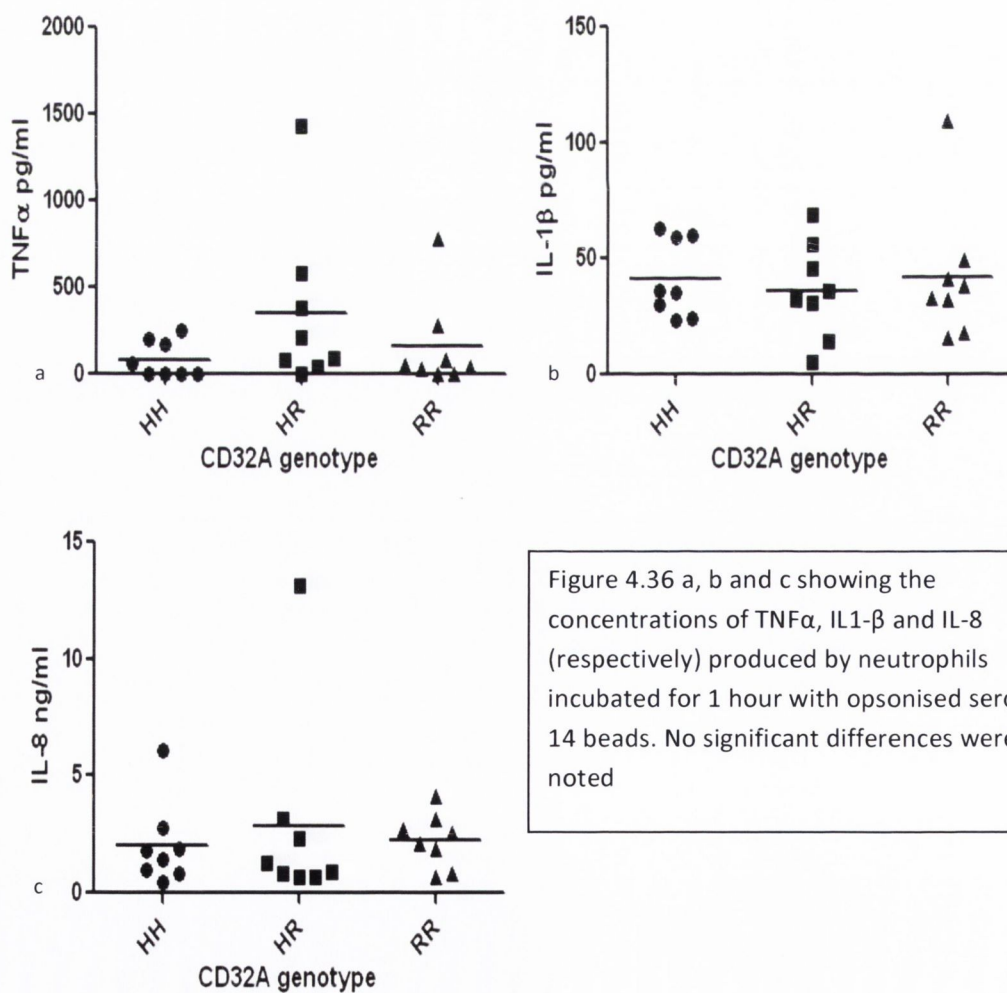


Figure 4.36 a, b and c showing the concentrations of TNF α , IL-1 β and IL-8 (respectively) produced by neutrophils incubated for 1 hour with opsonised serotype 14 beads. No significant differences were noted

4.3.15 Summary of results

1. Neutrophils from CD32A RR 131 bearing donors are less effective and efficient at phagocytosing pneumococcal target beads and bacteria
2. RR131 neutrophils produce significantly lower oxidative burst than other variants and also released less elastase and myeloperoxidase. In contrast CD35 upregulation was more pronounced in RR131 neutrophils.
3. No differences were noted in cytokine secretion or in the surface expression of CD11b and CD62L following pneumococcal target stimulation.

4.4 Discussion

In order to examine the functional importance of the polymorphic variants of CD32A an opsonophagocytic assay was set up. The purpose of this assay was to provide a measurement of phagocytosis under standard opsonising conditions with standard targets while varying the CD32A genotype borne on donor cells. In this study, a flow cytometric technique developed by Martinez *et al* for the measurement of functional anti-pneumococcal antibodies was adapted as the main assay (Martinez et al., 2006). Isolated neutrophils from healthy donors of known CD32A genotype were used instead of HL60 cells. A standard pooled high titre antipneumococcal antibody was used in the opsonising mix. The assay targets in the initial opsonophagocytic assay were serotype 9V and serotype 14 polysaccharide coated beads. These pneumococcal target beads were chosen because antipneumococcal antibodies were readily measured for standardisation purposes and because the bead based assay offered a relatively pure, opsonin dependent target. In particular the absence of innate immune activators such as LPS and other TLR agonists was desired. Several other studies have used similar assays in the evaluation of pneumococcal phagocytosis (Casado et al., 1993, Martinez et al., 2006, McCloskey and Salo, 2000, Prodan et al., 1995). These assays have differed in their findings of optimum opsonisation conditions.

Using serotype 9v and 14 beads, a phagocytosis time course was determined, using cells from a known heterozygote donor. In Figure 4.4 (page 121) the proportion of neutrophils phagocytosing beads initially increases rapidly before plateauing. A 1 hour time frame was arbitrarily chosen for efficient time management.

This bead based opsonophagocytic assay was first employed to determine the relative importance of antibody and complement to phagocytosis of pneumococcal polysaccharide coated beads. Unopsonised beads and beads opsonised with complement alone were not

phagocytosed efficiently (Figure 4.13a, page 134). Furthermore, in figure 4.13b, only a small amount of phagocytosis takes place in the presence of heat inactivated serum which contains antibody alone (10% or less). The combined importance of antibody and complement was confirmed by the use of a complement free intravenous immunoglobulin product. Supplementation of both IVIG and heat inactivated serum with rabbit complement re-established efficient phagocytosis with 30-45% of neutrophils phagocytosing beads.

Antibodies specific to the polysaccharide capsule of pneumococci have been shown to be essential for protection against pneumococcal disease. Opsonising antibodies activate complement system components (Fearon and Locksley, 1996, Walport, 2001b, Walport, 2001a). This results in optimal opsonisation and receptor mediated phagocytosis. This *in vitro* data is supported by the susceptibility of hypogammaglobulinaemic patients to infection with pneumococci and the reduction in infection susceptibility following replacement therapy (Maarschalk-Ellerbroek et al., 2011). Deficiencies of complement components can also predispose to infection with pneumococci (Yuste et al., 2008). However, studies in opsonophagocytic assays have been conflicting. The data presented here differs from the study of Rodriguez *et al* (Rodriguez et al., 1999). They suggested that phagocytosis of serotype 14 pneumococci was complement independent. However, they used a high cell:target ratio, which could favour opsonin independent phagocytosis and altered incubation temperature to facilitate cell attachment to target organisms. This suggests that *in vitro* manipulation of opsonophagocytic assay conditions can yield different results, however, using this bead based assay cold incubation and increased cell:target ratio did not reproduce complement independent phagocytosis (Figures 4.17a and b, page 139). Data presented here are more in keeping with the findings of Gardner *et al* who used chemiluminescence to identify that antibody and complement are important in phagocytosis (Gardner et al., 1982). This data also agrees with the necessity of complement and antibody for normal immune function *in vivo*.

The relative importance of antibody and complement was then evaluated using checkerboard dilutions (Figure 4.15, page 136). This data shows that a 1/16 dilution of IVIG is able to support efficient phagocytosis in the presence of complement. However, if complement is diluted beyond 1:2 the level of phagocytosis observed with 1:16 IVIG rapidly declines. Notably phagocytosis was still measurable at very dilute complement concentrations at high concentrations of IVIG. This and the results in heat inactivated serum, suggest that antibody alone can produce reproducible low level phagocytosis in this assay system. These findings support older studies that suggest that complement is particularly important in enhancing pneumococcal phagocytosis at lower specific antibody concentrations (Chudwin et al., 1983).

Polysaccharide capsular serotype has been shown to be a specific virulence factor and to have an impact on complement deposition and the requirement for antibody (Melin et al., 2010, Melin et al., 2009). With this phenotypic variability in mind, phagocytosis of all seven available bead serotypes was evaluated under a number of assay conditions (Figure 4.16, page 138). Serotype 14 beads demonstrated the highest levels of phagocytosis both in the presence and absence of complement. A recent study by Melin *et al* showed that serotype 23 clinical isolates were most susceptible to opsonophagocytosis by HL60 cells (Melin et al., 2010). Moderate susceptibility to opsonophagocytosis was demonstrated by serotype 23 pneumococci in this study. There are numerous differences between the cells and targets used in the respective opsonophagocytosis assays that could explain this difference. Notably serotype 14 and serotype 9v beads supported the most phagocytosis in the heat inactivated serum (Figure 4.16, page 138).

These results provide some reassurance that the assay system is suitable for the overall objective of evaluating phagocytosis due to different polymorphic variants of an IgG receptor. This data suggests that efficient phagocytosis is facilitated by a combination of antibody and complement. It seems likely in this bead based assay that antibody binding to the serotype specific polysaccharide coat facilitates complement activation and deposition. Phagocytosis then

occurs mediated by complement and immunoglobulin receptors (Hyams et al., 2010). The demonstrated reproducible low level phagocytosis suggested that IgG receptors were at least partly responsible. This is in keeping with data showing that blockade of the complement receptors CR1 and CR3 and the IgG receptors FcγRII (CD32) and FcγRIII (CD16) each partially reduced phagocytosis of pneumococci (Ali et al., 2003). In order to offer further evidence that Fc receptors play a role in mediating phagocytosis of beads in this assay, blockade experiments were set up. Considerable interassay variability was noted in this group of experiments (Figure 4.18a, b and c, page 140). However, a trend towards a statistically significant difference in the proportion of neutrophils phagocytosing serotype 14 beads was noted when a combination of anti-CD16, 32 and 64 monoclonal antibodies was pre incubated with neutrophils. A similar degree of inhibition of phagocytosis, by around 25% was noted using anti-CD32 alone (Figure 4.18a). Neither result was statistically significant. A peptide Fc receptor blocker was also used to block immunoglobulin receptors (Figure 4.18b). A small amount of dose dependent inhibition of phagocytosis was noted. Inhibition with monoclonal antibodies against complement receptors proved even more variable. A trend for inhibition of phagocytosis when neutrophils were pre-incubated with anti-CD11b was noted (Figure 4.18c).

These blockade experiments proved very variable. However, the reduction by 10-20% of phagocytosis by monoclonal anti-CD32, the anti-Fc receptor monoclonal mix, and the non specific Fc receptor blocker is similar to the proportion of phagocytosis observed when heat inactivated serum is used as an opsonin. It is important to note that the anti-CD32 monoclonal antibody used in these assays binds both CD32A and the related inhibitory molecule CD32B. CD32B is expressed on human neutrophils, although at much lower levels than CD32A (Su et al., 2007). It is possible that an interaction at this receptor is having an impact on result output. Further experimentation in this area was hampered by the lack of commercial availability of the differential antibodies.

These results suggest that the phagocytosis of serotype 14 beads is facilitated at least in part by IgG receptors, in particular CD32A. Although by no means technically perfect, these experiments gave us further confidence that our ultimate aim could be achieved using this assay system. It seems likely that Fc receptors are important in the phagocytosis of these beads in heat inactivated serum. Others have shown that, in the presence of complement, CD32A stimulation enhances CR3 (Cd11b/CD18) mediated phagocytosis (Huang et al., 2011). CD32A binding of opsonised antibody may also play an indirect role in enhancement of phagocytosis.

This bead based assay contains only pneumococcal polysaccharide. The addition of Toll-like receptor agonists LPS, peptidoglycan and PAMC3CSK4 to the opsonophagocytic assay did not significantly change phagocytosis in either the standard assay or the assay with heat inactivated serum (Figure 4.19 a and b, 142). The addition of TLR agonists has been shown to increase phagocytosis of pneumococci by murine microglial cells (Ribes et al., 2010). These experiments used a 24hour incubation time in comparison to 1hour in this assay. The results presented here suggest that additional stimulation of TLR receptors does not enhance phagocytosis of serotype 14 pneumococcal polysaccharide beads by neutrophils in this time frame.

The bead based assay examines the phagocytic response to opsonised pneumococcal polysaccharide residues. To complement this assay we established a similar assay using FITC labelled bacteria as targets. This assay behaved similarly to that developed by Gardner *et al*, demonstrating a considerable degree of complement dependence (Gardner et al., 1982) (Figure 4.20, page 144). However, once again a small amount of phagocytosis was reproducibly detected when heat inactivated serum was used as an opsonin.

In conclusion, two flow cytometric opsonophagocytic assays were established. Using pneumococcal polysaccharide beads and heat killed bacteria as targets, these assays provide reproducible results in the presence and absence of complement. Thus, the assays are of

potential use in the investigation of variations in Fc receptor function. The next step was to use the assays using standard opsonising sera while using donors of varying CD32A genotypes.

Figures 4.21 and 4.22 (pages 145 and 146) shows that the proportion of fresh RR131 neutrophils isolated from CD32A RR131 donors that phagocytosed opsonised serotype 14 polysaccharide coated beads was significantly less than HH131 or HR131 variants. This inferiority was consistent across a range of concentrations of the standard pooled antipneumococcal antibody that was used as an opsonin (Figure 4.23, page 146). Furthermore fewer RR131 neutrophils phagocytosed multiples of beads (Figure 4.24, page 147). These results suggest that RR131 variants are less efficient at phagocytosing serotype 14 polysaccharide labelled targets. HH variants are the most efficient. Opsonophagocytosis has been long established as a major defence mechanism against pneumococci (Vitharsson et al., 1994). Indeed, it is proposed that pneumococcal factors that impair host phagocytosis contribute to the pathogenicity of the organism (Martner et al., 2009, Hyams et al., 2010). With this in mind these results indicate that the presence of RR131 variant receptors on phagocytic cells may be a host factor that reduces phagocytosis of pneumococcal polysaccharide.

Having established that the RR131 genotype is associated with reduced phagocytosis of polysaccharide targets, experiments to determine if these results would also apply to whole bacterial targets were carried out. Significantly reduced proportions of RR131 neutrophils phagocytosed serotype 14 heat killed pneumococci across a range of antibody concentration compared with HR or HH131 variants (Figures 4.25-4.27, pages 148-149). These results echo those of Jansen *et al* in their finding of inferior phagocytosis of antibody and complement opsonised serotype 23F bacteria by RR131 neutrophils (these neutrophils also carried an FcgammaRIIIb NA2/NA2 haplotype)(Jansen et al., 1999). Figure 4.27 (page 149) also suggests that RR variants may require 6-8 times the antibody concentration of HH131 variants to achieve similar phagocytosis rates, this is even more significant than Jansen's data. Similar results have

been found with other encapsulated bacteria such as *Neisseria meningitidis* and group B streptococci (Sanders et al., 1995, Fijen et al., 2000). The results presented here extend these results, indicating, by the use of both whole bacteria and bead based assays, that opsonised pneumococcal polysaccharide is the specific target that leads to differences in CD32A dependent phagocytosis.

Although previous work has suggested that RR131 neutrophils phagocytose serotype 14 pneumococci less efficiently than other variants, the assay system used suggested that neutrophil phagocytosis of this pneumococcal strain was entirely complement independent (Rodriguez et al., 1999). Data presented here suggests otherwise. To explore this further, and to determine if CD32A polymorphic variation remained significant in the absence of complement, experiments in heat inactivated serum were carried out. Figures 4.28 a and b (page 151) shows that a much lower proportion of cells across all genotypes phagocytose serotype 14 beads and bacteria. Also, consistent with prior results, a significantly lower percentage of RR131 variants phagocytosed both beads and bacteria compared with other variants. These novel findings suggest that a small amount of phagocytosis is taking place completely independent of complement. This is likely to be mediated by IgG receptors including CD32A. It is interesting to note that the amount of bead phagocytosis achieved using heat inactivated serum corresponds well to the amount of inhibition of phagocytosis obtained with blockade of CD32A. The genotypic variation of phagocytosis provides further evidence of the importance of this receptor both in the presence and absence of complement. Recent data has examined the differential phagocytic capacity of CD32A compared with other IgG receptors, indicating that CD32A is the most important phagocytic receptor (Rivas-Fuentes et al., 2010). The data presented here does not examine the potential involvement of other receptors in pneumococcal phagocytosis. However, the burden of evidence suggests that variation in genotype has a significant *in vitro* impact.

The data presented thus far was suggestive of a relative phagocytic defect in CD32A RR131 bearing neutrophils. This is consistent with the presence of a partial immune deficiency. In bronchiectasis, ample evidence exists of a pro-inflammatory state contributing to lung damage (Shoemark et al., 2011, Courtney et al., 2008, Fuschillo et al., 2008). The next stage in the study was to examine neutrophils for activation markers that could indicate an inflammatory phenotype. Levels of CD11b, a component of complement receptor 3, and CD35, complement receptor 1 which are upregulated on neutrophil activation were examined by flow cytometry. This technique was also used to measure levels of L-selectin (CD62L) which is shed during neutrophil activation. CD11b was upregulated on exposure to the phagocytic stimulus, as expected (Crockard et al., 1992, Jones et al., 1994). This upregulation could be expected to facilitate attachment to complement opsonising products facilitating phagocytosis. CR3 plays an important role in adhesion of neutrophils to endothelial cells during inflammatory cell influx. No CD32A genotype dependent differences were noted with all variants increasing CD11b expression to a similar extent (page 153). Similarly complete shedding of CD62L was noted across all genotypes at the 1 hour time point, with no differences apparent between the groups (Figure 4.31, page 154). In contrast to these homogeneous findings, figure 4.30 (page 153) shows that expression of CD35 (CR1) was significantly higher in CD32A RR131 variants than HH131 variants. This difference is statistically subtle and there is considerable overlap between the genotypic groups. Nevertheless it is worthy of note. Murine studies have suggested that CD35 plays a role in the clearance of opsonised pneumococci (Li et al., 2010). It is possible that reduced phagocytosis by RR131 variant neutrophils and possible alteration in signal transduction pathways results in a compensatory increase in CD35 expression. Such speculation would require further detailed study and in particular the analysis of expression patterns of neutrophil activation markers over other time points would be required.

These results suggest that there are no dramatic genotype specific changes in neutrophil surface activation markers after exposure to serotype 14 beads for one hour. Other receptors or bacterial virulence factors may play a more crucial role in such changes, with variation in CD32A having less relevance.

Next, possible differences in oxidative burst generation were examined by measuring fluorescence changes in dihydroethidium (Figure 4.32, page 154). Hydroethidine is specifically oxidised by superoxide, the first NADPH oxidase product (Walrand et al., 2003). While the measurement of oxidative burst is a different outcome measure to bacterial killing assays, such assays correlate well using this bead based technology (Klein, 2006). As might be expected, more of the HH131 variant neutrophils generated a shift in immunofluorescence following incubation with, and phagocytosis of serotype 14 beads (Figure 4.32). HR131 cells generate intermediate oxidative burst, while RR131 generate the lowest levels. This correlation between amount of phagocytosis and oxidative burst has been observed previously (Casado et al., 1993, Klein, 2006). However, we have shown that phagocytosis and oxidative burst show considerable dependence on CD32A H131R variation. As expected, oxidative burst generation is lower in the genotypic group that phagocytosed fewer targets. This data may correlate with other studies in which defective oxidative burst has been identified in bronchiectatic neutrophils (King et al., 2009a, King et al., 2006b).

Again the data presented here fits in with the idea of a functional immune deficiency. However, murine data suggests that reduced phagocytosis may have an impact on the regulation of inflammation. Studies show that reactive oxygen species limit further neutrophil recruitment to the infected lung, an important step in the regulation of inflammation (Marriott et al., 2008). Therefore, the finding of reduced oxidative burst in RR131 variant neutrophils could facilitate inflammatory cell recruitment. This raises the possibility of RR131 variation being a double-edged sword, with reduced phagocytosis and enhanced inflammatory cell recruitment giving rise

to slow resolution of infection and ongoing stimulation of local inflammatory cells. This intriguing hypothesis may be worth exploring in further studies of bronchiectasis.

The final experiments in this part of the project examined some of the inflammatory products released from the phagocytosing neutrophils during the opsonophagocytic assay. Supernatants were examined for levels of elastase, myeloperoxidase, MMP-9, L-selectin, TNF α , IL-1 β and IL-8.

Activated neutrophils have cytokine expression profiles similar to monocytes and macrophages (Kasama et al., 2005). Secretion of proinflammatory cytokines such as TNF α and IL-1 β along with chemokines such as IL-8 has a potential important role in a number of inflammatory diseases (Kasama et al., 2005, Nathan, 2006). No genotype dependent differences in secretion of inflammatory cytokines were noted (Figures 4.36 a b and c, page 160). These experiments measured cytokine release after 1 hour of exposure to the phagocytic stimulus. Neutrophil cytokines are not pre-stored. Further experiments at extended time points would be required to determine whether the increased phagocytic activation produced variation in cytokine production at later stages. Increased levels of these cytokines have been noted in bronchiectasis, with a possible contribution to disease pathogenesis (Simpson et al., 2007). However, data presented here does not implicate CD32A genotypic variation in alteration of secretion of these cytokines.

Previous results indicated complete shedding of L-selectin over the duration of the 1 hour opsonophagocytic assay. As expected on the basis of this, no differences in L-selectin levels were detected between the genotypes (Figure 4.35b, page 159). Similarly no differences in MMP-9 were observed (Figure 4.35a). Again, caution is urged in the interpretation of these results because of the single time point used.

CD32A genotype dependent differences in levels of elastase and myeloperoxidase were noted (Figure 4.33 and Figure 4.34 respectively, page 158). CD32A RR131 variant neutrophils released

less elastase than HH and HR variants. RR131 neutrophils also produced less myeloperoxidase than HR variants. No significant difference in MPO production was noted between RR131 and HH131 neutrophils in terms of MPO production. Elastase and MPO are both contained preformed in neutrophil azurophilic granules (Nathan, 2006). As they have the same source, the similarity in results is unsurprising, although small numbers may be preventing reaching the same statistical conclusions. These important bactericidal mediators are also involved in tissue damage. Increases in elastase and myeloperoxidase in the bronchoalveolar lavage fluid of patients with bronchiectasis have been detected (Angrill et al., 2001). Such findings are representative of compartmentalised active neutrophilic inflammation in the lung. While RR131 variants in this in vivo assay produce less of these secreted inflammatory mediators, it is possible that when faced with a persistent infective burden which is poorly cleared overall, levels of elastase, myeloperoxidase and other inflammatory mediators may be increased. Thus the partial immune deficiency represented by CD32A RR131 variation can contribute in another way to the overall burden of inflammatory disease.

These findings add another layer of complexity to an emerging paradigm for a role of CD32A RR131 in bronchiectasis.

4.5 Conclusions

In this section we developed a versatile assay for the measurement of phagocytosis of pneumococcal targets. It was demonstrated that antibody and complement were required for efficient phagocytosis of both polysaccharide coated bead whole pneumococcal targets. However, evidence demonstrating that Fc receptors, and in particular CD32A was involved in the process of phagocytosis was generated.

Using this opsonophagocytic assay we demonstrated in a robust fashion that neutrophils bearing CD32A RR131 receptors were less capable of phagocytosing pneumococcal targets efficiently. It was also demonstrated that such cells generated a less significant oxidative burst and released less elastase and myeloperoxidase. These results suggest a relative functional defect in neutrophils. Surprisingly, when cell surface markers of activation and cytokine release were examined, with the exception of CD35 no differences were found. CD35 was noted to be expressed at higher levels on RR131 neutrophils. The reasons for this difference, which would need to be verified with further study, are unclear. It is interesting to speculate that it may be a compensatory mechanism in such cells.

These results provide evidence of a relative phagocytic defect, a subtle immune deficiency that could provide a functional explanation in bronchiectasis. However, experts suggest that neutrophils are not the first line immune defence cell in the lung, although there is little doubt that they are critical in bronchiectatic inflammation. Alveolar macrophages may be the key immune cell responding to the earliest stages of microbial insult in the lung. These early stages, at the interface between asymptomatic colonization and inflammatory infection may be important in the pathogenesis. Therefore, we next sought to examine the phagocytic capacity of peripheral blood monocytes as a model potentially reflective of alveolar macrophage function.

Chapter 5. The impact of CD32A polymorphic variation on monocyte phagocytosis

5.1 Introduction

5.1.1 Monocytes

Cells of the monocyte/macrophage lineage make up around 10% of circulating white cells. In the blood they exist as monocytes. Circulating monocytes have relatively short half lives in humans, around 3 days. These nondividing circulating macrophages were originally thought to be the source of more differentiated tissue macrophages and dendritic cells (Geissmann et al., 2010). Monocytes and macrophages have important functions in the maintenance of immunity, immune surveillance and the propagation and regulation of inflammation.

Peripheral blood monocytes in humans can be divided into two distinct subgroups on the basis of CD14 and CD16 expression (Yona and Jung, 2010). Classical monocytes are identified by high levels of CD14 expression, but absent CD16 (CD14^{high}, CD16⁻) and represent 90-95% of peripheral blood monocytes (Strauss-Ayali et al., 2007). A second group of 'non-classical' or 'proinflammatory' monocytes has also been recognised. These monocytes are smaller, express lower levels of CD14, but also express CD16 (CD14⁺CD16⁺). The nonclassical monocytes expand rapidly in response to systemic infection, including bacterial sepsis. They produce higher levels of TNF- α and lower levels of IL-10 on stimulation by TLR agonists than their classical counterparts (Strauss-Ayali et al., 2007). These non classical monocytes resemble more closely tissue macrophages and may represent a more mature monocyte phenotype (Gordon and Taylor, 2005). While nonclassical monocytes appear to have a role in the propagation of inflammation, classical monocytes may play a role in the downregulation of adaptive immunity (Gordon and Taylor, 2005). As is often the case in cellular phenotyping, many more subtle subgroups based on cell surface characteristics have been described including subgroups expressing CD64 and High HLA-DR expression (Strauss-Ayali et al., 2007). The functional consequences of these new discoveries are debated and remain to be elucidated.

Circulating monocytes express a variety of receptors on their surfaces (Yona and Jung, 2010). Monocytes of the classical type express CD32A as the predominant IgG receptor on the cell surface (Alevy et al., 1992, Strauss-Ayali et al., 2007). The more mature inflammatory monocytes also express the other IgG receptors CD16 and CD64. Differences in cell surface expression of CD16 and CD64 on peripheral blood monocytes have been observed in a variety of inflammatory diseases including rheumatoid arthritis (Laurent et al., 2011). CD32A expression also varies in peripheral blood monocytes across a number of diseases, but does not appear to be a defining characteristic of the various monocyte subgroups (Laurent et al., 2011, Hepburn et al., 2004). IgG receptors on monocytes facilitate binding of opsonised particles and immune complexes resulting in phagocytosis, generation of oxidative burst, monocyte activation and cytokine secretion (Hepburn et al., 2004). IgG receptor mediated phagocytosis with the production of reactive oxygen intermediaries within monocyte phagolysosomes results in killing of target pathogens contributing to adaptive immunity.

Monocytes also express pattern recognition receptors such as toll-like receptors and other scavenger receptor (Uematsu and Akira, 2006). Activation of TLRs on monocytes by pathogen associated molecular patterns results in the production of pro-inflammatory cytokines marking the early innate immune response (Chavez-Sanchez et al., 2010). The co-expression of receptors of the innate and adaptive immune systems suggests that monocytes and other lineage related cells can have an important function during several phases of an integrated immune response.

Peripheral blood monocytes have been considered to be a stage of intermediate development between progenitor cells and effector tissue macrophages. Recent evidence suggests that renewal of tissue macrophages does not rely solely on circulating monocytes (Auffray et al., 2009). However, during inflammatory insults and under the influence of pro-inflammatory cytokines elicited by a variety of pathogens, monocytes are recruited from the blood to the tissues (Serbina et al., 2008). In the tissues these blood derived monocytes contribute to the

pool of more differentiated and longer lived tissue macrophages and dendritic cells. The trafficking cues that lead to monocyte recruitment to tissues are, in general, poorly understood.

5.1.2 Macrophages

Macrophages are a group of cells that can be broadly described as tissue resident monocytes. They reside in various organs including the lung, skin, liver and brain. This tissue diversity belies extensive functional diversity among organ-specific macrophage subgroups. As the name macrophage or 'big-eater' suggests these cells are involved in the phagocytosis of microbes. However, they also play important roles in cytokine production, antigen presentation to T cells, the regulation of apoptosis, vascular homeostasis and tissue regeneration and repair (Stefater et al., 2011)

Resident macrophages can represent the first line of defence against microbial insult. A wide variety of cell surface receptors are expressed, enabling these cells to recognise and respond rapidly to pathogenic insult as part of the innate immune response (Gordon, 2007). Toll-like receptors and other collaborative scavenger receptors such as CD36 and MARCO (macrophage receptor collagenous) are involved in recognising pathogenic molecular motifs (Gordon, 2007). Signalling through these receptors results in the NF κ B mediated production of pro-inflammatory cytokines and the propagation of a targeted immune response (Gordon, 2007). Macrophage production of TNF, IL-1 and IL-12 results in inflammation, T cell activation and the enhancement of adaptive immune responses. These innate immune receptors enable macrophages to respond rapidly to infection. They can also survive longer at sites of inflammation than other cells such as neutrophils, making them the predominant cell of the late innate immune response.

Macrophages also express Fc receptors and complement receptors. These receptors permit the efficient phagocytosis of opsonised targets. Macrophages express all of the major classes of Fc gamma receptors (van Sorge et al., 2003). CD32A receptors are expressed at consistent levels and along with CD16 receptors are capable of mediating the phagocytosis of antibody coated targets (van Sorge et al., 2003). Phagocytosis results in macrophage activation and the stimulation of a killing oxidative burst and cytokine production. The tissue resident nature of macrophages means that they can be first line phagocytic cells with important roles in driving the subsequent immune response (Raley et al., 1999).

Like monocytes, macrophages are a heterogeneous group of cells. Macrophage heterogeneity is influenced by the both genetic background and by a variety of tissue specific factors (Mills et al., 2000). The state of activation of these cell populations are further influenced by their interaction with microbial products. Additionally T helper cell cytokine products can influence macrophage heterogeneity and activation status (Mills et al., 2000).

5.1.3 Macrophage activation

Macrophage activation has been conceptually divided into two types; 'classical' and 'alternative'. Classical activation occurs under the influence of the Th1 derived cytokines IFN γ and TNF α . Classical activation can also occur when cell surface TLRs or intracellular danger sensing receptors such as the NOD family of receptors encounter ligands (Mills et al., 2000). Classically activated macrophages produce high levels of IL-12 and IL-23, further promoting a Th1 cytokine response (Mills et al., 2000). These cells also express high levels of MHC II and co-stimulatory molecules such as CD80 and CD86, and produce proinflammatory cytokines including TNF, IL-1 and IL-6 (Martinez et al., 2008). Classically activated macrophages also produce large quantities

of reactive oxygen intermediaries, acting as effective killing machines when pathogens are phagocytosed (Martinez et al., 2008).

A number of other mediators result in the alternative activation of macrophages. These mediators inhibit the development of classically activated macrophages and impart more anti-inflammatory or immunomodulatory properties to the cell (Van Ginderachter et al., 2006). The prototypic cytokines resulting in alternative activation of macrophages are the Th2 cytokines IL-4 and IL-13. Exposure of macrophages to other anti-inflammatory cytokines such as IL-10 and TGF- β can also cause alternative activation, as can exposure to glucocorticoid hormones and apoptotic cells (Gough et al., 2001). The alternatively activated macrophages termed M2 cells are also heterogeneous, but further functional subdivision is controversial and may represent an over simplification of *in vivo* biology (Van Ginderachter et al., 2006). In general M2 cells exhibit strong phagocytic ability, but promote Th2 responses, secrete low levels of proinflammatory cytokines and higher levels of anti-inflammatory molecules such as IL-10 and TGF- β (Anderson et al., 2002). These alternatively activated cytokines reduce T cell proliferation and may have a more immunoregulatory role and a role in tissue repair. NF κ B linked receptors of the innate immune system are not thought to play an important role in M2 activation, however, these cells do express high levels of other non-opsonic pattern recognition receptors including the mannose receptor and DC-SIGN. These receptors may be important in normal physiological clearance of targets without coincident inflammation (Taylor et al., 2004). Macrophages belonging to the M2 family include those found in the airways, placenta and at immune privileged sites. These findings suggest that alternatively activated macrophages may be involved in protection against exuberant immune responses in these regions.

5.1.4 Macrophages in the lung

Macrophages play a role of emerging importance in lung immunity that may be relevant to the pathophysiology of a number of diseases. Alveolar macrophages represent the predominant immune cell in the lung airway (Wissinger et al., 2009). These cells act in concert with the various humoral lung defences such as lactoferrin, lysozyme, immunoglobulin and defensin (Lambrecht, 2006). Alveolar macrophages are critical to the barrier defence of the lung. Tight regulation of the immune system at this barrier is required to avoid collateral damage occurring during the continuous exposure to inhaled antigens. For this reason alveolar macrophages are generally in a quiescent state, producing relatively little cytokines in a manner allowing their classification as members of the M2 family. This quiescent state is maintained by TGF- β produced by the macrophages themselves and by other cells in the alveolar microenvironment (Takabayshi et al., 2006). This suppressive effect of TGF- β is maintained by the tight adherence of alveolar macrophages to alveolar epithelial cells with consequent expression of $\alpha\beta6$ -integrin, which acts as a TGF activating molecule. On exposure to pathogens, stimulation of pattern recognition and other macrophage cell surface receptors results in a loss of epithelial cells adherence with downregulation of $\alpha\beta6$ -integrin and reduced TGF-beta activation (Takabayshi et al., 2006). This removes the brake on activation of resident alveolar macrophages allowing them to take on a more pro-inflammatory phenotype with the production of IL-6 and TNF- α (Takabayshi et al., 2006). Additionally these activated alveolar macrophages can phagocytose more efficiently, produce a vigorous oxidative burst and produce a variety of chemokines recruiting inflammatory monocytes and neutrophils to the site of lung inflammation (Lambrecht, 2006). The activated alveolar macrophages and recruited inflammatory monocytes promote dendritic cell and T cell function propagating the adaptive immune response. Late in the evolving immune response as pathogen is cleared T cell IFN γ induced MMP-9 production again favours TGF- β activation promoting alveolar macrophage adherence and the expression of $\alpha\beta6$ -integrin

(Takabayshi et al., 2006). This mechanism puts the brakes on the local inflammatory response re-establishing normal homeostasis. Alveolar macrophages are important regulators of lung immunity and represent the first line of defence of the cellular immune system in the lung. Alveolar macrophages have been examined in the setting of chronic obstructive pulmonary disease. In fact alveolar macrophages can account for many of the inflammatory changes observed in the COPD lung (Barnes, 2004). Numbers of alveolar macrophages are increased in the lung of smokers and more profoundly increased in patients with COPD (Magno and Di Stefano, 2007). Survival of these cells may be increased by cigarette smoke (Marques et al., 1997). Alveolar macrophages may be at the centre of a vicious cycle of airway inflammation with pro-inflammatory cytokines, cigarette smoke and oxidative stress facilitating further macrophage activation and the release of other inflammatory cytokines, chemokines and enzymes to upregulate the inflammatory response in the lung (Barnes, 2004). This upregulated inflammatory response may further be contributed to by bacterial pathogens. Taylor *et al* suggest that monocyte derived macrophages from COPD patients have impaired phagocytosis of *Strep. Pneumoniae* and *H. influenzae* (Taylor et al., 2010). Other authors have demonstrated reduced clearance of *H. influenzae* by alveolar macrophages from the lungs of smokers (Martí-Llitas et al., 2009). This impairment of phagocytic function in the lung of smokers is, at the same time, coupled with an upregulation of inflammation (Hodge et al., 2007). This uncoupling of infective and inflammatory pathways may be central to the pathogenesis of airway obstruction and recurrent infection in COPD.

Alveolar macrophages are also important for the removal of apoptotic cells in the lung, a process termed efferocytosis (Tauber, 2003). Failure to remove apoptotic cells can result in dysregulated homeostasis and inflammation. Apoptotic cells are increased in a number of chronic lung diseases, particularly CF and non-CF bronchiectasis (Tauber, 2003, Vandivier et al., 2002, Vandivier et al., 2006). This poses an additional problem for alveolar macrophages in their

function as potential regulators of inflammation (Vandivier et al., 2006). The phagocytosis of apoptotic cells by alveolar macrophages actively suppresses inflammation by promoting the secretion of TGF- β (Huynh et al., 2002). Furthermore the clearance of apoptotic cells in models of pulmonary injury resolution is associated with the release of a number of growth factors initiating tissue repair (Morimoto et al., 2001). The phagocytosis of apoptotic neutrophils may be less efficient at suppressing inflammation than other cell types (Fadok et al., 2001). Failure of adequate clearance of apoptotic cells can also result in secondary necrosis with subsequent inflammation (Haslett, 1999). Apoptotic cells are increased in the airways of patients with CF and with COPD (Vandivier et al., 2002). In relation to COPD, cigarette smoke suppresses efferocytosis *in vitro* and *in vivo*, while in CF increased levels of neutrophil elastase reduces efferocytosis (Vandivier et al., 2002, Hodge et al., 2003, Kirkham et al., 2004). In chronic lung inflammation this failure of efficient clearance of apoptotic cells may result in the propagation of inflammation and inefficient repair promoting further disease. Such mechanisms may also be relevant to bronchiectasis.

Cells of the monocyte lineage have not been studied extensively in bronchiectasis. Studies in bronchiectatic sputa have suggested monocyte recruitment to the lung is a feature of bronchiectatic inflammation (Owen et al., 1992). While neutrophils are the most common cell type in the inflamed airway lumen, macrophages and lymphocytes are the predominant cell wall infiltrate (Lapa e Silva et al., 1989, Gaga et al., 1998). Macrophage activation is facilitated in bronchiectasis by interactions with infecting and colonising pathogens (Gaga et al., 1998). Infection or colonisation with organisms such as, biofilm forming *H. Influenzae* or *Strep. Pneumoniae*, allows stimulation of the production of proinflammatory cytokines via pattern recognition, scavenger, complement and Fc receptors (Gaga et al., 1998). This results in the development of an inflammatory phenotype in alveolar macrophages with the secretion of cytokines such as IL-8 and TNF- α . These proinflammatory cytokines can recruit other monocytes

and neutrophils from the circulation (Simpson et al., 2007). Some commentators have reported increased levels of apoptotic neutrophils in the airways of bronchiectatic patients, in keeping with findings in CF and COPD (Vandivier et al., 2006). Others have published conflicting data (Watt et al., 2004). The high levels of necrotic neutrophils observed in bronchiectatic airways may be as a direct consequence of failure of apoptotic clearance (Simpson et al., 2007). Ongoing airway inflammation caused by continued bacterial stimulation and poor clearance of apoptotic cells may further impair the function of airway neutrophils and macrophages perpetuating the inflammatory cycle. Alveolar macrophages may be central to the initiation of the inflammatory cycle in bronchiectasis, however it seems clear that an initial, and perhaps ongoing infective insult is required to kick start the pathways.

5.1.5 Aims

- To establish a whole blood assay of monocyte phagocyte function
- To examine the effect of the CD32A H131R polymorphism on phagocytic activity
- To examine the effect of the CD32A H131R polymorphism on monocyte cytokine secretion.

5.2 Materials and Methods

5.2.1 Development of the whole blood monocyte assay

In order to examine the response of mononuclear cells to stimulation by pneumococcal targets, a whole blood phagocytosis assay was set up. It was anticipated that this assay could fulfil all the requirements of the previous neutrophil isolation assay, using a smaller volume of whole blood. The assay went through a number of developmental stages.

5.2.2 Washing steps are critical to the assay

Initial experiments were conducted as follows.

- 9mls of Lithium Heparin whole blood was obtained from volunteers
- Cells were separated from plasma (500g, 5 minutes) and resuspended in opsonophagocytic assay buffer.
- The opsonophagocytic assay was carried out as previously.

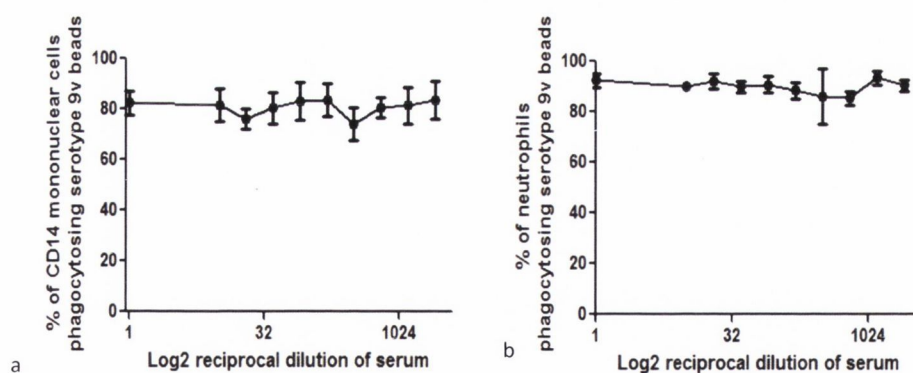


Figure 5.1 showing CD14 mononuclear cell (a) and neutrophil (b) phagocytosis in whole blood assay when clots formed due to inadequate washing

Using this method it became readily apparent that more phagocytosis than had been expected was observed. Furthermore, the formation of clots was apparent at the end of 1hr OPA incubations. Only small numbers of mononuclear cells were obtained. Finally, it was not possible to titrate out the effect of the opsonising antibodies on either neutrophil or mononuclear cell phagocytosis of serotype 9v polysaccharide coated beads (Figure 5.1).

It was felt that inadequate washing was allowing donor clotting factors to enter the OPA assay mixture. This permits clot formation, and probably facilitates phagocytosis. Furthermore, it seems likely that, using this technique, donor antibodies could be included in the OPA mix, potentially confounding the results. Increasing the number of washes with HBSS did not resolve the clotting issue.

To overcome this, a series of large volume washes in a Lithium Heparin containing solution were included. This resulted in a phagocytosis protocol described as follows

- 9mls Lithium Heparin whole blood was obtained from volunteers
- 3mls of Lithium heparin (LiH) whole blood was obtained for enumeration of neutrophils and monocytes.
- Cells were separated from plasma (500g, 5 minutes) and resuspended in HBSS containing LiH (HBSS/LiH was obtained by rinsing Lithium heparin (Vacurette, GBO, Gloucester UK) tubes with HBSS (without calcium/magnesium)).
- This solution was further made up to 20mls with HBSS/LiH, before centrifugation at 500g for 5 minutes.
- This was repeated 3 times
- The final samples was resuspended in OPA

- Samples were diluted in OPA to give a summed total concentration of neutrophils and monocytes of 5×10^6 cells/ml (or 5000 cells/ μ l)
- 10 μ l of whole blood and a further 40 μ l of OPA buffer were then added to the serotype 9V beads. Thus, approximately 50000 phagocytic cells were added to each 100 μ l opsonophagocytosis mix.
- Phagocytosis was allowed to progress as described previously for 1 hour and for 4 hours in selected assays.
- Control data was gathered as described previously
- Following phagocytosis, lysis of red cells was carried out using 2mls of BD FACSLyse (BD Biosciences, Oxford, UK)
- Cells were then washed twice in BD FACSFlow (BD Biosciences, Oxford, UK)

Using this technique a reproducible phagocytosis assay was established. Clots were not observed, titration of opsonising antibodies produced the desired effect, and yield of monocytes was good. The reasons for use of both serotype 9v beads are described later.

5.2.3 Overcoming potential problems caused by two cell populations of interest

In the neutrophil assay a cell: target ratio of 1:4 was used. The whole blood assay has two cell populations of interest, neutrophils and mononuclear cells. This could lead to potential variability. Therefore it was decided that if the ratio of neutrophils to monocytes was outside the range 7:1 – 11:1 the sample would be rejected. Samples that were rejected had a repeat FBC after one week and if satisfactory the experiment proceeded. One subject had a persistent mild monocytosis over the course of three weeks and therefore samples were not analysed.

5.2.4 Staining of mononuclear cells is desirable

Mononuclear cells can be identified by their characteristic position on the forward scatter, side scatter plot on whole blood analysis. However, after 1 hour stimulation with polysaccharide beads the mononuclear population has spread out. This is likely to be due to mononuclear cells increasing in complexity after phagocytosis of bead and moving towards the neutrophil position on the flow cytometer (Figure 5.2).

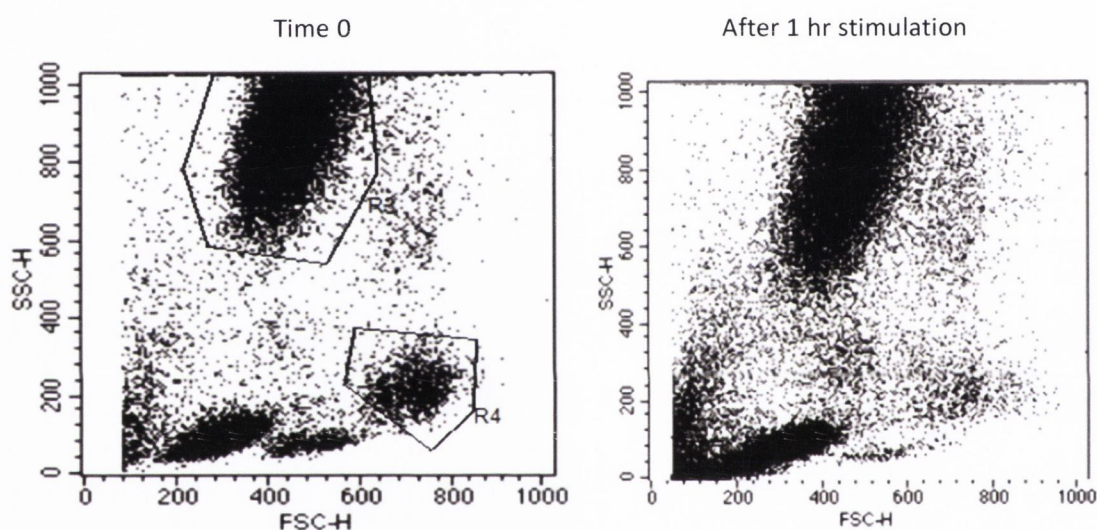


Figure 5.2 shows the a distinctive mononuclear cell population described by the gate R4 which disappears after 1 hour stimulation with opsonised pneumococcal bead targets

To ensure that we were examining a well defined monocyte population it was decided to include a staining step for CD14, after the opsonophagocytosis assay and before cell lysis.

- OPA assay mixes were removed from the 96well plate and placed in labelled falcon tubes (BD Biosciences, Oxford, UK)

- 5ul of Anti-CD14-APC (BD Biosciences, Oxford, UK) was added to the OPA mix, vortexed gently and incubated for 20 minutes in the dark. Cell lysis and washes were performed as previously described (Section 5.2.2, page 187)

Samples were then analysed on a FACS Calibur flow cytometer as described previously.

Neutrophils were identified by their forward and side scatter characteristics, monocytes were gated on by their characteristic side scatter position and CD14 positive status (Figure 5.3).

Sample acquisition was considered complete when 1000 cells were collected in the mononuclear cell gate. Figure 5.4 compares neutrophil and CD14 mononuclear cell phagocytosis of serotype 9v beads.

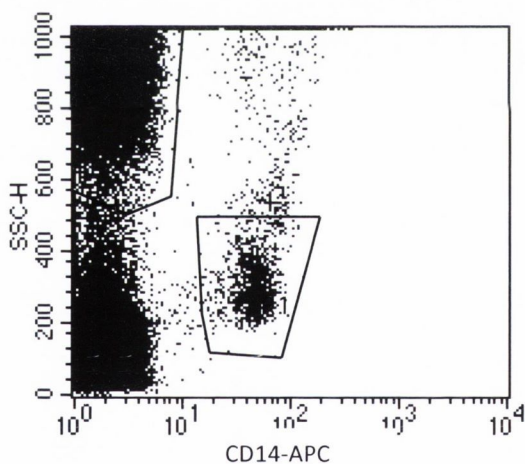


Figure 5.3 showing a discrete population of CD14 mononuclear cells identified by combining side scatter and CD14 characteristics

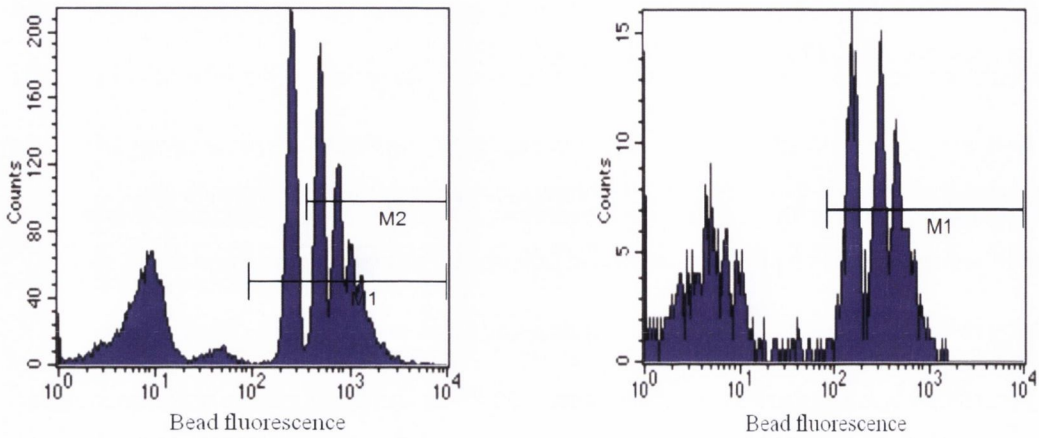


Figure 5.4 showing neutrophil (left) and CD14 mononuclear cell phagocytosis (right) of serotype 9v polysaccharide conjugated beads. Note the different Y axis scales with much fewer mononuclear cells being acquired during the assay

5.2.5 Mononuclear cell oxidative burst

Oxidative burst in mononuclear cells was measured using dihydrorhodamine (DHR) as a substrate. This assay was used because the emission spectra of the serotype 9v beads and the hydroethidine stain used in chapter 4 were not compatible. The DHR assay was adapted from the established oxidative burst assay used for the diagnosis of chronic granulomatous disease in the clinical immunology laboratory and published previously (Walrand et al., 2003). Briefly:

- 10mg/ml stock solution of dihydrorhodamine (Sigma Aldrich, Dorset, UK) was made up in DMSO
- 10 μ l aliquots were protected from light and frozen at -80°C until use
- Immediately prior to use aliquots were brought to room temperature and a final dilution of 2 μ l of DHR in 20ml of HBSS (with calcium and magnesium) was made

- 2.5 μ l of dihydrorhodamine was added to chosen wells 10 minutes after the OPA incubations had commenced (in dark). A negative control well was included in each run containing cells not exposed to the opsonic stimulus. Compensation was carried out on each run using single colour compensation. Phagocytosis was not measured on oxidative burst samples.
- Oxidative burst was measured as the proportion of cells with a right shift in immunofluorescence (beyond negative control) at completion of the 1 hour opsonophagocytosis assay. Samples were read immediately.

5.2.6 Measurement of whole bacterial phagocytosis in neutrophils and monocytes using a whole blood phagocytosis method.

- Serotype 9v bacteria (kind gift of Dr. B McConnell) were grown, killed and labelled as described previously in section 4.2.12 (page 128)
- Opsonophagocytic assay using bacterial targets were carried out according to the method described in section 4.2.12. Cell: target ratio was maintained at 1:4
- For bacterial phagocytosis samples were assessed after 1 hour
- Results were analysed using Cell quest analysis software after acquisition on a BD FACSCalibur flow cytometer

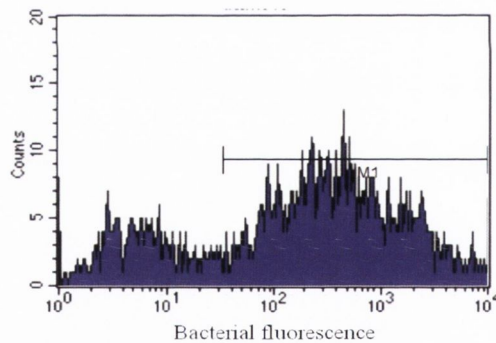


Figure 5.5 showing phagocytosis of serotype 9v FITC labelled bacteria by CD14 mononuclear cells in the whole blood assay

5.2.7 Measurement of intracellular cytokines by flow cytometric analysis of whole blood mononuclear cells.

The measurement of intracellular cytokines was carried out by adapting the procedure laid out in the BD application note to the opsonophagocytic assay (Biosciences, 2011).

- Wells were set up in the standard 96 well plate opsonophagocytosis assay for cytokine staining
- Stimulation with beads and positive controls was carried out in the presence of Brefeldin A which inhibits cytokine transport
- 2 μ l of a 0.5 μ g/ml solution of Brefeldin A (Sigma Aldrich, Dorset, UK) was added to chosen experimental wells
- Positive controls for each cytokine stain were established using LPS. These were included in each assay run. Negative controls were also included in which stimulating beads or bacteria were omitted. Serum controls in which antipneumococcal serum and complement was included in the absence of stimulating beads formed a baseline for the assay. In positive control wells LPS was added to make a final concentration in 100 μ l of 100ng/ml.
- Incubation was carried out at 37 $^{\circ}$ c in a 7% CO₂ incubator for 4 hours
- Following incubation cell surface staining for cell surface CD14 was carried out as previously described (section 5.4)
- After cell surface antibody staining red cell lysis was carried out using 2mls of 1x BD FACSLYSE (BD Biosciences, Oxford, UK). Lysis solutions were incubated for 10 minutes at room temperature in the dark.
- The lysis solutions were centrifuged at 500g for 5 minutes. The supernatant was carefully removed.

- Cell permeabilisation was carried out by adding 500µl of 1X FACS Permeabilizing Solution (BD Biosciences, Oxford, UK) to each experimental tube. Tubes were then vortexed gently to resuspend the pellet. Permeabilisation was carried out for 10minutes at room temperature in the dark. After permeabilisation cells are ready for intracellular staining
- Cells were then washed in 4mls PBS, with centrifugation at 500g for 5 minutes. The supernatant was removed.
- FITC labelled anti-cytokine antibodies were then added to selected wells in bead assays. Anti-IL-1, Anti-IL6 and Anti-TNF antibodies were chosen for this part of the study (Table 5.1).
- Permeabilised cells were incubated with anticytokine antibodies for 30 minutes at room temperature in the dark
- Cells were washed in 4mls of FACSFlow before resuspension in 0.5mls FACS Flow for cytometric analysis (Figures 5.6, 5.7 and 5.8)
- Samples were acquired immediately on a FACS Calibur flow cytometer, following suitable compensation and analysed using BD CellQuest software
- Experimental data was presented as the relative mean fluorescence intensity when compared with the positive (LPS) control. Using this internal control corrected for some of the internal assay variability.

Antibody	Fluorochrome	Clone	Catalogue no.
Anti-CD14	APC	MSE2	BD Pharmingen 555399
Anti-IL1 β	FITC	AS10	BD Fastimmune 340515
Anti-IL-6	FITC	AS12	BD Fastimmune 340526
Anti-TNF α	FITC	6401.1111	BD Fastimmune 340511

Table 5.1 showing antibodies used in the detection of intracellular cytokines in the whole blood phagocytosis assay

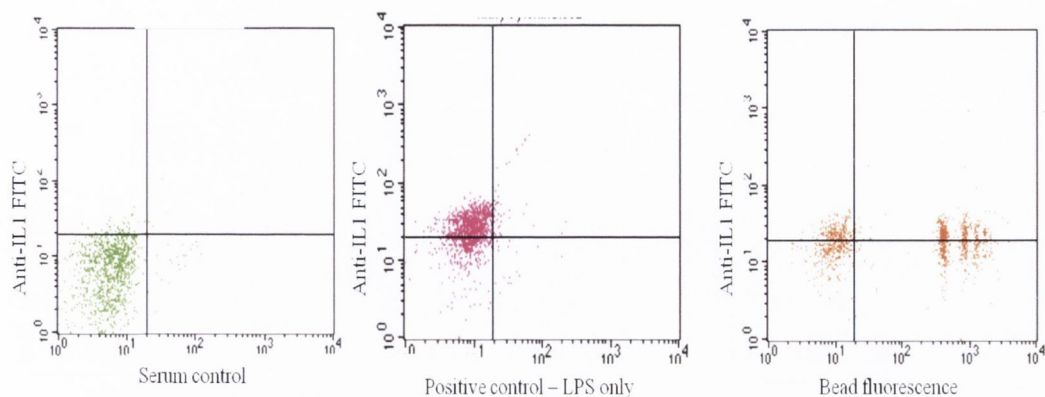


Figure 5.6 showing intracellular IL-1 staining for negative (serum) control (left panel), following 4 hours of stimulation with LPS (middle panel) and following stimulation with serotype 9v polysaccharide beads (right panel)

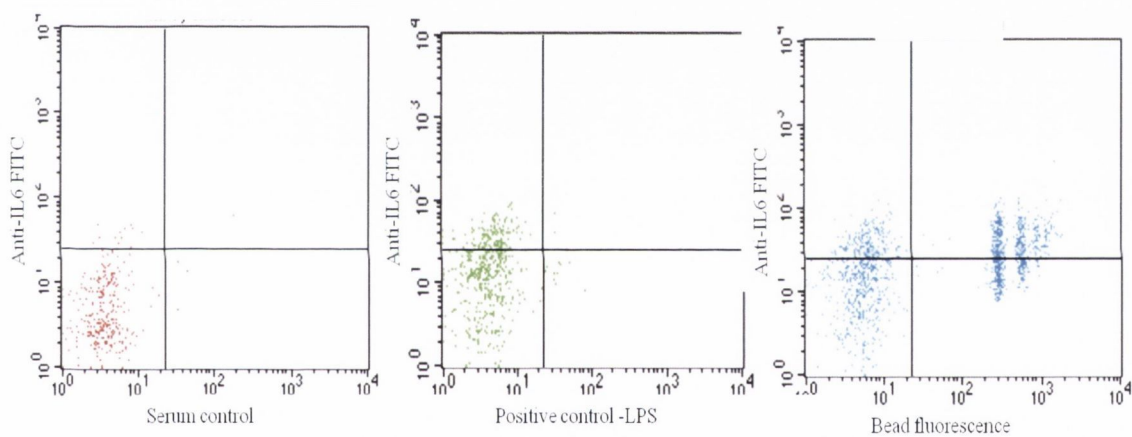


Figure 5.7 showing intracellular IL-6 staining for negative (serum) control (left panel), following 4 hours of stimulation with LPS (middle panel) and following stimulation with serotype 9v polysaccharide beads (right panel). Beads stimulate slightly higher IL6 secretion than LPS

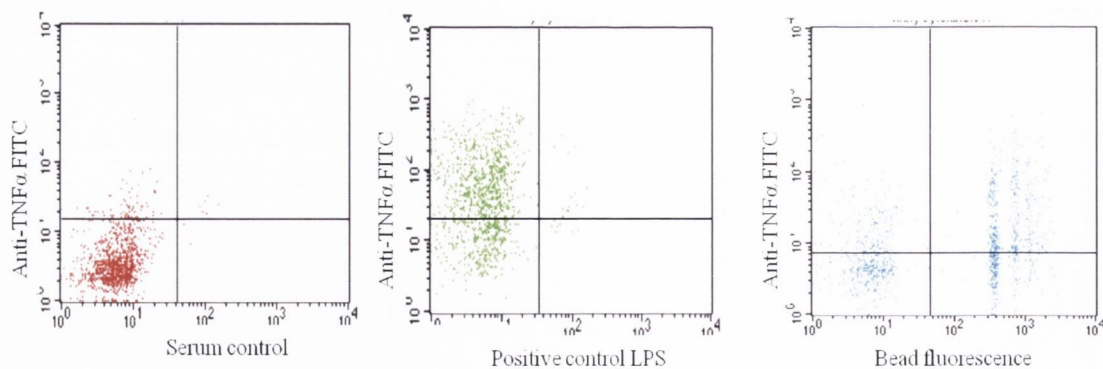


Figure 5.8 showing intracellular TNF- α staining for negative (serum) control (left panel), following 4 hours of stimulation with LPS (middle panel) and following stimulation with serotype 9v polysaccharide beads (right panel).

5.2.8 Analysis

Data was presented using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and was analysed using Graph Pad Prism or SPSS (Version 20, IBM, CA, USA). Standard error bars are presented unless otherwise specified. Continuous variables were analysed using one way ANOVA/Tukey's multiple comparison test. Non parametric data was analysed by U testing or Kruskal-Wallis for multiple comparisons. $\alpha=0.05$ for all comparisons.

5.3 Results

5.3.1 Time course for phagocytosis of serotype 14 and serotype 9v beads in whole blood.

A whole blood assay for phagocytosis allows the simultaneous measurement of phagocytosis by CD14 mononuclear cells and neutrophils. Phagocytosis of serotype 9v and serotype 14 polysaccharide coated beads preopsonised with serum and complement was examined at various time points over four hours (Figure 5.9a and 5.9b respectively). Results indicate that under these assay conditions a higher proportion of neutrophils phagocytose beads than monocytes. As in the isolated neutrophil opsonophagocytic assay phagocytosis occurs rapidly in the early stages of the assay and plateaus as time progresses.

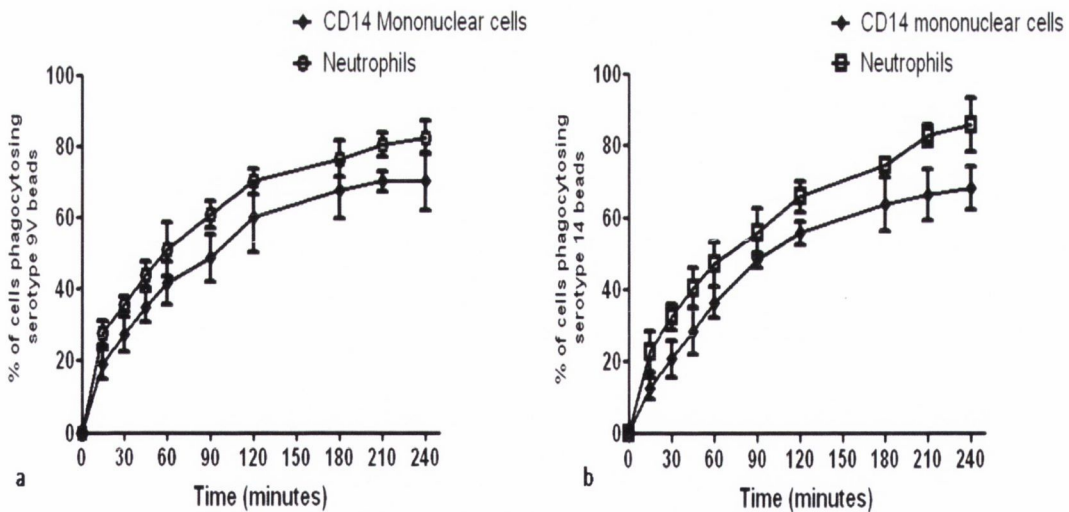


Figure 5.9 showing the percentage of CD14 mononuclear cells and neutrophils that phagocytose opsonised serotype 9v (Figure 5.9a) and serotype 14 (Figure 5.9b) coated beads over a series of time points

5.3.2 Serotype 9v and serotype 14 beads require antibody and complement for efficient phagocytosis in whole blood.

Results from the neutrophil opsonophagocytic assay suggest that efficient phagocytosis of serotype 14 beads requires antibody and complement. To determine whether this optimum opsonising mix is required in whole blood, and in particular is needed for phagocytosis by CD14 mononuclear cells, experiments based on different opsonising mixes were carried out.

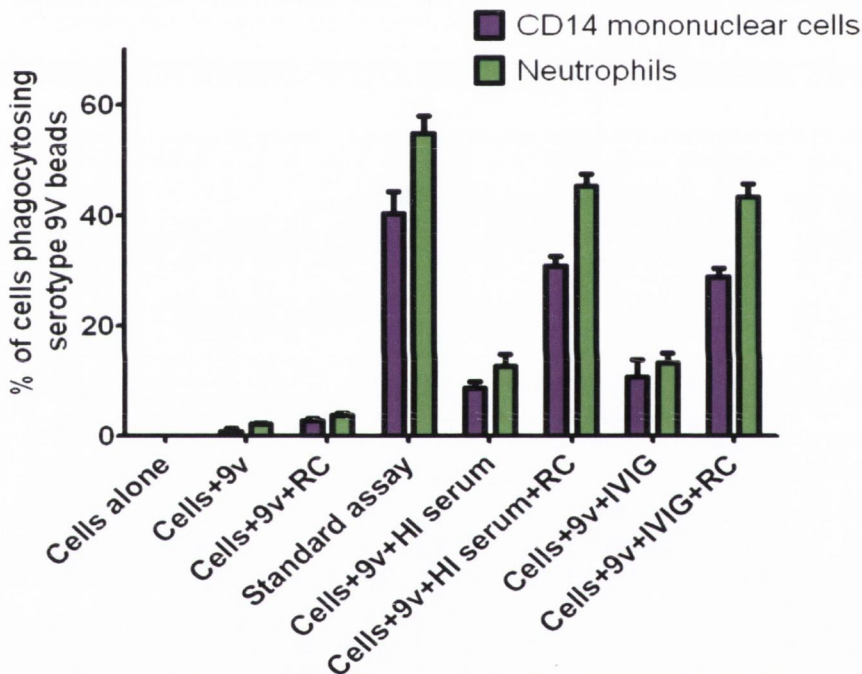


Figure 5.10 showing the percentage of CD14 mononuclear cells or neutrophils that phagocytose serotype 9v beads under different opsonisation conditions (n=3)

Serotype 9v beads require antibody and complement for efficient phagocytosis by both neutrophils and CD14 mononuclear cells. Neutrophils are better at phagocytosing beads under all assay conditions (Figure 5.10). However, as observed with the antibody assay there is a degree of phagocytosis that occurs in the presence of antibody and absence of complement.

A series of identical experiments were carried out with serotype 14 beads. Results showed a surprising magnitude of opsonin independent phagocytosis for monocytes, but not for neutrophils in the whole blood assay (Figure 5.11). It is apparent that 10-15% of CD14 mononuclear cells are capable of phagocytosing serotype 14 beads in the absence of antibody or complement. It is interesting that this is not the case with serotype 9v beads.

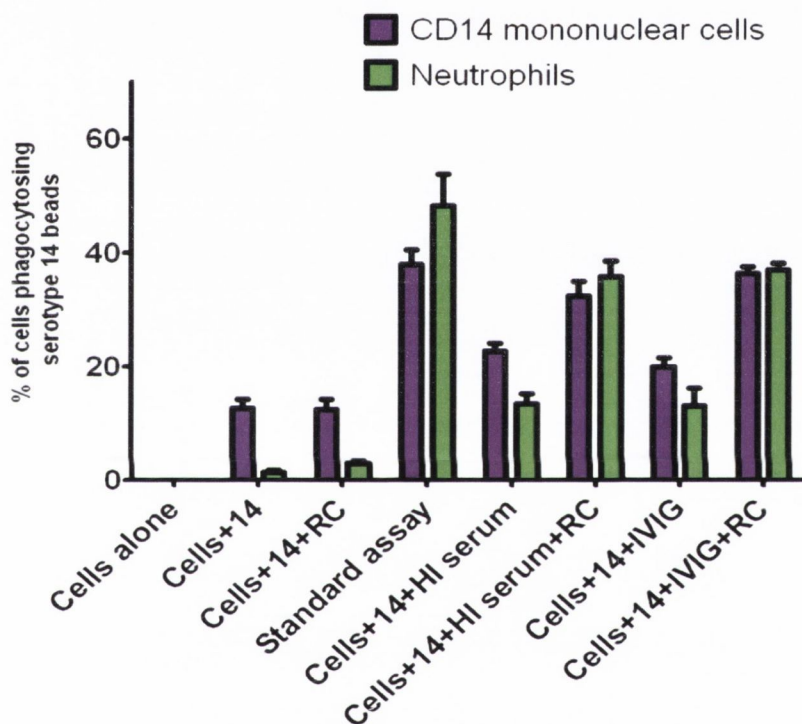


Figure 5.11 showing the percentage of CD14 mononuclear cells or neutrophils that phagocytose serotype 14 beads under different opsonisation conditions. Antibody and complement is required for efficient phagocytosis. Non specific phagocytosis by monocytes is noted on incubation with non opsonised serotype 14 beads

5.3.3 Phagocytosis of serotype 14 and 9v bacteria by CD14+ mononuclear cells in whole blood also requires antibody and complement

As with the neutrophil bead based opsonophagocytic assay we set up a complementary whole blood bacterial phagocytosis assay using FITC labeled serotype 9v and serotype 14 bacteria. For both serotype 9v and serotype 14 bacteria antibody and complement is required for efficient phagocytosis (Figures 5.12 and 5.13). In contrast to the bead based whole blood assay (Figure 5.11), significant opsonin independent phagocytosis of serotype 14 bacteria is not observed

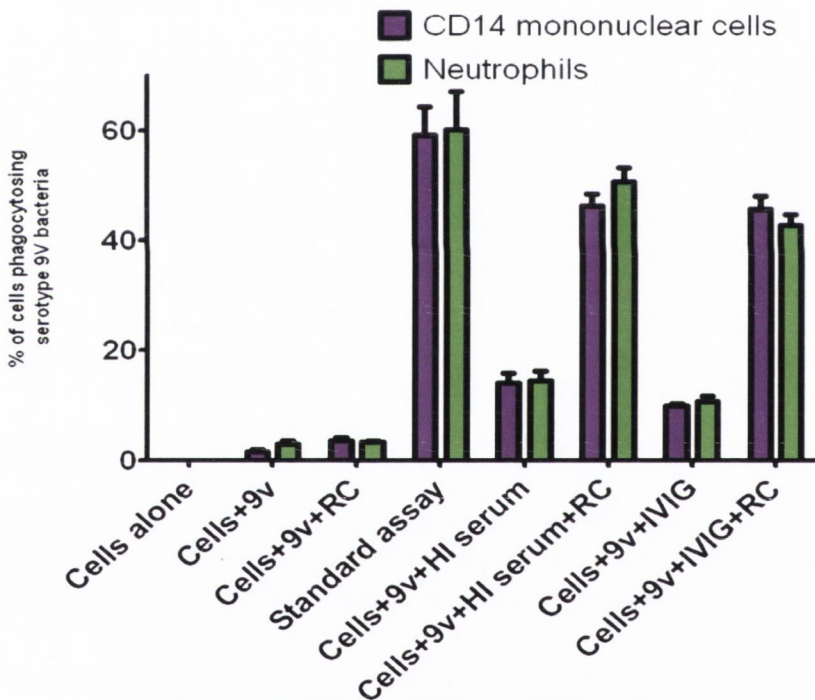


Figure 5.12 showing the percentage of CD14 mononuclear cells or neutrophils that phagocytose serotype 9v bacteria under different opsonisation conditions. Antibody and complement is required for efficient phagocytosis

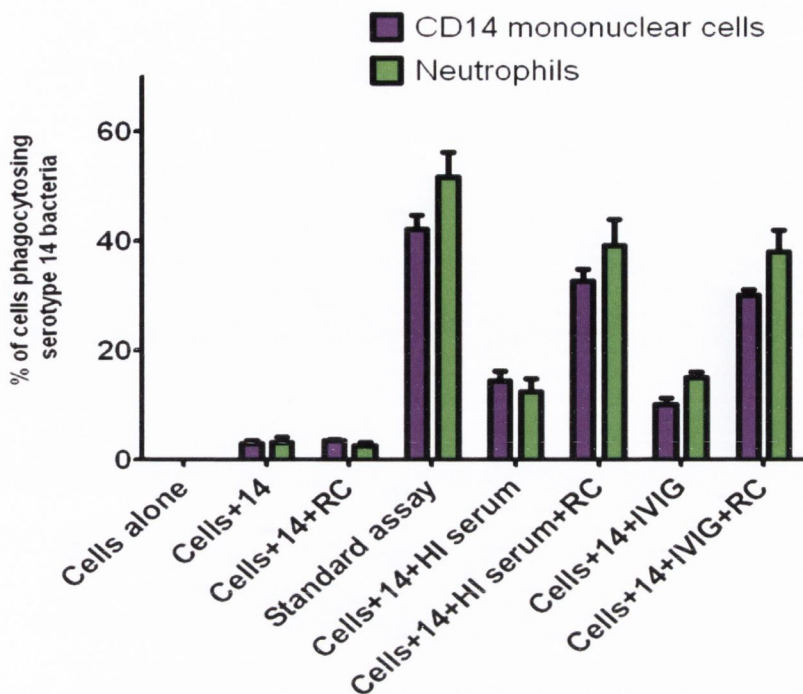


Figure 5.13 showing the percentage of CD14 mononuclear cells or neutrophils that phagocytose serotype 14 bacteria under different opsonisation conditions. Antibody and complement is required for efficient phagocytosis. Minimal opsonin independent phagocytosis is noted (n=3).

The fact that no opsonin independent phagocytosis of serotype 14 bacteria (as opposed to beads) was noted may suggest that the observation with beads is due to a non specific interaction between serotype 14 beads and monocytes. Further experimentation will be required to elucidate this.

Given the potential confounding influence of the non specific phagocytosis of serotype 14 beads by CD14 mononuclear cells subsequent examination of genotype dependent differences in whole blood phagocytosis used serotype 9v beads only. Both serotypes of bacteria were examined.

5.3.4 CD32A genotype affects CD14 mononuclear cell and neutrophil phagocytosis of opsonised serotype 9v beads

The whole blood phagocytosis assay was used to assess CD32A genotype dependent differences in serotype 9v bead phagocytosis in neutrophils and CD14 mononuclear cells. Significantly fewer CD14 mononuclear cells bearing the homozygote RR131 variant CD32A receptors phagocytose serotype 9v beads at 1hour than other genotypes ($p < 0.01$ vs HR, $P < 0.001$ vs HH)(Figures 5.14a and 5.15). Simultaneous measurement of neutrophil phagocytosis shows similar results (Figure 5.14b).

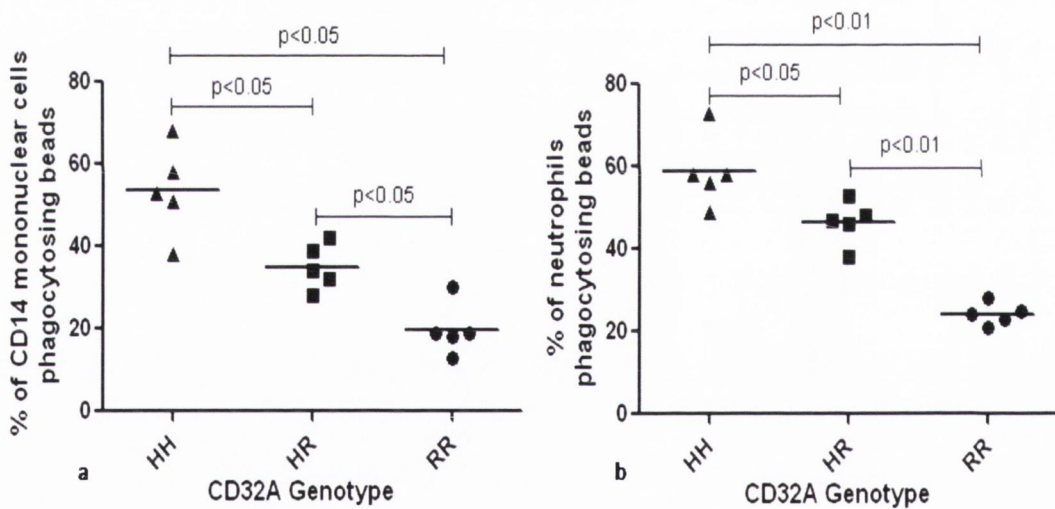


Figure 5.14 showing the proportion of CD14 mononuclear cells a) and neutrophils b) from donors of known CD32A genotype that phagocytose opsonised serotype 9v beads. RR donors exhibit reduced phagocytosis.

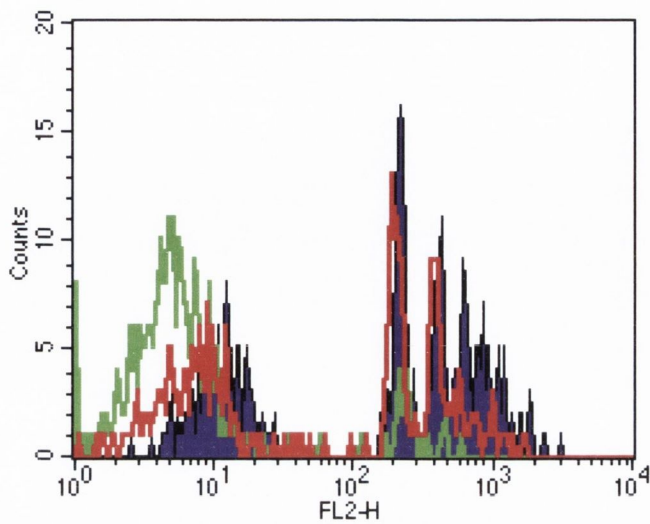


Figure 5.15 showing CD14 mononuclear cell phagocytosis of serotype 9v beads according to donor CD32A genotype. HH131 cells are shown in solid purple, HR131 in red and RR131 cells in green.

In further results that echo those from the isolated neutrophil phagocytosis assay, the proportion of neutrophils and CD14 mononuclear cells that phagocytose multiple beads is also reduced in the R131 variant group (Figure 5.16a and b). Similarly, across a range of serum dilutions differences in the proportion of CD14 mononuclear cells and neutrophils of different genotypes that phagocytose beads are clear (Figure 5.17a and b).

These results indicate that phagocytosis of serotype 9V beads is critically dependent on Fc receptor polymorphisms in terms of the percentage of cells phagocytosing, number of beads phagocytosed and dilution of antibody required to maintain efficient phagocytosis.

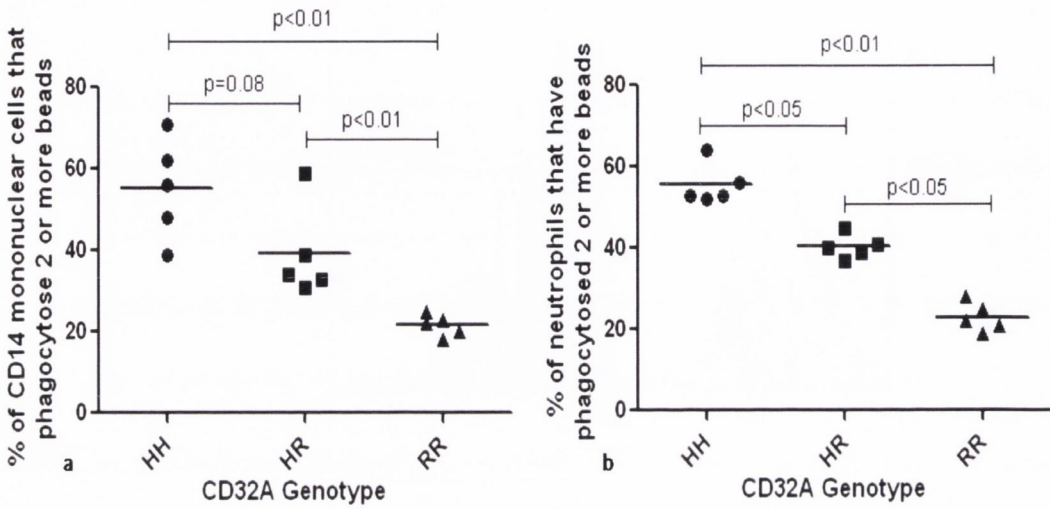


Figure 5.16 showing the proportion of CD14 mononuclear cells a) and neutrophils b) from donors of known CD32A genotype that phagocytose multiple opsonised serotype 9v beads. RR donors exhibit reduced phagocytosis efficiency

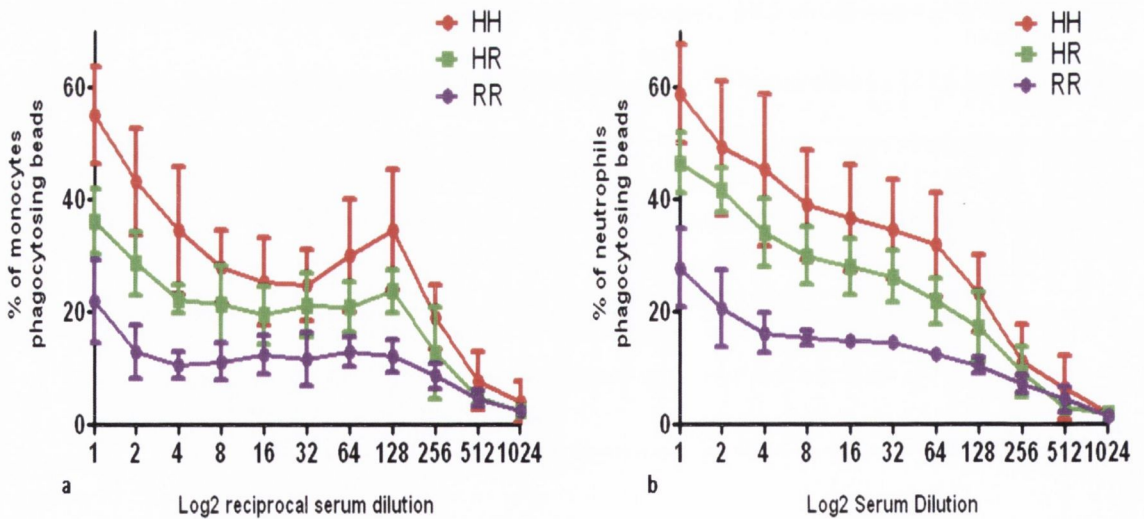


Figure 5.17 showing the proportion of CD14 mononuclear cells a) and neutrophils b) of known CD32A genotype that phagocytose serotype 9V beads across a range of dilutions of opsonising serum. RR donors exhibit reduced phagocytosis.

5.3.5 There are no CD32A dependent differences in the proportion of CD14 mononuclear cells that have phagocytosed beads at the 4 hour time point.

In order to establish if genotype dependent differences in phagocytosis in whole blood were maintained over a longer time frame, a 4 hour opsonophagocytic assay was carried out. At the four hour time point no difference in the proportion of CD14 mononuclear cells phagocytosing beads was detected between the varying donor genotypes (Figure 5.18a). Measurement of neutrophil phagocytosis did not demonstrate significant differences between HH and HR genotypes (Figure 5.18b). However, even at this time point, a lower proportion of RR131 donor neutrophils were observed to phagocytose beads.

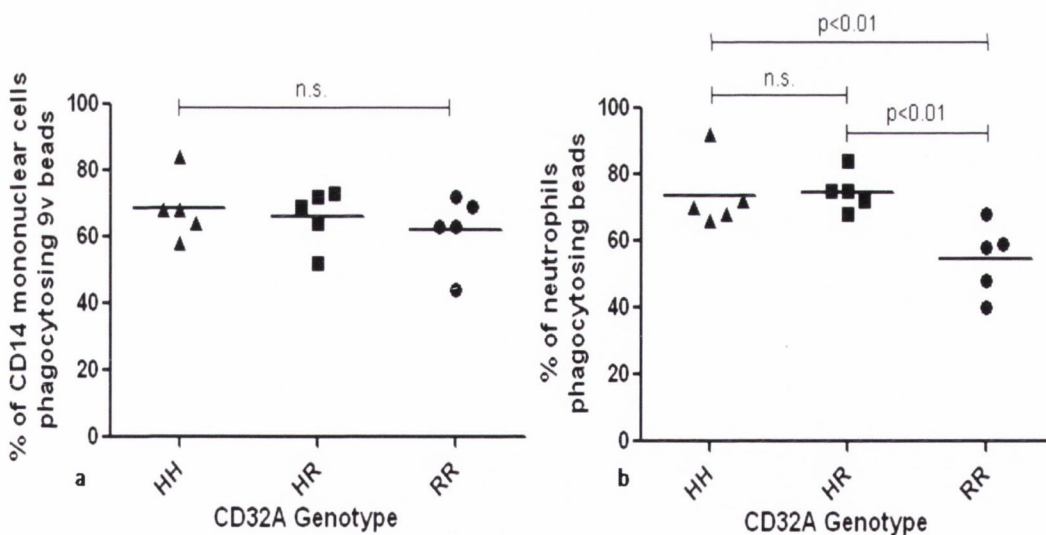


Figure 5.18 showing the proportion of CD14 mononuclear cells a) and neutrophils b) from donors of known CD32A genotype that phagocytose serotype 9V beads at a four hour time point. In CD14 mononuclear cells no difference is apparent. In neutrophils, fewer RR 131 cells have phagocytosed beads ($p < 0.01$) than other groups.

This result suggests that polymorphisms in CD32A may influence the efficiency of early phase of phagocytosis in CD14 mononuclear cells. This early phase, and the speed of response to a potentially pathogenic micro-organism in the lungs may be particularly important.

5.3.6 A significantly lower proportion RR131 variant donor cells phagocytose serotype 9v and serotype 14 bacteria than other variants.

In order to extend the results of the bead assay, FITC labeled heat killed highly encapsulated bacteria of serotypes 14 and 9v were prepared for use in the whole blood opsonophagocytic assay. When incubated with both neutrophils and CD14 mononuclear cells from donors of known CD32A genotype, RR131 bearing cells consistently produced lower levels of phagocytosis with both serotypes (Figure 5.19). There was no significant difference between HH and HR variants with either bacterial serotype. However, in each group of assays a higher proportion of HH131 donor cells phagocytosed bacteria. While not reaching statistical significance this suggests that HH131 neutrophils and CD14 mononuclear cells may be marginally superior to heterozygote cells in terms of phagocytosis.

The results obtained using pneumococci as targets in this whole blood assay replicate, for both neutrophils and CD14 mononuclear cells, the by now familiar pattern of inferior phagocytosis by RR131 bearing phagocytes.

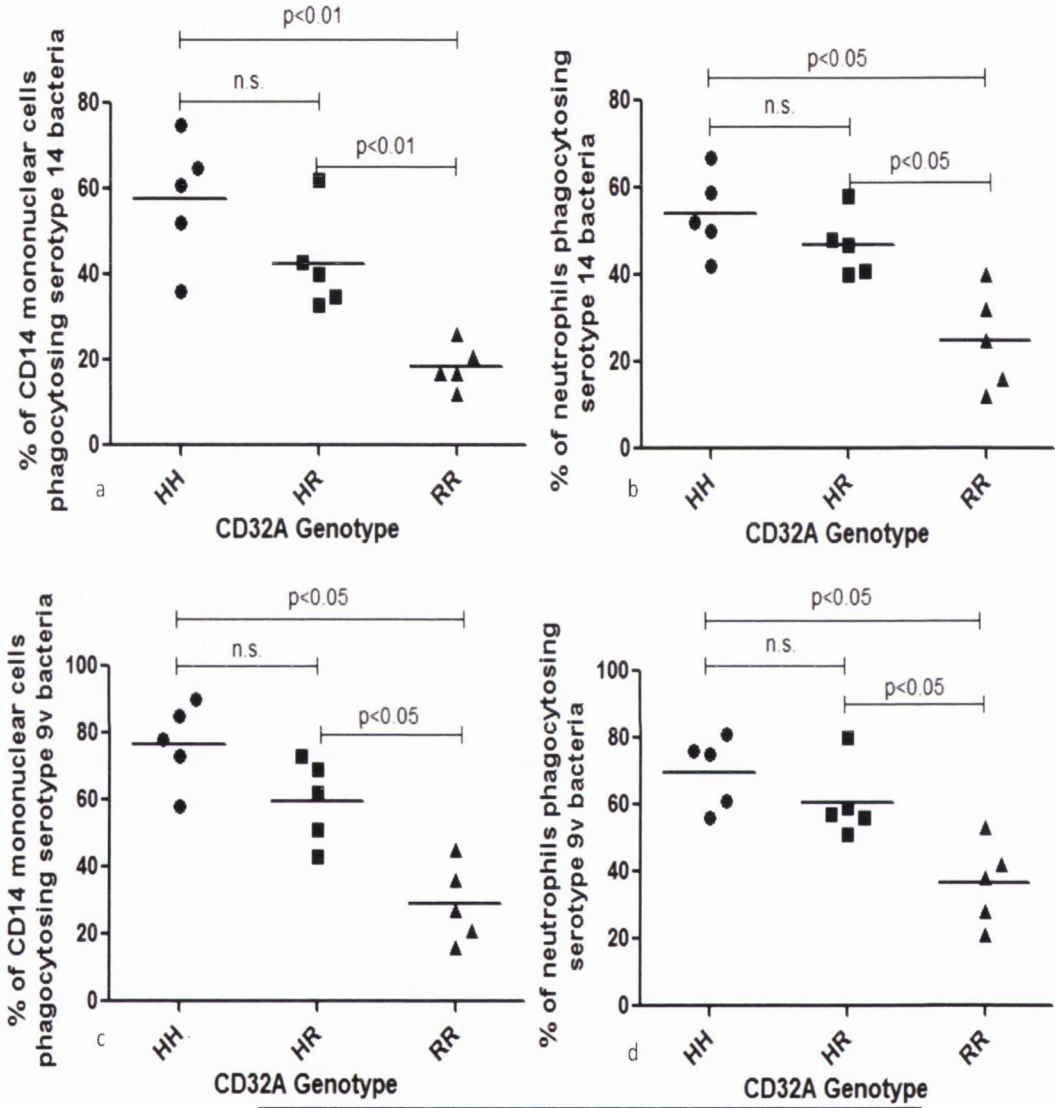


Figure 5.19 showing the percentage of CD14 mononuclear cells or neutrophils that phagocytose serotype 14 bacteria (panels a and b) or serotype 9V bacteria (panels c and d). Cells from RR131 donors produce inferior phagocytosis

5.3.7 No CD32A genotype dependent differences are noted in the oxidative burst generated in CD14 mononuclear cells by serotype 9v polysaccharide coated beads

A variant of the oxidative burst assay used with the isolated neutrophils was used in the whole blood assay. Activation of oxidative burst pathways, results in the generation of hydrogen peroxide which can oxidize non fluorescent dihydrorhodamine to green fluorescent rhodamine 123 detectable on flow cytometry. Dihydrorhodamine was added to the opsonophagocytic assay mix and the proportion of CD14 mononuclear cells staining positive after a 1 hour incubation was assessed. No significant differences were noted between the various CD32A genotype groups (Figure 5.20). A general trend to a reduced oxidative burst in CD14 mononuclear cells from R131 donors was observed. Although this trend did not reach statistical significance, it echoes results seen in the neutrophil phagocytosis assay. It seems likely that extension of this small cohort could well validate these findings further.

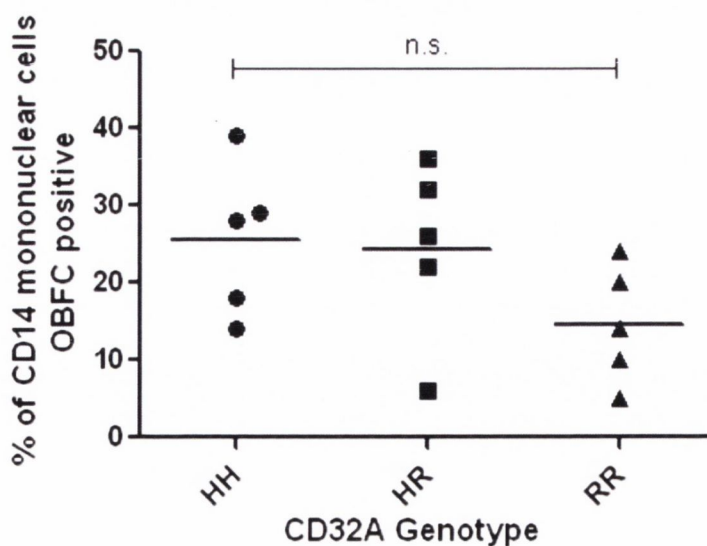


Figure 5.20 showing the percentage of CD14 mononuclear cells staining positive for oxidative burst. No significant difference is noted between the groups

5.3.8 No significant CD32A genotype dependent differences are noted in the amount of intracellular IL-1, IL-6 or TNF α detected in CD14 mononuclear cells.

Activated macrophages are important sources of cytokines which can drive the immune response. Macrophages produce large amounts of pro-inflammatory cytokines on appropriate stimulation which can recruit other inflammatory cells, stimulate T cell activation and drive the adaptive immune response. Differences in the activation of macrophages by opsonised targets via Fc receptors have the potential to influence downstream cytokine production potentially leading to an important general effect on systemic immunity. To determine whether such an effect was apparent, intracellular cytokines were examined.

Levels of intracellular IL-1, IL-6 and TNF α were measured in peripheral blood monocytes from donors of known CD32A genotype were measured after a 4 hour incubation with opsonised serotype 9v beads. Levels of intracellular cytokine staining were compared to incubation of cells with LPS alone. No significant genotype dependent differences in the production of intracellular IL-1 or TNF α by CD14 mononuclear cells were noted in this whole blood assay (Figures 5.21-5.22). A trend towards increased intracellular IL-6 staining was noted in CD14 mononuclear cells from RR131 donors stimulated with both opsonised serotype 9v bacteria and serotype 9v beads (Figure 5.23). This, however, did not reach the threshold for significance.

Using this assay and time scale no genotype dependent differences in cytokine secretion were noted. However, given the magnitude and reproducibility of differences in phagocytosis due to variation at CD32A it is hard to envisage that this would not have some effect on macrophage function and downstream signaling. Further experiments should look at cytokine secretion at more prolonged time points which may show differences. Assessment of the apoptotic response

of macrophages might also show important differences that could have an impact on inflammatory responses.

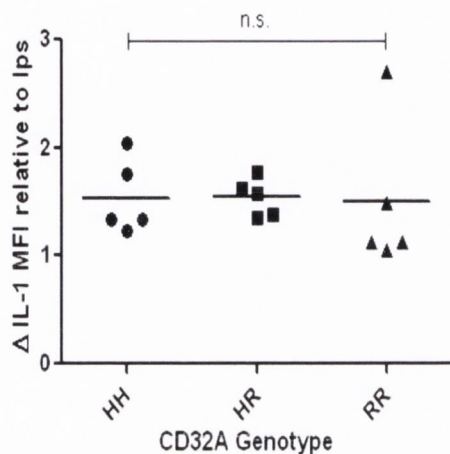


Figure 5.21 showing the mean fluorescence intensity of intracellular IL-1 staining (relative to LPS control) of CD14 cells of known CD32A genotype on stimulation

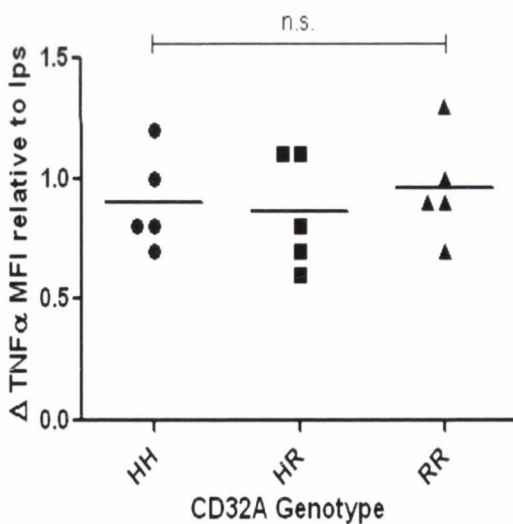


Figure 5.22 showing the mean fluorescence intensity of intracellular TNFα staining (relative to LPS control) of CD14 mononuclear cells from donors of known CD32A genotype on stimulation with opsonised serotype 9V beads

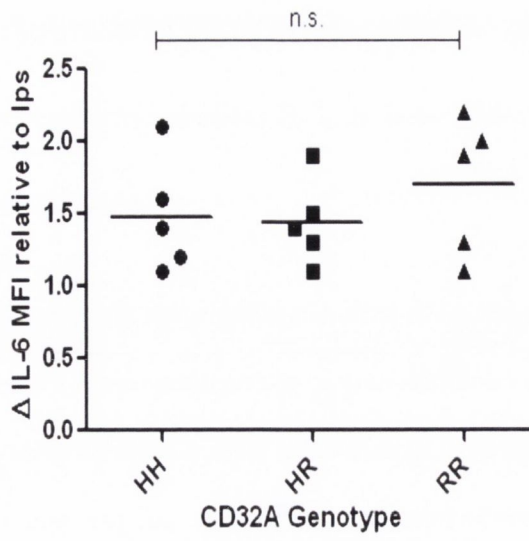


Figure 5.23 showing the mean fluorescence intensity of intracellular IL-6 staining (relative to LPS control) of CD14 mononuclear cells from donors of known CD32A genotype on stimulation with opsonised serotype 9V beads

5.4 Discussion

Cells of the monocyte lineage are central to the immune response in the lung. To build on the results generated in the neutrophil opsonophagocytosis assay, phagocytosis in peripheral blood CD14 mononuclear cells was examined. The cells arise from the same precursors as alveolar macrophages and both cell types express CD32A on the cell surface.

A flow cytometric opsonophagocytosis assay was established. This assay had the advantage of allowing assessment of phagocytosis of targets in both neutrophils and CD14 mononuclear cells simultaneously. Others have used similar assay techniques using whole blood preparations with differing methods of monocyte identification (Chaka et al., 1995, Prodan et al., 1995). This project expanded on these basic techniques allowing the assessment of oxidative burst and intracellular cytokine production.

A marginally higher proportion of neutrophils phagocytosed serotype 14 and 9v beads in comparison to CD14+ mononuclear cells (Figure 5.9a and b, page 197). This was consistent across time points and experiments. Others have demonstrated a superiority of neutrophils for the phagocytosis of group B streptococci using flow cytometric techniques confirming historical data (McCloskey and Salo, 2000, Steigbigel et al., 1974). There are a number of possible explanations for this observation. The difference may be intrinsic to the assay; in the whole blood assay neutrophils are in excess and are more likely to encounter and phagocytose opsonised targets. Alternatively, the difference in surface receptor profiles between the two lineages, or levels of intrinsic activation of the cells may be responsible. However, the differences demonstrated in this assay system are minimal and suggest that peripheral blood CD14 mononuclear cells are capable of phagocytosing pneumococcal polysaccharide coated targets.

Examination of the opsonising conditions required for phagocytosis of serotype 9v beads by CD14 mononuclear cells and neutrophils demonstrated a requirement for complement and antibody to achieve optimal phagocytosis (Figure 5.10, page 198). These results echo those found in the neutrophil assay. However, as observed with the neutrophil assay, a small but reproducible amount of phagocytosis was observed with antibody alone. With serotype 9v beads minimal opsonin independent phagocytosis was observed (Figure 5.10).

Similar experiments were carried out using serotype 14 beads. These showed some unexpected results. Figure 5.11 (page 199) indicates that in keeping with previous findings a combination of opsonising complement and antibody is required for optimal phagocytosis in CD14 mononuclear cells and neutrophils in whole blood. However, it was apparent that a proportion of CD14 mononuclear cells were capable of phagocytosing serotype 14 beads in the absence of opsonins. This was not the case with serotype 9v beads (Figure 5.10, page 198). These results were consistent both within and across bead batches (data not shown).

Opsonin independent phagocytosis is thought to be important in the early defence against infection and in the young (Chattopadhyay et al., 2011, Miyata and van Eeden, 2011).

Monocytes possess scavenger receptors on their surface which can initiate phagocytic pathways (Parod and Brain, 1986). Phagocytosis can be mediated by members of the SR-A family (including MARCO), SIGN-R1, mannose receptors or integrins on monocyte and macrophage surfaces (Ofek et al., 1995). SIGN-R1 can bind capsular polysaccharide of different serotypes (Kang et al., 2004). Interestingly, these experiments demonstrated that polysaccharide 14 was the most effectively recognised by SIGN R1. SR-A and MARCO also bind pneumococci although the molecular target of these interactions is not clear (Paterson and Orihuela, 2010). Hence, while the observations detailed here may be assay specific, the opsonin independent phagocytosis of serotype 14 beads may reflect the function of monocyte cell surface pattern recognition molecules.

Bacterial phagocytosis in the whole blood assay was more efficient for both serotypes than bead phagocytosis. Attempts to reproduce the significant opsonin independent phagocytosis observed with serotype 14 beads with serotype 14 bacteria were unsuccessful (Figure 5.13, page 201). The reason for this is unclear but could be explained by the apparent relative increase in affinity for serotype 14 polysaccharide compared with other serotypes shown in Kang's experiments (Kang et al., 2004). Another possible explanation could include differences in the distribution, concentration and configuration of serotype 14 polysaccharide on the bead surface as opposed to bacteria. Other investigators have demonstrated that pneumococcal polysaccharide interaction with mannose receptors on macrophages may be conformation dependent (Zamze et al., 2002). Alternatively this interaction could be non-specific. Further experimentation will be required to determine the significance, if any, of these results.

This group of experiments demonstrates that opsonisation by antibody and complement of serotype 14 and 9v beads and bacteria is required for efficient phagocytosis by both CD14 mononuclear cells and neutrophils (Figure 5.12 and 5.13, pages 200-201). These results supported the use of conditions similar to those used in the neutrophil OPA.

Serotype 9v beads were used for subsequent experiments examining CD32A genotype dependent differences in phagocytosis. This avoided the potential confounding influence of opsonin independent phagocytosis. Results presented in figures 5.14-5.17 (pages 202-204) show that the proportion of CD14 mononuclear cells that phagocytose serotype 9v beads varies depending on CD32A genotype. Significantly less CD14 mononuclear cells bearing RR131 receptors phagocytose serotype 9v beads than other genotypes across a range of dilutions. Neutrophils in this whole blood assay follow a similar pattern. Results for both cell types are similar to findings for the neutrophil opsonophagocytic assay. RR131 bearing neutrophils and CD14 mononuclear cells also phagocytose fewer beads than other genotypes. These results

suggest that CD32A polymorphisms affect the phagocytic capacity of neutrophils and CD14 mononuclear cells with respect to serotype 9v targets in whole blood.

In this set of experiments we extended observations on phagocytosis out to 4 hours. At this time point RR131 bearing neutrophils still exhibited a relative deficiency in phagocytosis (Figure 5.18, page 205). However, no difference in the proportion of CD14 mononuclear cells phagocytosing serotype 9v beads at this time point was detected. This suggests that CD32A receptor genotypic variation may be particularly important in CD14 mononuclear cells in the time period immediately after exposure to opsonised targets.

It is well established, and illustrated again in the present study that opsonisation improves phagocytosis. Opsonisation allows phagocytosis to take place quickly. Antibody and complement deficiencies result in slow phagocytosis and susceptibility to infection. This factor of the speed of the immune response is an understudied area, but likely to be of significance. These experiments suggest that CD14 mononuclear cells with differing CD32A genotypic variants are capable of similar levels of phagocytosis over 4 hours, despite clear differences after 1 hour. The impact of such a disparity is unclear. However, it is generally accepted that a slow rate of phagocytosis allows bacterial numbers to increase, while increased phagocytosis rates permit increased bacterial clearance (Anand et al., 2007). Opsonic defects lead to reduced phagocytosis and reduced rates of phagocytosis and clinically manifest as susceptibility to infection. It is apparent that CD32A H131R receptor variation can also reduce the rate of phagocytosis. It is interesting to speculate that defective early phagocytosis by RR131 bearing CD14 mononuclear cells allows an increase in bacteria that facilitating the establishment of overt clinical infection. These experiments use inert polysaccharide beads and killed bacteria, the impact of an actively replicating bacterial population on these findings and on apoptosis and necrosis of phagocytosing cells is not established.

Whole blood phagocytosis assays were also carried out using serotype 14 and serotype 9v bacteria. These results confirmed that phagocytosis in RR131 variant neutrophils and CD14 mononuclear cells was reduced compared to other genotypic variants (Figure 5.19, page 208). This whole blood assay provides additional confirmation of the findings of the neutrophil OPA. It shows that RR131 variant neutrophils have reductions in the magnitude and efficiency of the phagocytosis of serotype 9v beads and bacteria. This extends the previous findings to another pneumococcal serotype. This is strong evidence that the observation of a relative phagocytosis defect in neutrophils with RR131 variant receptors may not be serotype specific. Further experiments will be needed to see if such results apply to other encapsulated bacterial species.

These results provide proof in principle that the phagocytic capacity of cells of the monocyte/macrophage lineage is affected by CD32A H131R polymorphisms. This has potential implications for pneumococcal disease in the lung, where alveolar macrophages play a role in defence against infection and regulation of inflammation.

Monocytes generate an oxidative burst on phagocytosing targets. Dihydrorhodamine was used as an indicator of the presence of an oxidative burst. This choice of this dye allowed compatibility with other fluorescent stains. In contrast to hydroethidium used in the neutrophil OPA which measures superoxide production, dihydrorhodamine is more sensitive to the production of hydrogen peroxide (Walrand et al., 2003). These subtle differences in sensitivity for different components of the oxidative burst assay were not expected to influence results for these OPA experiments.

No statistically significant genotype dependent differences were observed in oxidative burst in CD14 mononuclear cells. However, a trend towards reduced oxidative burst was noted in RR131 bearing cells (Figure 5.20, page 210). These results suggest that efficient monocyte phagocytosis of pneumococcal targets promotes an enhanced oxidative burst. The finding of genotype

dependent differences in oxidative burst supports work suggesting that CD32A is the dominant Fc receptor involved in NADPH activation.

Cytokine production by monocytes and macrophages is important in driving the immune response. Monocytes have been shown to produce pro-inflammatory cytokines when exposed to pneumococci (Anand et al., 2007). Murine studies have shown rapid production of IL-1 β , IL6 and TNF α by murine alveolar macrophages when stimulated with pneumococci (Anand et al., 2007). Monocytes stimulated with pneumococci can drive Th1 and Th17 responses in human cells (Olliver et al., 2011). Monocyte cytokine secretion after exposure to pneumococcal stimuli may be important in lung immunity and inflammation. CD14 mononuclear cells were examined for genotype dependent differences in cytokine production in whole blood. These experiments indicated that both 9v polysaccharide coated beads and bacteria could elicit production of proinflammatory cytokines IL-1, IL-6 and TNF- α (Figures 5.21, 5.22, 5.23, pages 2011-212). Intracellular cytokine staining was more intense when bacterial targets were used. No genotype dependent differences in the production of proinflammatory cytokines were identified.

There are a number of possible explanations for this result. Cytokine production was measured at four hours. This may not be a long enough incubation to allow differences in cytokine production to be measured. Data generated by stimulating macrophages with the 23 valent pneumovax vaccine noted a peak cytokine response at 24 hours with minimal responses at 4 hours (Hong et al., 2010). Notably these experiments did not include an opsonised phagocytic stimulus, relying instead on polysaccharide and adjuvant stimulation of innate immune receptors. Opsonisation with IgG2 appears to be a particularly potent stimulus of monocyte TNF- α and IL-6 production (Foreback et al., 1997). Alternatively, the measured equivalence of pneumococcal phagocytosis at 4 hours between the genotypes could amount to an equivalence

of the stimulus for cytokine production. A third explanation is that the stimulus for cytokine production occurs independently of CD32A and is therefore unaffected by variation in the receptor. However, others have observed cytokine production on immune complex CD32A mediated peripheral blood monocyte stimulation (Mathsson et al., 2006, Mullazehi et al., 2006). In contrast recent data has suggested a disconnect between CD32A activation and secretion of pro-inflammatory cytokines. Dai et al observed that differential signalling pathways between CD64 and CD32A resulted in clear differences in function in human monocyte cell lines (Dai et al., 2009). CD64 stimulation produced higher levels of pro-inflammatory cytokines after immune complex stimulation (measured at 24 hours). In contrast, CD32A stimulation resulted in the generation of NADPH oxidase products, enhanced antigen presentation but impaired cytokine secretion. This raises the possibility that despite its importance in phagocytosis, CD32A does not play a dominant role in cytokine production in human monocytes. If this is the case, it is unsurprising that CD32A genotypic variation would not give rise to differences in the secretion of proinflammatory cytokines. Further studies using extended incubations would be required to exclude any influence of CD32A variation on CD14 mononuclear cell cytokine production.

Evidence presented here demonstrates differences in phagocytosis and oxidative burst between CD32A genotypes in CD14 mononuclear cells. This difference in phagocytosis, particularly in the early phase may have further functional impact.

Apoptosis of alveolar macrophages is important in the regulation of inflammation in the lung in pneumococcal infection (Dockrell et al., 2003). Phagocytosis of apoptotic macrophages reduces expression of proinflammatory cytokines and reduces neutrophil recruitment and may contribute to successful resolution of inflammation (Marriott et al., 2006). Reduced alveolar macrophage apoptosis is associated with increased inflammation (Marriott et al., 2006). Studies in paediatric asthmatics have linked reduced phagocytosis with increased alveolar macrophage apoptosis (Fitzpatrick et al., 2008). This increased macrophage apoptosis may have an

immunoregulatory effect. Studies have linked phagocytosis of pneumococci with the induction of apoptotic pathways (Marriott et al., 2004). It is interesting to speculate that a reduction in phagocytic capacity in RR131 bearing macrophages might propagate a knock on reduction in macrophage apoptosis. This could predispose to increased inflammation and a failure to clear pathogenic organisms. One extension of experiments presented in this project would be to examine for genotype dependent differences in apoptosis of CD14 mononuclear cells after exposure to pneumococcal targets.

One of the major limitations of this section of the project is the use of peripheral blood CD14 mononuclear cells as a surrogate for lung macrophages. Caution should be exerted in linking the findings presented here directly to lung pathology in the form of bronchiectasis. Further studies using cultured and primary alveolar macrophages, while challenging and not without limitations, are required to confirm the potential clinical relevance of the relative phagocytic defect presented here.

5.5 Conclusion

In summary significant defects have been documented in the phagocytic capacity of both neutrophils and monocytes from individuals with polymorphisms in CD32A. Taken together with the previously documented association between bronchiectasis, and its severity, with the same polymorphisms of CD32A, these findings give a plausible mechanistic explanation for this disease association.

Chapter 6. Mannose binding lectin variation in bronchiectasis

6.1 Introduction

6.1.1 Complement

The complement system is the major humoral component of the innate immune system (Du Clos T.W., 2008). This tightly regulated group of proteins is composed of the classical, alternative and lectin cascades (Figure 6.1). Complement has a number of important immune functions that can be divided into three major components; initiation of an inflammatory response, opsonisation of foreign material, clearance of apoptotic cells and lysis of susceptible cells (Du Clos T.W., 2008).

The classical pathway is initiated mainly by immune complexes, while alternative and lectin pathways are triggered by bacterial cell wall components (Du Clos T.W., 2008). Amplification of the protein cascades at a number of steps enables the production of a rapid inflammatory response. In addition the presence of complement receptors on a variety of cells of the adaptive immune system provides an important link with the innate immune system.

Mannose binding lectin is a member of the collectin family of plasma proteins (Abbas et al., 2007). The collectins, which also include the pulmonary surfactant components SP-A and SP-D, are a family of soluble pattern recognition molecules characterised by a calcium dependent lectin head connected to a collagen like tail (Seaton et al., 2010). MBL binds to mannose and fucose on bacterial cell surfaces, functioning as an opsonin and initiating the lectin complement pathway (Du Clos T.W., 2008). This molecule represents an important part of an innate immune microbial recognition pathway.

MBL is mainly produced in the liver (Jack et al., 2001a). Levels rise during infection and inflammation, therefore it acts as an acute phase protein. The MBL structural unit is a homotrimer of peptides associated in a tightly coiled collagen like triple helix, similar to C1q (Figure 6.2) (Turner and Hamvas, 2000). Each subunit possesses a lectin head which recognises pathogen oligosaccharides (Holmskov et al., 2003). MBL may also be involved in the recognition

of apoptotic and necrotic cells (Nauta et al., 2003). Circulating MBL exists in higher order multimers of the homotrimer. Higher order multimers bind pathogen more efficiently allowing optimal activation of mannose associated serine proteases (MASP) and initiation of the lectin complement cascade. The six-subunit form is the major circulating form (Wong and Sim, 1997). Forms with fewer subunits bind less avidly to carbohydrate surfaces and are defective in complement activation (Lu et al., 1990).

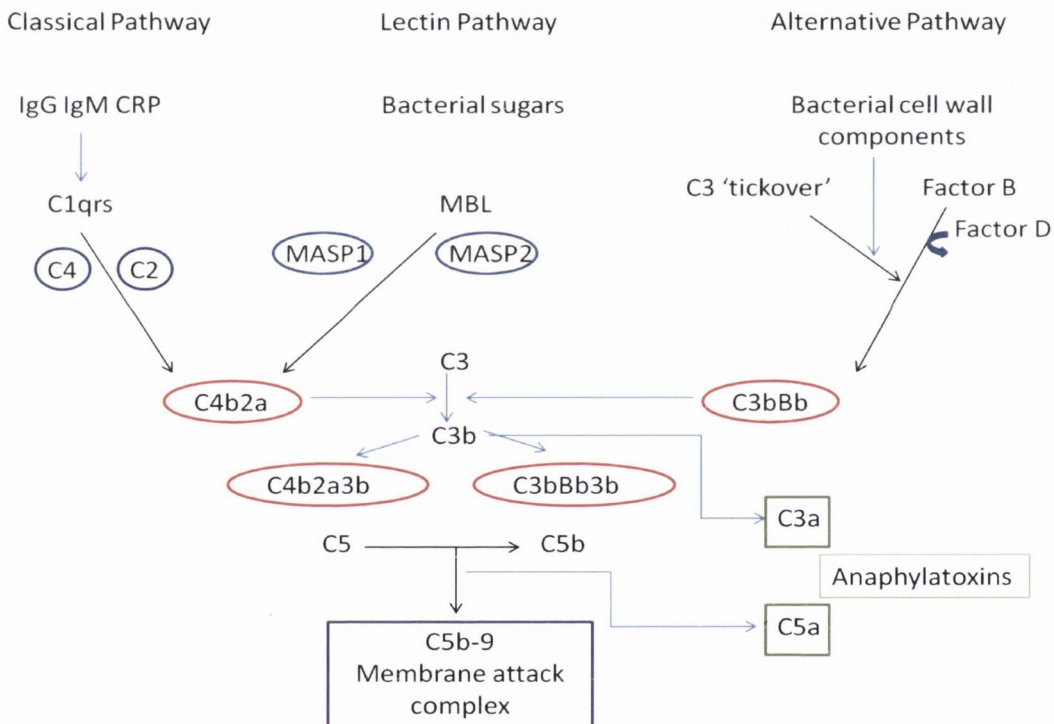


Figure 6.1 showing the classical, lectin and alternative cascades that make up the complement system of proteins. MBL is the initial component of the lectin cascade.

6.1.2 MBL genetics

Serum MBL concentrations vary considerably. This variability is largely dependent on genetic factors. MBL is encoded by the gene MBL2 on chromosome 10. MBL2 is comprised of four exons (Figure 6.2). Three common missense mutations in exon 1 result in considerable variability in serum levels. These mutations, at codon 52 (Arg-Cys), codon 54 (Gly-Asp) and codon 57 (Gly-Glu) result in disruption of the coiled collagen-like triple helix. This leads to failure of the normal multimerisation of the MBL structural subunit. Low order oligomers are inefficient at activating the complement cascade and are more rapidly degraded (Roos et al., 2004). Wild type MBL is designated A with the various mutants designated B (codon 54), C (codon 57) and D (codon 52) in order of their discovery (Sastry et al., 1989). These mutant alleles can be collectively referred to as O. MBL serum levels exhibit considerable variation within each genotype some, of which is due to additional polymorphisms in the promoter (H/L and X/Y variants) and 5' untranslated (P/Q variant) region of the MBL2 gene. Promoter polymorphisms have a particular influence on

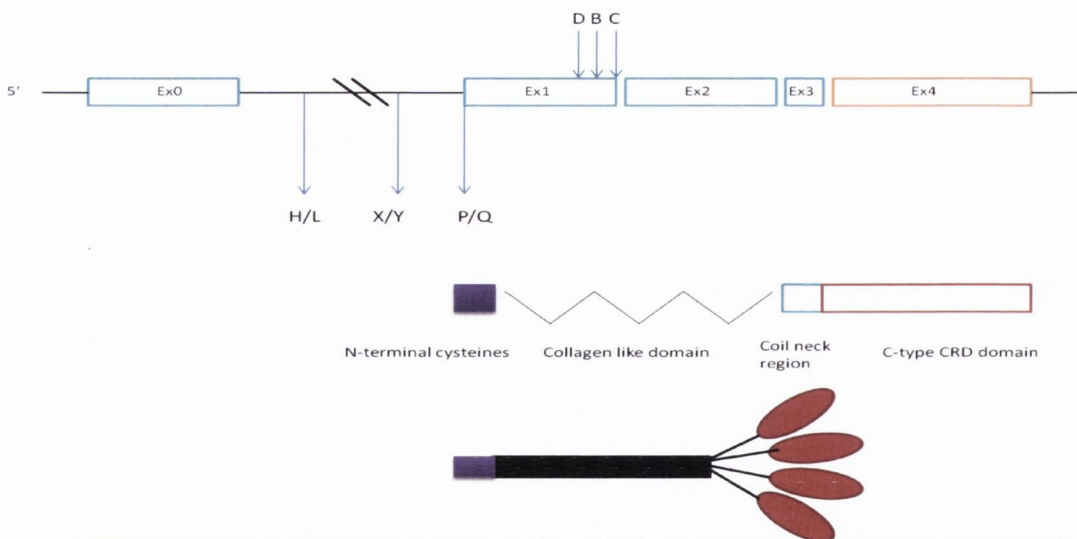


Figure 6.2 showing MBL gene (top), polypeptide (middle) and oligomeric structure. The top panel indicated the position of the main promoter (H/L, X/Y and P/Q) and exonic (B.C.D) variants. The lower panel shows a tetrameric form of MBL.

MBL levels in the presence of a wild-type (A) coding region. HYP A, LYP A, LYQA and LXPA are associated with high, intermediate, low-intermediate and low MBL levels respectively. In

addition to these wild type haplotypes, only three variant haplotypes, LYPB, LYQC and HYPD are observed. In mutant homozygotes or compound heterozygotes functional circulating MBL is essentially absent.

MBL genotype is also subject to considerable ethnic variation, due to the difference in the frequency of inheritance of mutant variants in different populations. In Caucasians the prevalence of the wild type genotype (A/A) is 60-70% while MBL heterozygosity (A/O) is 30-40%. Mutant homozygosity (O/O) is less common 0-10% (Madsen et al., 1998, Mead et al., 1997, Minchinton et al., 2002). Variant alleles are found in even higher proportions in some ethnic groups. The high prevalence of MBL variants has led to speculation about the potential for an associated survival advantage to counterbalance the immunological disadvantages of low MBL levels (Bouwman et al., 2006). Hypotheses suggest that low MBL levels may reduce immune mediated tissue damage in children during inflammatory insults or that high MBL levels facilitate the uptake of intracellular pathogens. Advances are needed in our understanding of the reasons for genetic variability at MBL2.

6.1.3 MBL activation

MBL is the only member of the collectin family capable of activating complement. Complement is an important innate immune defence system. Activation of the complement cascade has numerous outcomes including inflammation, opsonisation and cell lysis. High order MBL oligomers associate with MBL-associated serine proteases (MASP) 1, 2 and 3, and the non protease Map19 in the circulation. Binding of the C-type lectin head of MBL to pathogen carbohydrate motifs induces a conformational change in MBL resulting in MASP activation. The MASPs are homologous to C1r and C1s. Although exact mechanisms are undetermined evidence suggests that MASP-2 plays the central role in downstream complement activation. MASP-2 activation results in cleavage of C4, ultimately producing C4b2a which is a C3 convertase (Figure

6.1, page 219). This results in C3 activation and the generation of the anaphylatoxins C3a and C5a. C5b is also generated, which initiates formation of the membrane attack complex. MASPs are also subject to genetic heterogeneity which may impact on the overall integrity of the lectin pathway of complement (Thiel et al., 2009).

MBL can also facilitate phagocytosis. By complement activation MBL can result in opsonic C3b deposition on the microbial surface allowing phagocytosis, or, phagocytosis can occur through an intrinsic opsonic effect of MBL itself. MBL can function in isolation as an opsonin with *N. Meningitidis*, influenza A and mycobacteria (Hartshorn et al., 1993, Jack et al., 2001b, Polotsky et al., 1997). Alternately MBL may act to enhance phagocytic uptake of organisms in conjunction with other opsonins (Ghiran et al., 2000, Levitz et al., 1993).

MBL may have a role in the modulation of the inflammatory response although reports are conflicting. MBL has been shown to inhibit monocyte TNF- α release upon stimulation with cryptococcal membrane glycoprotein, while increasing MBL concentrations resulted in increased TNF α and IL-6 release from *Leishmania chagasi* exposed monocytes (Ghezzi et al., 1998, Santos et al., 2001). Studies of MBL activation with *Neisseria* noted an initial brisk increase in pro-inflammatory cytokines (Jack et al., 2001b). However, at higher MBL concentrations, towards physiological upper limits, monocyte cytokine production was suppressed. While MBL may be able to modulate inflammation its effects may be complex and differ depending on infecting organism and time point in the illness.

6.1.4 Measurement of MBL; defining deficiency

Measurement of MBL in diagnostic laboratories has proven controversial. A variety of different assay systems have been used (Bay and Garred, 2009, Forster-Waldl et al., 2003, Gadjeva et al., 2004). Many modern assays measure higher order serum MBL oligomers by ELISA (Forster-Waldl et al., 2003). These quantitative assays measure functional high order MBL excluding the measurement of the lower order oligomers associated with variant alleles. Authors argue that these assays are superior to the measurement of monomeric MBL which can overestimate the MBL functional levels and are less cumbersome and time consuming than pure functional lysis assays (Forster-Waldl et al., 2003). Genotype specific reference ranges have not been established. Additionally, the serum definitions of MBL deficiency and insufficiency have not been established. Furthermore, whether analysis of serum MBL levels or genetic analysis or a combination of both is the optimum way to study MBL in association with disease has not been determined.

6.1.5 MBL deficiency and disease

MBL variant genotypes are common in Caucasians (Garred, 2008). Low serum levels of MBL are also common. Additionally absence of detectable circulating functional MBL is observed not infrequently (Minchinton et al., 2002). MBL levels and variant MBL haplotypes have been studied in a wide variety of disease states (Alipour et al., 2009, Altorjay et al., 2010, Bouwman et al., 2005, Brouwer et al., 2008, Chapman et al., 2010, Eagan et al., 2010, Frakking et al., 2011, Garred et al., 1997, Gordon et al., 2006, Graudal et al., 2000, Hegele et al., 2000, Hoeflich et al., 2009, Jacobsen et al., 2001, Minchinton et al., 2002, Muhlebach et al., 2006, Nisihara et al., 2010, Peterslund et al., 2001, Santos et al., 2001, Yuen et al., 1999). Both increased and decreased MBL level have been associated with disease.

MBL deficiency was first associated with clinical disease in 1968 in a child suffering from dermatitis, diarrhoea and recurrent bacterial infections. Defective phagocytosis of yeast and bacterial particles was identified and ultimately shown to be due to MBL polymorphisms (Sumiya et al., 1991). The importance of innate immune mechanisms in the response to infection has led investigators to question whether low or absent MBL levels are associated with infection susceptibility.

MBL deficiency has been associated with a number of bacterial infections. A large UK study suggested an association of MBL deficiency and early childhood meningococcal disease (Hibberd et al., 1999). However, others have not found this association (Garred et al., 1993). Similarly, controversy exists over an association with invasive pneumococcal disease (Roy et al., 2002, Kronborg et al., 2002). Murine and *in vitro* studies suggest MBL deficiency may also have a role in susceptibility to *Staph aureus* however definitive *in vivo* evidence is not yet available. Other studies indicate that MBL deficiency may be important when immunity is compromised by a number of different mechanisms, for example during early immune system maturation (Auriti et al., 2010). MBL deficiency has been associated with infection in the setting of stem cell transplant, post chemotherapy, post surgery and in Down's syndrome (Mullighan et al., 2002, Nisihara et al., Peterslund et al., 2001). In liver transplantation the MBL status of the donor determines the risk of life threatening infection, suggesting the major importance of hepatic MBL in post transplant host defence (Bouwman et al., 2005). MBL deficiency has also been identified as a poor prognostic factor in paediatric oncology patients (Frakking et al.). However, this association between MBL deficiency and reduced event free survival was not completely explained by infection susceptibility. Frakking et al hypothesised that poor clearance of apoptotic cells in MBL deficient paediatric oncology patients could play a role in early relapse thus reducing event free survival (Frakking et al., 2011). These data suggest that MBL deficiency may be associated with bacterial infection.

MBL has been shown to bind to a number of viruses and may play a role in innate immunity to viral infection. *In vitro* data demonstrating binding of MBL to the HIV envelope protein gp120 and inhibiting HIV entry into cultured lymphoblasts led to speculation of a disease modifying role in HIV (Ezekowitz et al., 1989). However serum MBL levels may not reach high enough concentrations in humans to effect viral neutralisation (Ying et al., 2004). *In vivo*, a number of studies suggest that MBL deficiency increases the risk of HIV acquisition, including acquisition by vertical transmission (Boniotto et al., 2000, Garred et al., 1997, Nielsen et al., 1995, Prohaszka et al., 1997). Other studies do not demonstrate an association (Malik et al., 2003, McBride et al., 1998). Conflicting data also exists regarding disease progression. Maas et al demonstrated a delay in the development of AIDS from HIV seroconversion (Maas et al., 1998). However, Garred et al noted reduced survival times after the development of AIDS possibly due to an increase in opportunistic infection (Garred et al., 1997). The involvement of MBL in HIV pathogenesis and progression is complex and deserves continued scrutiny. B variant MBL alleles have been associated with progression to cirrhosis and the development of spontaneous bacterial peritonitis in chronic hepatitis B (Yuen et al., 1999). Association studies with hepatitis C remain inconclusive. *In vitro* and murine studies have demonstrated a potential role for MBL in influenza A and herpes simplex 2 infection, although human association studies are at present lacking (Chang et al., 2010). Small studies have associated MBL deficiency with susceptibility to fungal pathogens such as *aspergillus fumigatus* in immunocompromised patients (Chang et al., 2010). Mannan, to which MBL binds, is an important cell wall component in many fungi (Chang et al., 2010).

The putative role for MBL in immunity to infection and in modulation of the inflammatory response has led to examination of the importance of MBL status in sepsis. Studies in the adult and paediatric setting have shown that MBL variant alleles were associated with severity of sepsis (Fidler et al., 2004, Garred et al., 2003, Gordon et al., 2006). In contrast Huh *et al* did not

note a clear association of sepsis syndromes with MBL variants but did observe low serum MBL levels to be an independent risk factor for mortality in an adult cohort (Huh et al., 2009).

Investigators have examined how MBL may contribute to lung defence. MBL is present in infected airways (Fidler et al., 2009). In particular MBL was detected in the airways of the acutely inflamed lung rather than in chronically diseased airways (Fidler et al., 2009). Debate remains over whether this represents a true defence mechanism or bystander serum leak into airways due to increased permeability during acute infection (Jambo and Gordon, 2009). Variation in MBL2 has been identified as an important disease modifying gene in cystic fibrosis. Low MBL levels and variant genomes were associated with a more rapid decline in lung function, earlier *P. Aeruginosa* colonisation and poorer survival (Garred et al., 1999, Drumm et al., 2005, Muhlebach et al., 2006).

Chronic obstructive pulmonary disease (COPD) is a smoking related chronic lung disease characterised by airflow limitation due to chronic bronchitis and emphysema. Worldwide it is the fourth leading cause of death. COPD is associated with infective exacerbations resulting in increased mortality and morbidity. Yang *et al* found an association of the B variant allele with hospital admissions with an infective exacerbation of COPD (Yang et al., 2003). Other variant alleles were not examined. A second recent study demonstrated an association between recurrent infective exacerbations and MBL B and XA alleles (Lin et al., 2011a). In contrast Eagan *et al* found no association with low serum MBL levels and a variety of markers of severity in COPD (Eagan et al.).

MBL status has been examined before in bronchiectasis. Kilpatrick *et al* examined serum MBL in bronchiectasis and found no association with low MBL. MBL genotype was not examined. They did find lower levels of another collectin molecule, L-ficolin in the bronchiectasis cohort when compared to disease and healthy control groups (Kilpatrick et al., 2009). Whether measurement

of MBL levels alone is sufficient in the examination of the relationship between MBL and an inflammatory disease such as bronchiectasis is open to debate.

MBL, like other complement components, has a role in the clearance of apoptotic cells.

Apoptotic cells express increased amounts of fucose and other carbohydrate moieties which MBL is capable of binding. Low MBL could therefore result in impaired apoptotic cell clearance, increasing the risk of autoimmune disease. Alternately, high MBL could exacerbate inflammation after initial tissue damage, although there is scant evidence to support this side of the 'double edged sword'. In SLE MBL deficiency and MBL variant alleles have been associated with the disease itself and with indices of severity such as renal involvement, thrombosis and infection susceptibility (Garred et al., 2001), (Ohlenschlaeger et al., 2004). MBL gene variants may be minor susceptibility factors for rheumatoid arthritis predisposing to early disease (Graudal et al., 2000, Jacobsen et al., 2001).

In coronary artery disease an interesting picture is emerging. MBL deficiency haplotypes are associated with an increased risk of early atherosclerosis and increased carotid plaque area (Alipour et al., 2009, Hegele et al., 2000). However, MBL functional deficiency is associated in improved mortality in patients with coronary artery occlusion undergoing angioplasty (Haahr-Pedersen et al., 2009). Additionally high serum MBL levels are associated with increased risk of post angioplasty cardiac dysfunction possibly due to increased complement mediated tissue damage during the ischaemia-reperfusion process (Trendelenburg et al.).

There are numerous strands of evidence supporting the relevance of MBL and MBL deficiency in a variety of conditions. However, it is apparent that the majority of people with low MBL levels; sometimes termed insufficient as opposed to deficient, are healthy. Analysis of the literature demonstrates that abnormalities in the MBL pathway are clinically important in those with concomitant immune defects. Indeed the accepted clinical phenotype of MBL deficiency is not

associated with a particular pathogen or condition. MBL deficiency appears to manifest as recurrent infective episodes of unknown aetiology beginning at 3-6 months (Haahr-Pedersen et al., 2009, Klein, 2005, Koch et al., 2001). These MBL deficient children rarely require hospital admission and continue to thrive but do require regular medical attention and multiple courses of antibiotics. These infections resolve at school age. Such cases highlight the importance of MBL in the 'window of vulnerability' during which the immune system is developing. The relevance of deficiency in otherwise healthy adults is hotly debated. A recent study by Hoeflich *et al* demonstrated that the frequency of MBL deficiency was higher in subjects with recurrent infections than controls in the absence of other immune deficiency (Hoeflich et al., 2009). However, a large population study did not find any association of MBL deficiency with mortality and morbidity in adults. The relevance of MBL is developing but as yet such examination lacks a routine indication in healthy adults.

6.1.6 Aims

1. To establish the frequency of MBL polymorphic variants in a healthy Irish control population
2. To determine a reference range for serum MBL levels that could be used to define deficiency
3. To determine if there is any association between MBL genetic variation and bronchiectasis

6.2 Materials and methods

6.2.1 MBL serum level measurement

Two different MBL assays were evaluated for this project. A C4 deposition ELISA measuring functional MBL (Sanquin Diagnostics, Belgium) and an ELISA measuring higher order MBL oligomers (Bioporto Diagnostics, Denmark). High order oligomers represent functional MBL capable of binding bacterial sugar moieties and initiating the complement cascade (Minchinton et al., 2002). Details of the evaluation are not presented in this project, however, no significant difference was noted between the assays. With this in mind this project proceeded using the more cost and time efficient Bioporto assay. Samples were processed using MBL Oligomer Elisa Kits (Bioporto Diagnostics, Denmark) according to manufacturer's instructions. Samples were processed in duplicate with the average optical density obtained from a Labsystems Multiskan EX (ThermoScientific, NC, USA).

6.2.2 MBL genotyping

Genomic DNA was obtained from patients and controls as described previously. Flanking SNP sequences for the six MBL SNPs of interest were obtained from DbSNP (Table 6.1, page 232 - 233). MBL genotyping was carried out commercially using a competitive allele specific PCR SNP (KASPR) coupled with a FRET based genotyping system with assays being designed and validated by Kbiosciences (Kbiosciences, Hoddesdon, UK). Genotyping data was viewed graphically using SNPview (Kbiosciences, Hoddesdon, UK). Random internal control samples were included to ensure that data received was of good quality (10 repeat samples for controls and 10 for patient samples). Reproducibility of these randomly distributed internal repeat samples was 100%; no discordance was identified. Further reassurance regarding the quality of the results was

obtained from the agreement of haplotypes identified with previously published results, and the general agreement of genotype frequencies of controls with published observations in other Northern European populations.

6.2.3 Analysis

Data was presented graphically using GraphPad Prism 5.0 (GraphPad Software, CA, USA) and was analysed statistically using Graph Pad Prism or SPSS (Version 20, IBM, CA, USA).

MBL2PQ	CGCCAGGTGTCTAGGCACAGATGAACCCCTCCTTAGGATCCCCACTGCTCAT
	CATAGTGCCCTACCTTTGTAAAGTACTAGTCACGCAGTGTCACAAGGAATGT
	TTACTTTTCCAAATCCYCAGCTAGAGGCCAGGGATGGGTCATCTATTTCTAT
	ATAGCCTKMACCCAGATTGTAGGACAGAGGRCTRCT [C/T] GGTAATATG
	TGTTTCATTAACYGAGATTAACCTTCCCTGAGTTTTCTCACACCAAGGTGAGG
	ACCATGTCCCTGTTTCCATCACTCCCTCTCCTTCTCCTGAGTATGGTGGCAG
	CRTCTTACTCAGAAACTGTGACCTGTGAGGATGCCCAAAGACCTGCCT
MBL2XY	AACAGGGACA TGGTCTCAC CTTGGTGTGA GAAACTCAG GGAAGGTAA TCTCAGTTAA
	TGAACACATA TTTACCGAGC ATGCCCTCG TCCTACAATC TGGGTGCAGG CTATATAGAA
	ATAGATGACC CATCCCTGGC CTCTAGCTGG GGATTGGAA AAGTAAACAT TCCTTGTGAC
	ACTGCGTGAC TAGTACTTTA ACAAAGGTAG GCACTATGAT GAGCAGTGGG GATCCTAAGG
	AGGGGTTCAT CTGTGCCTAG ACACCTGGCG TTGCTGCTGG AAGACTATAA ACATGCTTTC
	S
	GTGGCAGTGA GAACAAATGG GACCGTGCAT TGCCAGCTGG CTTATGCCTG TTAGCTTAGG
	GAATGAGAAT GAGTGGAAAC CCAGGTGTCT GTAGGAGCTT CCTCTTCTC TCCATGTCCT
	CTTCGGGTCC ATCTGCCACC TGAATCCCAT CTTGTATCT GGGCAGCTGA TTCCCCTCCA
	GGACCTGTTA ACTATCATTT ACTCATTGGC AGGCCCTGAG CTGAAAGCTG GTGATCCAAA
	GAGGAAGGTC TCAACCTCAG ATCAACCTCA ACCTTAGTCA CCAACCCAGC CCAGAATTA
MBL2H/L	ATTGCCAGCT GGCTTATGCC TGTTAGCTTA GGAATGAGA ATGAGTGGAA ACCCAGGTGT
	CTGTAGGAGC TTCCTCTTTC TCTCCATGTC CTCTCGGGT CCATCTGCCA CCTGAATCCC
	ATCTTTGTAT CTGGGCAGCT GATTCCCCTC CAGGACCTGT TAACTATCAT TTACTIONT
	GCAGGCCCTG AGCTGAAAGC TGGTGATCCA AAGAGGAAGG TCTCAACCTC AGATCAACCT
	CAACCTTAGT CACCAACCCA GCCCAGAATT AACTGGAGTT TGCTTCCCCT TGGTGTTTTA
	S
	ACAGGCTTGC CTGGGTAAGC ATTTTCTCTG GAAATTTCTT ACTACGTTGG CCCTGGCAGA
	GTGCTAAATA CCTCTCTACT TTCTGGCAGG AATCCCCTAT GTTCTGTGAG TCAAAAACCA
	CTGGCAATTA GAAACTTGC CTTGAATCCT CCTCCCATT CTCAGTATTA ACCTTTCATT
	TGTTCTTCAG AGCTTCTTTC CAGCTATTGC CCCCTAAGAA ACCTCAGCAG CCTAGCCCCA
	TAGGTCTAAG CCTCCAAGTT TCCCTTGA ACAAAGACA GTCTCCACTG CAGATGAACA
MBL2D	CCAAATCCCC AGCTAGAGGC CAGGGATGGG TCATCTATTT CTATATAGCC TGCACCCAGA
	TTGTAGGACA GAGGGCATGC TCGGTAATA TGTGTTTATT AACTGAGATT AACCTTCCCT
	GAGTTTTCTC ACACCAAGGT GAGGACCATG TCCCTGTTTC CATCACTCCC TCTCCTTCTC
	CTGAGTATGG TGGCAGCGTC TTACTIONTACT ACTGTGACCT GTGAGGATGC CAAAAGACC
	TGCCCTGCAG TGATTGCCTG TAGCTCTCCA GGCATCAACG GCTTCCCAGG CAAAGATGGG
	Y
	GTGATGGCAC CAAGGGAGAA AAGGGGGAAC CAGGTACGTG TTGGGCTGTT CTGTCTCTGC
	AATCTTTAC CTTCCAGAGG AAACCTGCCTG GGGATATGAG GAGACTGATG TCCTATTTGA
	GTATATTTT CTCAACTATA CTGTAACCTA AACAGAGAT TCAGCTCTCA TTCTCTGGCA
	TCTCAGAATT CCACACAGCA GTTTGTGACT AATAGTTGTC TTGCCAGCCC AGGAAAGTGG
	CCCACAGGTC AGGCCATCCC GTGGGACACA GGATGAATTT TTCTTCTCTG GGTCATTGTC

MBL2C	AGGCCAGGGA TGGGTCATCT ATTTCTATAT AGCCTGCACC CAGATTGTAG GACAGAGGRC
	ATRCTYGGTA AATATGTGTT CATTAAACYGA GATTAACCTT CCCTGAGTTT TCTCACACCA
	AGGTGAGGAC CATGTCCCTG TTTCCATCAC TCCCTCTCCT TCTCCTGAGT ATGGTGGCAG
	CGTCTTACTC AGAAACTGTG ACCTGTGAGG ATGCCCAAAA GACCTGCCCT GCRGTGATTG
	CCTGTAGCTC TCCAGGCATC AACGGCTTCC CAGGCAAAGA TGGGYGTGAT GRCACCAAGG
	R
	AGAAAAGGGG GAACCAGGTA CGTGTGGGC TGTCTGTCT CTGCAATTCT TTACCTTCCA
	GAGGAAACTG CCTGGGGATA TGAGGAGACT GATGTCCTAT TTGAGTATAT TTTTCTCAAC
	TATACTRTAA CTCAAACAG AGATTGAGCT CTCATTCTCT GGCATCTCAG AATTCCACAC
	AGCAGTTTGT GACTAATAGT TGTCTTGCCA GCCCAGGAAA GTGGCCACACA GGTGAGGCCA
	TCCCGTGGGA CACAGGATGA ATTTTCTTCT TCTGGGTCAT TGTCATGTCA GACCCCTATT
MBL2B	AGGCATAAGC CAGCTGGCAA TGCACGGTCC CATTTGTTCT CACTGCCACC GAAAGCATGT
	TTATAGTCTT CCAGCAGCAA CGCCAGGTGT CTAGGCACAG ATGAACCCCT CCTTAGGATC
	CCCCTGCTC ATCATAGTGC CTACCTTGT TAAAGTACTA GTCACGCAGT GTCACAAGGA
	ATGTTTACTT TTCCAAATCC CCAGCTAGAG GCCAGGGATG GGTCATCTAT TTCTATATAG
	CCTGCACCCA GATTGTAGGA CAGAGGGCAT GCTCGGTAAT TATGTGTTCA TTAAGTGA
	TTAACCTTCC CTGAGTTTTT TCACACCAAG GTGAGGACCA TGTCCCTGTT TCCATCACTC
	CCTCTCCTTC TCCTGAGTAT GGTGGCAGCG TCTTACTCAG AAAGTGTGAC CTGTGAGGAT
	GCCCAAAGA CCTGCCCTGC AGTGATTGCC TGTAGCTCTC CAGGCATCAA CGGCTTCCCA
	GGCAAAGATG GCGTGATG
	R
	CACCAAGGGA GAAAAGGGGG AACCAGGTAC GTGTTGGGCT GTTCTGTCTC TGCAATTCTT
	TACCTTCCAG AGGAAACTGC CTGGGGATAT GAGGAGACTG ATGTCCTATT TGAGTATATT
	TTTCTCAACT ATACTGTAAC TCAAACAGA GATTGAGCTC TCATTCTCTG GCATCTCAGA
	ATTCCACACA GCAGTTTGTG ACTAATAGTT GTCTTGCCAG CCCAGGAAAG TGGCCACAG
	GTCAGGCCAT CCCGTGGGAC ACAGGATGAA TTTTCTTCT CTGGGTCATt gtcagtcag
	accctattc acttcagtag gtagggcacc aggttcaaga ggccaagaa gagatggagt

Table 6.1 showing flanking DNA sequences for MBL polymorphisms obtained from dbSNP (page 236-237)(www.ncbi.nlm.nih.gov/projects/SNP)

6.3 Results

6.3.1 MBL haplotype frequencies in a healthy Irish population

In order to determine the MBL2 haplotype in an Irish control population, single nucleotide polymorphisms were analysed by competitive allele specific PCR (n=75). Polymorphisms were examined at codons 54, 57 and 52 (variants B, C and D respectively) of exon 1 of the MBL2 gene. Additionally, variation in the promoter region was examined; H/L, X/Y and P/Q. Internal repeats were used as a quality control to ensure result specificity. No variation in internal control samples was detected. In keeping with published literature, seven haplotypes were identified in the control population (Garred, 2008). Four of these haplotypes are associated with normal MBL levels; HYPA, LYQA, LYPA and LXPA. A further three haplotypes are associated with low levels of functional MBL; LYPB, LYQC and HYPD (Garred, 2008). Haplotype results from the Irish control population were comparable to published Caucasian Spanish and Danish control populations (Figure 6.3) (Garred, 2008, Villarreal et al., 2001). Results were also similar to a control population from North Africa, but demonstrated clear differences with haplotype frequencies from Sub-Saharan African controls.

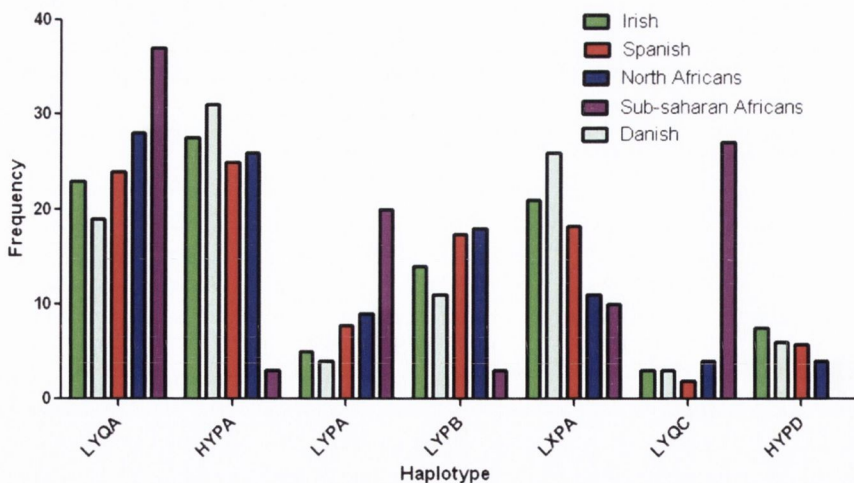


Figure 6.3 showing the frequency of MBL2 haplotypes in healthy Irish controls (n=75). Results are compared to published haplotype frequencies from other control populations from Europe and Africa

6.3.2 There is no difference in the prevalence of MBL deficient genotypes between patients and unmatched controls

MBL haplotypes were also identified in our cohort of 101 Irish patients with bronchiectasis and compared to the control population. By convention, sufficient haplotypes are termed A, and deficient haplotypes termed O. Using this nomenclature the MBL genotype may be described as AA; homozygote for sufficient haplotypes, AO; heterozygote and OO representing homozygosity for deficient haplotypes or compound heterozygosity for deficient haplotypes. Using this simplified nomenclature, MBL genotype in patients and controls were compared. No significant difference in the prevalence of MBL deficient genotypes was identified (Figure 6.4, χ^2 test $p > 0.05$).

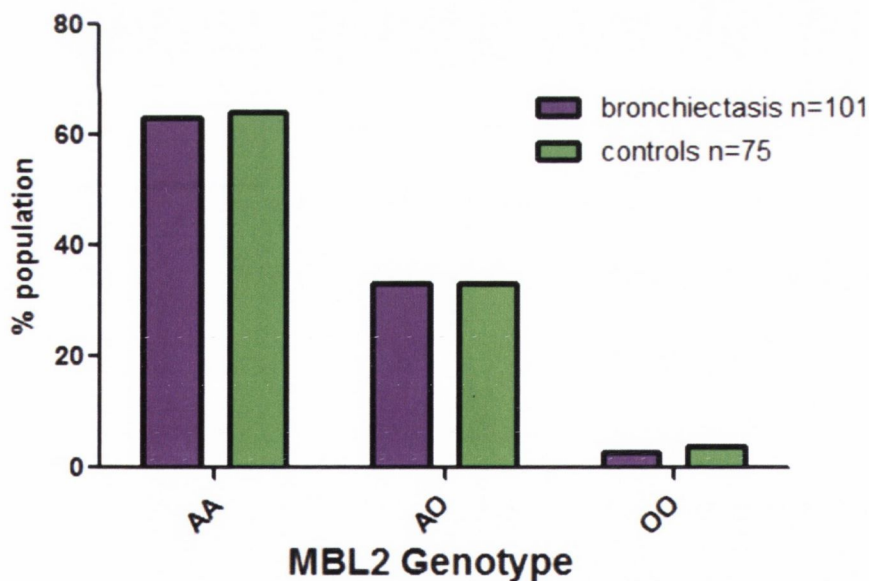


Figure 6.4 showing the prevalence of MBL sufficient (AA) and deficient (AO/OO) genotypes in patients with bronchiectasis and with controls. No significant difference was noted.

To look for a haplotype association with bronchiectasis in more detail, the prevalence of individual haplotypes in bronchiectasis and control populations was also compared. Once again no significant difference in haplotype prevalence was noted (Figure 6.5, χ^2 $p > 0.05$).

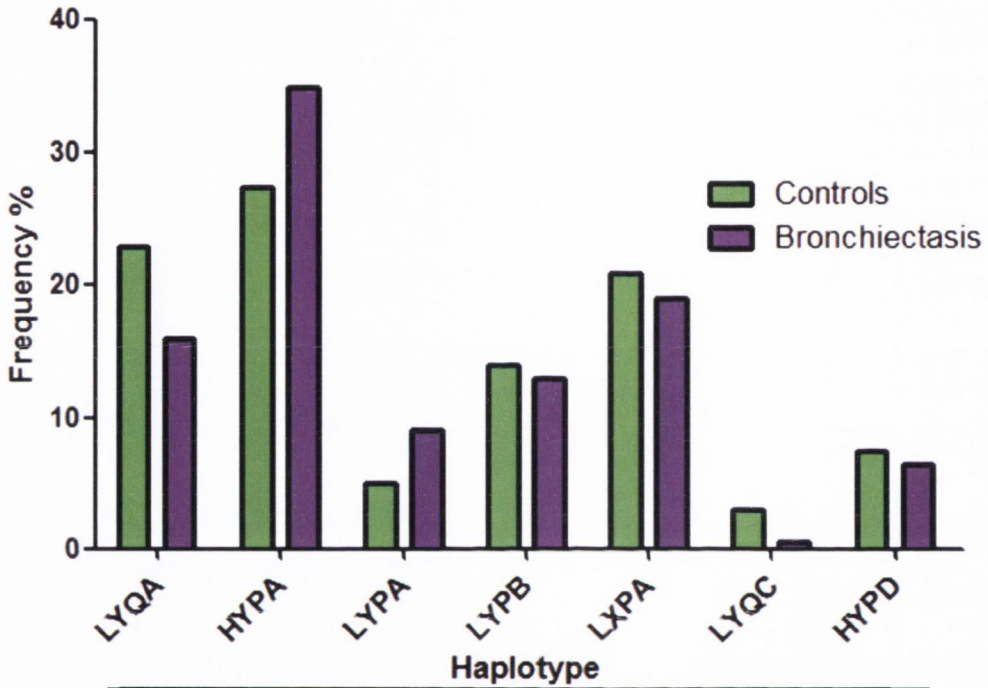


Figure 6.5 showing MBL2 haplotype frequency in bronchiectasis and control populations. No significant differences are observed.

6.3.3 Serum levels of MBL are reduced in healthy Irish controls expressing deficiency haplotypes

Analysis of MBL2 haplotypes is expensive, time consuming and requires specialist equipment. Measurement of serum MBL levels by ELISA, while still expensive, is more widely available. There also remains considerable debate as to which is the best way of determining clinically relevant MBL function (Minchinton et al., 2002). To determine if measurement of oligomeric MBL by ELISA could be used as a surrogate marker for MBL deficiency haplotypes, serum levels were assessed in healthy controls (n=75). As expected controls carrying insufficiency haplotypes (AO/OO) expressed levels of MBL significantly lower than the AA group (Figure 6.6).

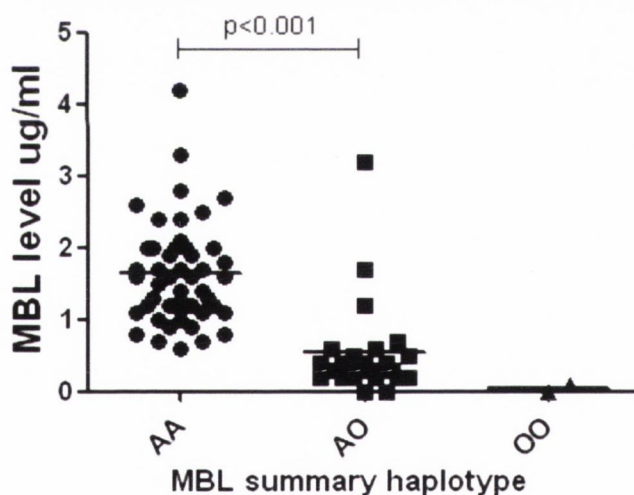


Figure 6.6 showing serum MBL levels in healthy Irish controls divided by summary haplotypes. As expected MBL levels are higher in the AA group.

Of the control population, 48 (64%) carry wild type MBL2 haplotypes, 27 (33%) carry one deficiency haplotype, and 2 (3%) carry two deficiency haplotypes. The combination of two deficiency haplotypes is rare in the healthy Irish population. Examination in more detail of the haplotype makeup of the wild type Irish population shows the impact of promoter variation on

MBL levels in patients carrying two wild type (AA) MBL haplotypes (Figure 6.7). The presence of the HY promoter results in higher MBL levels, the presence of LX alleles results in lower MBL alleles, while LY gives intermediate levels. In particular highest levels are associated with HY homozygosity (HYA/HYA) while lowest levels are associated with LX homozygosity (LXA/LXA). While levels vary considerably within the AA group due to promoter variation, MBL levels are still higher than those in groups from heterozygote or homozygote mutant haplotypes, but several do fall outside the manufacturer's normal reference range for MBL sufficiency of >1.0ug/ml.

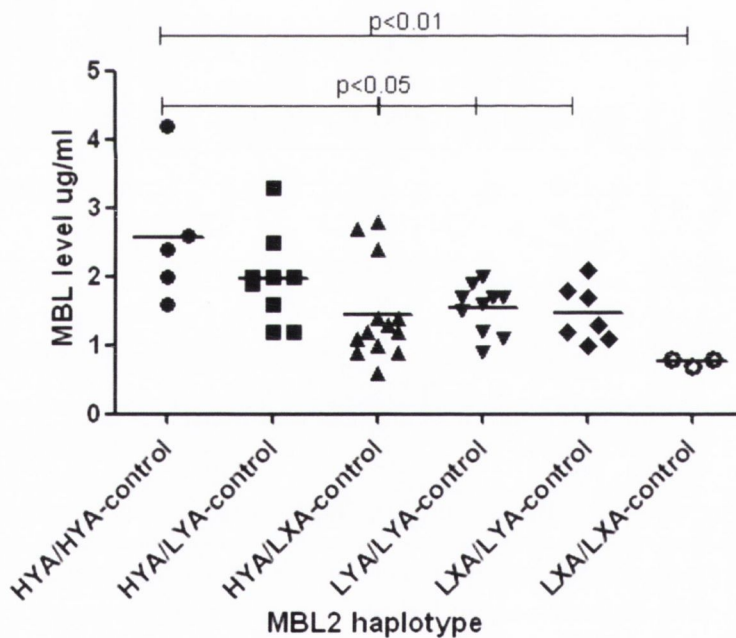


Figure 6.7 showing the variation in serum MBL levels within the control population carrying wild type haplotypes. The presence of HY promoter polymorphisms results in higher MBL levels, with LX polymorphisms associated with reduced MBL levels

6.3.4 Examination of serum MBL levels is a clinically useful way of distinguishing between MBL sufficient (AA) and insufficient haplotypes (AO/OO) in controls

A specific reference range for MBL in the Irish population has not been published. To calculate a normal range, two statistical methods were used. The first standard method used was to calculate +/- two standard deviations from the mean for the AA population (Examining the AA population alone allows us to examine a unimodal distribution. This calculation was based on log transformed values to resolve skewness). This gave a normal range of MBL levels in healthy controls of AA genotype as being 0.64-3.55ug/ml. This crude method suggests that levels below 0.64ug/ml are not consistent with MBL sufficient genotypes.

A more informative method of examination of the discriminative ability of the MBL oligomeric ELISA is the generation of a ROC curve (Figure 6.8 and Table 6.2). This gives insight into the sensitivity and specificity of a test to discriminate between two states, in this case MBL sufficient genotypes and MBL deficient genotypes.

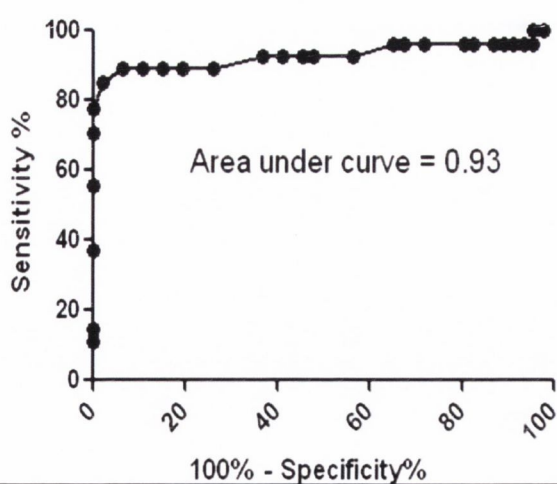


Figure 6.8 showing a ROC curve based on analysis of the ability to discriminate between sufficient (AA) and insufficient (AA/OO) genotypes.

For the control cohort the area under the ROC curve is 0.93. A perfect discriminatory test would give a result of 1, while a test that has no discriminatory ability would give an area under the curve of 0.5. Essentially the value of 0.93 indicates that a randomly selected patient will have a lower MBL value than 93% of controls. Using this data sensitivity and specificity values at various cut-off points were obtained, some of which are presented in Table 6.1.

Cutoff $\mu\text{g/ml}$	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.5500	77.78	57.74% to 91.38%	100.0	92.29% to 100.0%	
< 0.6500	85.19	66.27% to 95.81%	97.83	88.47% to 99.94%	39.19
< 0.7500	88.89	70.84% to 97.65%	93.48	82.10% to 98.63%	13.63
< 0.8500	88.89	70.84% to 97.65%	89.13	76.43% to 96.38%	8.18
< 0.9500	88.89	70.84% to 97.65%	84.78	71.13% to 93.66%	5.84
< 1.050	88.89	70.84% to 97.65%	80.43	66.09% to 90.64%	4.54
< 1.150	88.89	70.84% to 97.65%	73.91	58.87% to 85.73%	3.41
< 1.250	92.59	75.71% to 99.09%	63.04	47.55% to 76.79%	2.51

Table 6.2 showing sensitivities and specificities of the ability of various cut-off values for discrimination between MBL sufficient (AA) and insufficient genotypes (AO). Highlighted is a possible cut-off value of 0.75 $\mu\text{g/ml}$

These results suggest that creating a cut-off value of 0.75 $\mu\text{g/ml}$ would allow identification of MBL deficient genotypes with a sensitivity of 89% and a specificity of 93% in this control cohort. However, further knowledge of the behavior of MBL in bronchiectasis and other disease settings is required to determine how broadly applicable this cut-off level is.

6.3.5 MBL levels are higher in wild-type (AA) patients with bronchiectasis than controls

MBL serum levels were evaluated in the cohort of 101 Irish patients with bronchiectasis to determine if levels were altered in the disease. Results were divided on the basis of the summary genotypes AA, AO and OO as shown in figure 6.9.

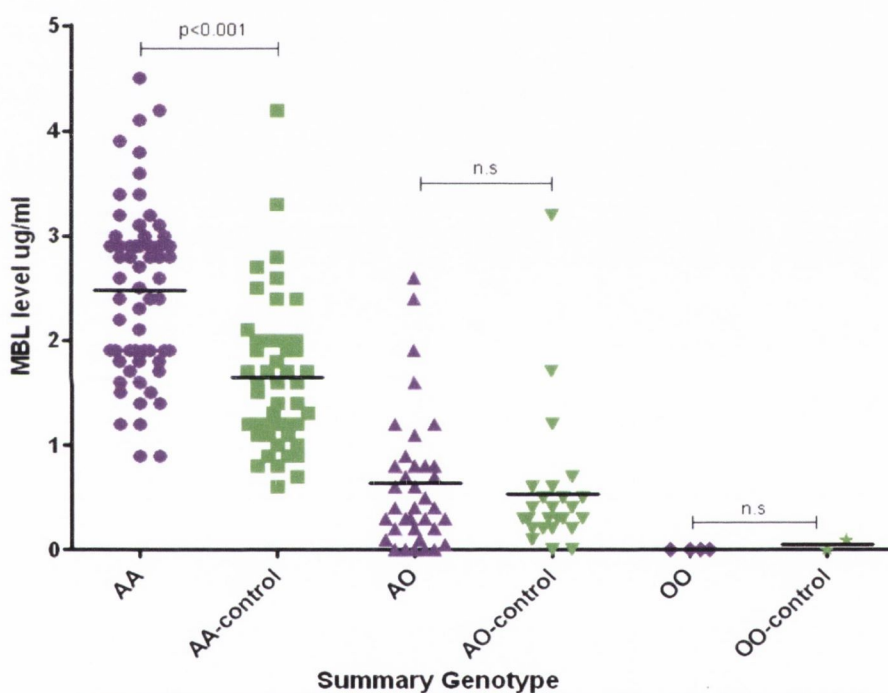


Figure 6.9 showing MBL levels in 101 patients and 75 controls subdivided by MBL2 summary genotype. Patients (purple) with sufficient genotypes (AA) have higher MBL levels than controls ($p < 0.001$, Mann-Whitney).

Bronchiectasis patients (in purple) with wild type (AA) genotypes compatible with MBL sufficiency had significantly higher levels of serum MBL than control counterparts. In contrast, no significant difference was detected in AO genotype patients and AO controls. Additionally no

significant difference was detectable between MBL levels in OO genotype patients and controls, although numbers were very small and thus the ability to detect a difference was limited.

6.3.6 Serum MBL levels in bronchiectasis can distinguish between sufficient (AA) and deficient (AO/OO) genotypes

Using MBL serum levels from bronchiectasis patients a ROC curve was constructed to determine a cut-off for distinguishing between MBL sufficient and deficient genotypes in the disease population (Figure 6.10). This gave a different MBL level for genotype discrimination in

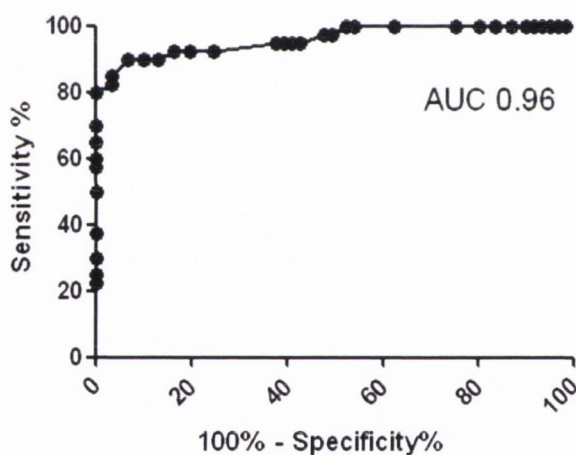


Figure 6.10 showing a ROC curve based on analysis of the ability to discriminate between sufficient AA and insufficient AO/OO genotypes in the disease population

bronchiectasis. While in the control population a serum MBL cut-off of $0.75\mu\text{g/ml}$ was obtained, in the disease cohort using similar methodology the cut-off is considerably higher. Serum MBL levels less than $1.3\mu\text{g/ml}$ will identify MBL deficient genotypes with a sensitivity of 90% and a specificity of 94%. Clearly the presence of a disease process, probably related to

inflammation induced MBL levels, has impacted on the discriminatory ability of the test, shifting the range that is associated with genotypic normality. The elevation in MBL levels observed in the AA genotypic group may be related to ongoing inflammation in the disease cohort, warranting further examination.

6.3.7 CRP levels are higher in the MBL deficient group

MBL can function as an acute phase reactant. The observation that MBL levels are higher in wild-type bronchiectasis patients than in wild type controls therefore has a number of potential explanations. It could indicate that AA patients have worse bronchiectasis and higher levels of inflammatory indices. Alternatively, the absence of raised MBL levels in AO patients may be due to an inability to produce an acute phase response with MBL. To examine this further we examined CRP and α 1 antitrypsin as other acute phase reactants in patients and controls.

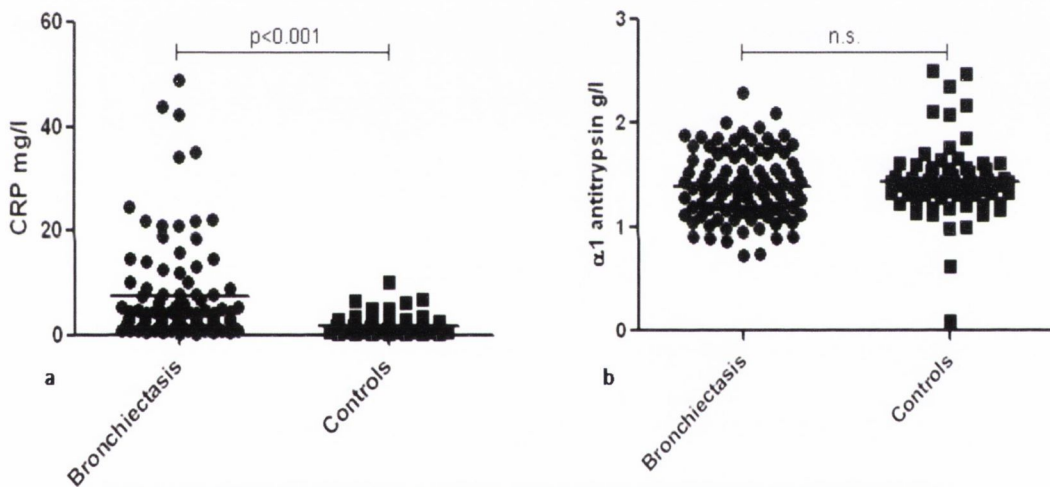


Figure 6.11 showing a) CRP and b) α 1 antitrypsin levels in patients with bronchiectasis ($n=101$) and controls ($n=75$). Patients with bronchiectasis have significantly higher levels of CRP than unmatched controls (Mann-Whitney, $p < 0.001$). In contrast, no difference in α 1 antitrypsin levels between disease and normal populations was noted (Mann-Whitney $p < 0.05$).

Figure 6.11a shows CRP levels taken from patients with bronchiectasis during a period of clinical stability and healthy controls. CRP levels in bronchiectatic patients are significantly higher than those of unmatched healthy controls. In contrast, no difference was observed in α 1 antitrypsin levels in the two groups (Figure 6.11b). These results suggest that bronchiectasis patients have higher acute inflammatory indices in terms of CRP than unmatched healthy individuals. This may

reflect an ongoing inflammatory insult in such patients. This is not reflected in differences in $\alpha 1$ antitrypsin, which is known to be a much less sensitive marker of inflammation.

To determine if the higher CRP levels observed in the bronchiectasis cohort were related to the higher MBL levels observed in the AA genotype group, CRP data was broken down into MBL genotypic variants. In contrast to the data on MBL levels (Figure 6.9), these results suggest that higher levels of CRP and hence inflammation are found in the AO/OO patient subgroup (Figure 6.12).

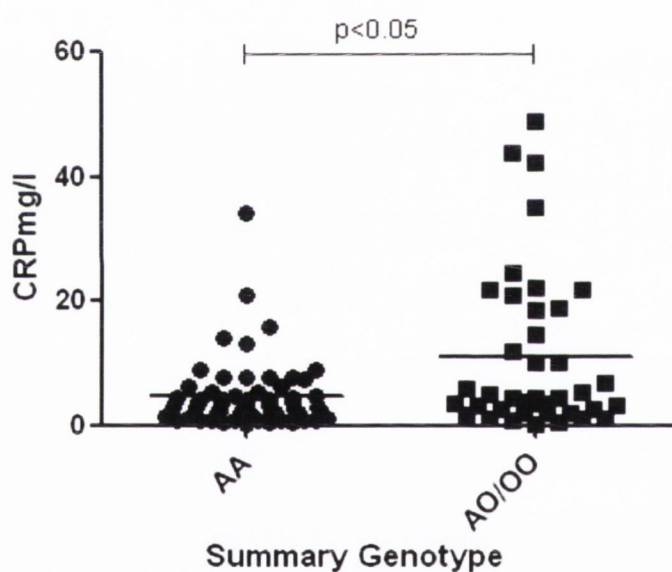


Figure 6.12 showing CRP levels in patients with bronchiectasis grouped into MBL summary genotype. Levels in the MBL insufficient group are significantly higher than in the AA group.

The control cohort is not significantly different from the patient cohort in terms of gender, however, there is an age discrepancy. To determine whether age had an influence on MBL or CRP levels we compared results to a smaller cohort of age matched controls, without evidence

of inflammatory disease. These results showed that age matched controls had MBL and CRP levels comparable with unmatched controls (Figure 6.13). Levels of MBL in these healthy older subjects were significantly lower than in patients with bronchiectasis ($p < 0.05$). Similarly CRP levels were lower among these healthy subjects than patients with bronchiectasis ($p < 0.05$).

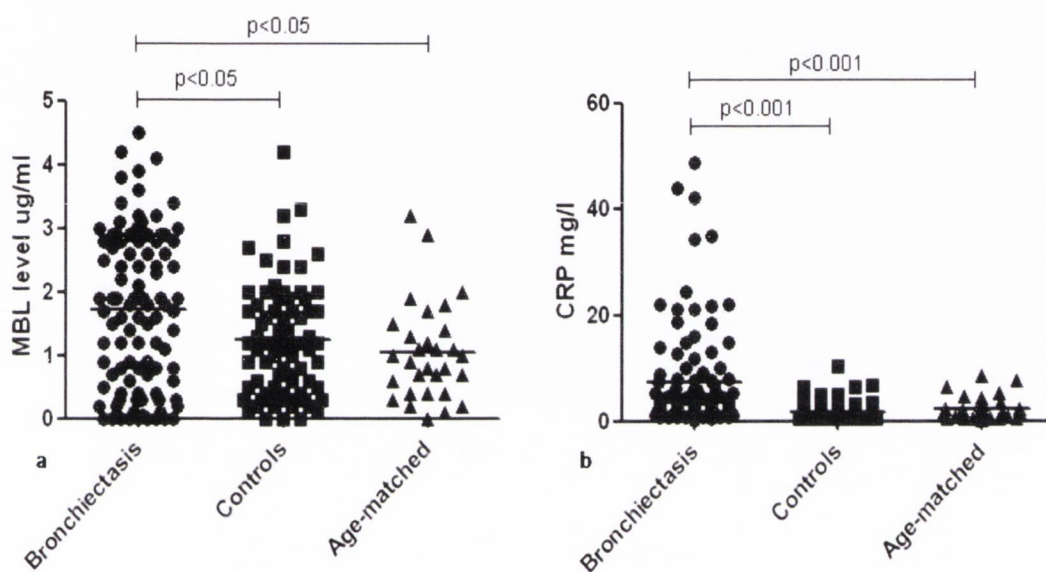


Figure 6.13 showing MBL a) and CRP b) levels for bronchiectasis patients (n=101), controls (n=75) and a smaller group of age-matched controls (n=30)

This suggests that the differences in MBL and CRP levels between control cohorts and the patient group are related to the presence of disease as opposed to age changes.

6.3.8 MBL levels in bronchiectasis do not correlate well with other acute inflammatory markers

MBL is an acute phase protein, levels of which are thought to rise during inflammation. CRP and α 1 antitrypsin can also act as acute phase proteins. However, this study has demonstrated that CRP levels are higher in the group of bronchiectasis patients expressing the MBL AO deficiency genotype. Correlations of CRP with α 1 antitrypsin, and MBL with CRP and α 1 antitrypsin were carried out. Log CRP has a weak correlation with α 1 antitrypsin (Figure 6.14).

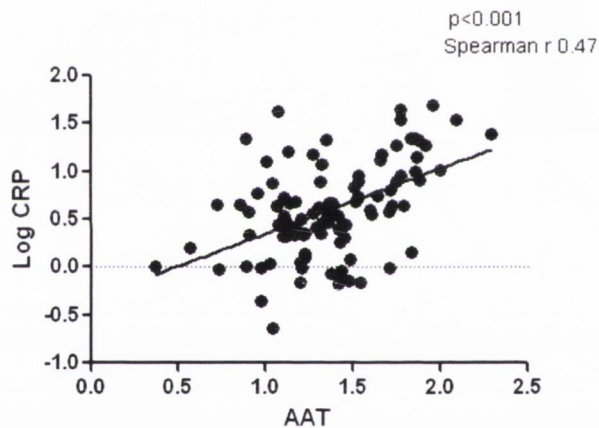


Figure 6.14 showing a weak correlation between α 1 antitrypsin (AAT) and log CRP. There is a significant correlation ($p < 0.001$) but the strength of the relationship is weak

If CRP is accepted as the prototypic acute phase protein in respiratory disease, this result suggests that α 1 antitrypsin operates as acute phase reactant, but imperfectly so. In contrast MBL levels do not correlate with either CRP or α 1 antitrypsin (Figure 6.15a and b). These results were not unexpected, given the disconnect between MBL genotype and CRP levels already presented. Results were further subdivided on the basis of MBL genotype. MBL levels of patients with the AA MBL genotype did not correlate with logCRP levels (Figure 6.16a), however, patients

with the promoter/exon 1 genotype combination YA/YA (ie any combination of HYPA, LYPA and LYQA haplotypes) did demonstrate a weak correlation with logCRP (Spearman's r 0.42). This weak correlation suggests that patients with YA/YA haplotype MBL variants may have increased MBL levels when CRP is elevated. In contrast other haplotype subgroups may not increase MBL levels during periods of inflammation.

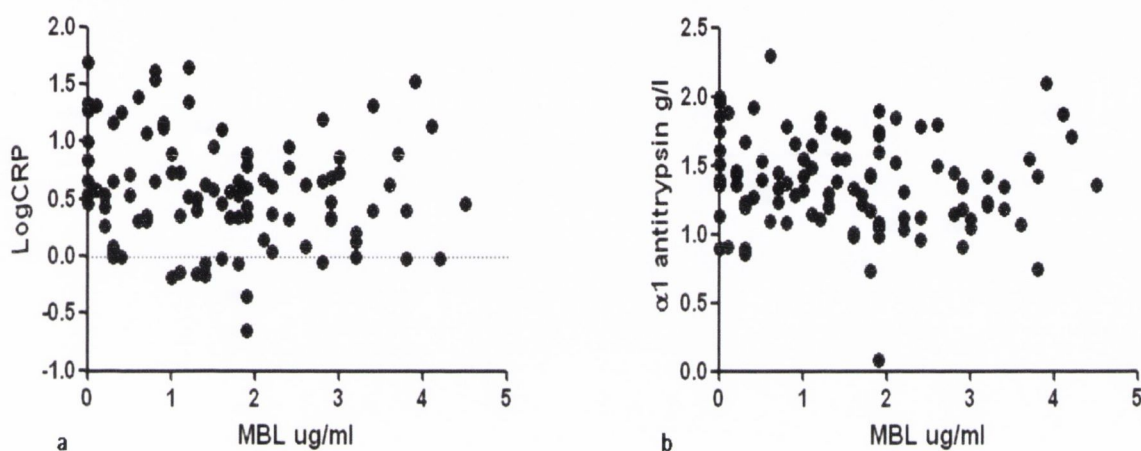


Figure 6.15 showing no significant correlation of MBL levels with a) logCRP or b) α 1 antitrypsin. Transformation of MBL does not affect this result.

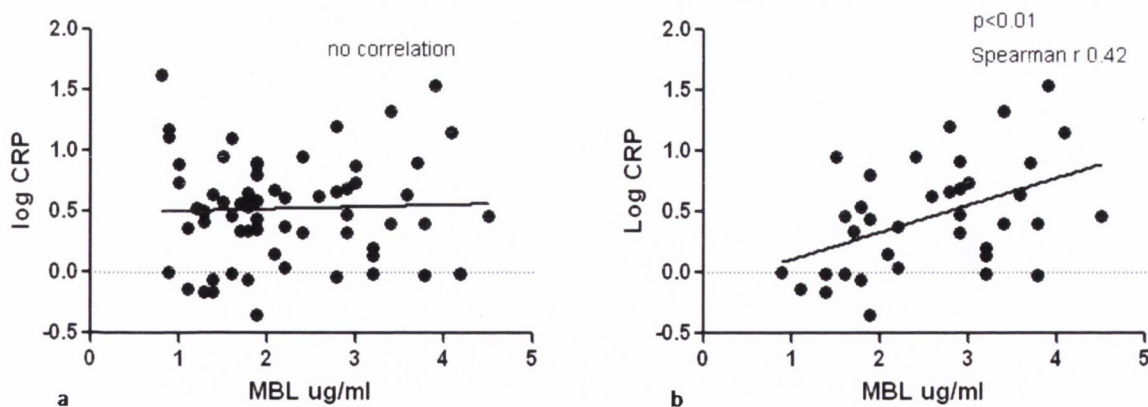


Figure 6.16 a) showing a lack of correlation between serum MBL and logCRP levels of patients with bronchiectasis of AA genotype. Figure 6.16 b) shows a weak correlation of MBL and logCRP in serum from patients homozygous for the wild type A allele and the Y promoter (Spearman $r=0.42$) (genotypes are homozygous for HYPA, LYPA, LYQA).

6.3.9 Low serum MBL levels in bronchiectasis are weakly associated with daily production of purulent sputum

Clinical association studies were carried out to determine if serum MBL levels are associated with clinical parameters of severity in bronchiectasis, including daily production of purulent sputum, infective exacerbations and hospital admissions. MBL levels were assessed at three levels – absolute deficiency (levels $<0.2\mu\text{g/ml}$), reduced (level $<0.7\mu\text{g/ml}$), reduced in disease (levels $<1.3\mu\text{g/ml}$). The ‘reduced’ and ‘reduced in disease’ levels were chosen on the basis of the ROC curves in disease and control groups.

Results (Table 6.3) indicate that absolute MBL deficiency is associated with daily production of purulent sputum ($p<0.01$). However, absolute MBL deficiency did not have a significant impact on other clinical parameters. Furthermore at less profoundly reduced MBL levels no association with any clinical parameter was observed.

Serum MBL	Reported exacerbations/year		<i>p</i> value	Admissions over 5 years		<i>p</i> value	Reported production of daily purulent sputum		<i>p</i> value
	0-3	4+		0	1+		No	Yes	
<0.2	7	6	1.00	5	8	0.23	3	10	0.01
<0.8	16	14	1.00	16	14	0.83	10	20	0.08
<1.3	23	21	0.83	25	20	1.00	16	28	0.07

Table 6.3 showing clinical parameters of bronchiectasis patients ($n=101$) at different serum levels of MBL. A weak association of absolute MBL deficiency with daily production of purulent sputum is noted ($p<0.01$, Fisher’s Exact Test).

Similarly when analysis is carried out on the basis of haplotype, findings are not consistent. The MBL sufficient haplotype LYQA is protective against daily purulent sputum ($p<0.05$) and hospital admission ($p<0.05$). No other haplotype associations are noted (Table 6.4).

Haplotype	Reported exacerbations/year		<i>p</i> value	Admissions over 5 years		<i>p</i> value	Reported production of daily purulent sputum		<i>p</i> value
	0-3	4+		0	1+		No	Yes	
HYP A	41 (20)	32 (16)	0.56	37 (18)	36 (18)	0.46	31 (15.5)	42 (21)	0.37
LXP A	22 (11)	17 (8.5)	0.86	18 (9)	21 (10.5)	0.28	18 (9)	21 (10)	1.00
LYQ A	18 (9)	14 (7)	0.85	24 (12)	8 (4)	0.02	21 (10)	11 (5.5)	0.04
LYP A	9 (4.5)	10 (5)	0.63	11 (5)	8 (4)	0.81	10 (5)	9 (4.5)	0.81
LYP B	13 (6)	13 (6)	0.83	13 (6)	13 (6)	0.67	10 (5)	16 (8)	0.40
HYP D	5 (2.5)	7 (3.5)	0.55	7 (3.5)	5 (2.5)	1.00	7 (3.5)	5 (2.5)	0.56
LYQ C	1 (0.5)	0	<i>n.d.</i>	1 (0.5)	0	<i>n.d.</i>	0	1 (0.5)	<i>n.d.</i>
Total	109 (53.5)	93 (46.5)		111 (55)	91 (45)		97 (48)	105 (52)	

Table 6.4 showing number (%) of haplotypes from 101 patients with bronchiectasis (202 haplotypes) associated with the clinical parameters of reported exacerbations/year, hospital admissions with infective exacerbations of bronchiectasis over 5 years and reported daily production of purulent sputum. LYQA is associated with lower levels of hospital admission over 5 years ($p=0.02$) and purulent sputum production ($p=0.04$). (p values, Fisher's Exact Test, *n.d.* = not done)

Other MBL sufficient haplotypes HYP A, LXP A and LYP A are not significantly associated with these markers of clinical severity. Furthermore, association with LYQ A and protection against self reported infective exacerbations was not observed. If, however, a correction for multiple comparisons, such as Bonferroni's correction, is included in the statistical analysis the altered α value (α/n , where n is the number of statistical tests applied to the population) renders these results insignificant.

6.3.10 Low serum MBL levels are not associated with more severe bronchiectasis on CT grading

Serum MBL cut-off level ug/ml	Mean bronchiectasis CT score in patients with levels below cut-off (+/- sd)	Mean bronchiectasis CT score in patients with levels above cut-off (+/- sd)	P value (Mann-Whitney)
0.2	10.3 (+/- 5.91)	9.2 (+/- 6.31)	n.s.
0.8	9.6 (+/- 5.74)	9.5 (+/- 6.82)	n.s.
1.3	9.2 (+/- 5.71)	9.7 (+/- 7.11)	n.s.

Table 6.5 showing mean bronchiectasis CT scores among groups of patients with different levels of MBL. No significant (n.s.) is observed.

Sum bronchiectasis scores were available on 93 of the 101 patients with bronchiectasis. The mean CT score in bronchiectasis patients subdivided on the basis of serum MBL level did not differ significantly (Table 6.5).

6.3.11 MBL haplotype is not associated with radiological severity

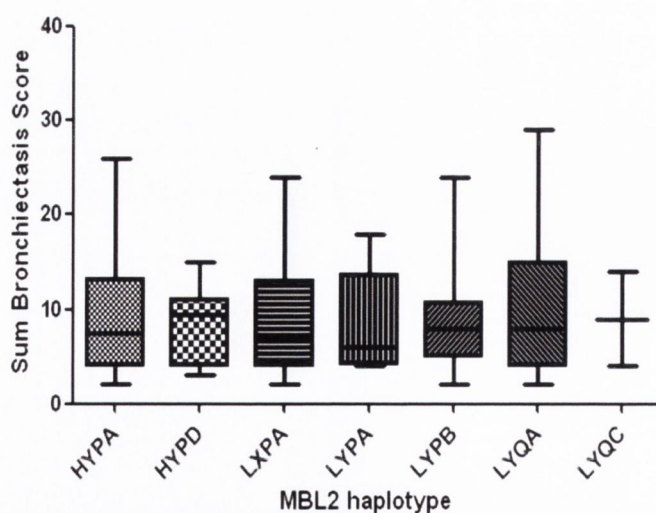


Figure 6.17 showing box-whisker plot of bronchiectasis scores available on 93 bronchiectasis patients of known MBL2 haplotype (total haplotypes = 186). No significant differences are observed (Kruskall-Wallis, $p > 0.05$)

Analysis of Bronchiectasis CT scan scores on the basis of MBL haplotypes reveals no significant difference in mean bronchiectasis scores between the various MBL groups (Figure 6.17). In addition to the serological evidence, these results suggest that MBL in isolation has little impact on the presence or severity of bronchiectasis.

6.4 Discussion

Deficiency of mannose binding lectin has been associated with a number of disease processes. Research interest centres on its potential role in infection susceptibility, making it a good candidate gene for bronchiectasis.

MBL has not been studied extensively in an Irish context. Initially the frequency of different haplotypes was examined in healthy Irish controls (Figure 6.3, page 238). This showed that the haplotype distribution of the Irish population was similar to other Northern European populations (Garred, 2008, Garred et al., 2009, Villarreal et al., 2001). This indicated a variant haplotype frequency of 23%, broadly in line with comparable populations. The MBL pathway probably represents the most evolutionarily ancient pathway of complement activation (Boldt et al., 2010). It may be suspected that such an ancient pathway resulting in a varied global distribution of genotypes may be due to selective pressure. However, Verdu *et al* argue that such changes are due to population migration and evolutionarily neutral population drift alone (Verdu et al., 2006). This point is often put forward as an argument for the immunological redundancy of MBL2 variation. Nevertheless, the consistency of these results with similar populations provided reassurance about the robust nature of the commercial genotyping assay.

As expected, when MBL levels in the healthy control population were examined, concentrations varied with genotype as previously documented (Figures 6.6 and 6.7, pages 241 and 242).

Subjects homozygous for wild-type AA alleles had highest MBL levels, while those with low MBL levels had low or absent levels. These findings allowed development of a population based normal range (Figure 6.8, Table 6.2, pages 243-244). Based on ROC curve analysis for discrimination between AA and AO/OO genotypes an optimum cut-off of 0.75µg/ml was determined. Such results, while uninteresting from a research point of view, are important to

the function of the immunological laboratory. These data have allowed local reference ranges to be reset to more accurately reflect the local population.

Examination of MBL genotype frequency in the context of bronchiectasis proved disappointing. No significant difference in the distribution of summary haplotypes or haplotype frequencies was noted between patients and controls (Figures 6.4 and 6.5, pages 239 and 240). Variant MBL polymorphisms have been shown to be important in lung disease. Two studies have identified links between deficiency genotypes and infective sequelae in COPD (Lin et al., 2011b, Yang et al., 2003). Evidence is also present for an association with invasive pneumococcal disease (Valles et al., 2010). However, the validity of such 'genotype only' studies have been questioned (Eisen, 2010). This is because, as is apparent in Figure 6.4, some individuals with genotypes associated with deficiency can still produce significant amounts of MBL. Therefore studies relying on gene associations alone may underestimate disease associations (Eisen, 2010). Serum levels of MBL were therefore assessed in patients with bronchiectasis.

In contrast to expectations, MBL levels were found to be higher in patients than controls (Figure 6.9, page 245). Analysis of summary genotypes indicated that this difference was specific to the AA genotype. In the AO group no difference was noted in serum MBL levels between patients with bronchiectasis and controls. The patient cohort showed no evidence of association of low serum MBL levels with disease. This finding of an increase of MBL levels in bronchiectasis should not come as a surprise as MBL is an acute phase reactant (Thiel et al., 1992). The observations presented in the present study have a number of possible explanations. Patients in the AA bronchiectasis group could have more significant bronchiectatic change than those in the AO/OO group; this would be contrary to expectations. However, when more severe inflammatory disease is present MBL levels can be elevated (Serag Esmat 2012). Alternatively an age related change in MBL is possible as the control group was not age matched. However, MBL

is thought to fall slightly with age. A more likely explanation is that the presence of bronchiectasis is triggering an inflammation related acute phase response that is only observed in patients with sufficient genotypes. This finding has been observed previously in patients with community acquired pneumonia (Herpers et al., 2009b).

In the setting of bronchiectasis MBL still retained a good discriminatory ability to identify sufficient and insufficient genotypes (Figure 6.10, page 246). However, the elevation in mean MBL levels in the bronchiectasis group has resulted in a change in the optimum discriminatory cut-off. Clearly the use of a single reference range calculated in this way is not suitable to define MBL deficiency, as disease specific changes in MBL will have an impact on the decision threshold. This has led some authors to use arbitrary cut-offs at a level beyond which they believe MBL serum deficiency may become more clinically significant (Eisen, 2010). These findings suggest that in other inflammatory diseases, levels of MBL would have to be used with caution to define deficiency status.

To determine whether the increased levels of MBL seen in the patient group were indeed due to the presence of ongoing low grade inflammation the levels of other acute phase reactants were assessed. Given the presence of an intercurrent inflammatory disease it is not surprising that levels of CRP are higher in bronchiectasis than controls (Figure 6.11a, page 247). This is in contrast to α 1 antitrypsin levels which were not different in the two populations (Figure 6.11b, page 247). However, when the patient cohort is examined in terms of summary MBL genotype, significantly higher levels of CRP are found in the AO/OO group (Figure 6.12, page 248). In an attempt to exclude possible age related differences a small age and gender matched cohort was obtained and MBL and CRP levels assessed (Figure 6.13, page 249). These results do not have the back-up security of the MBL genotyping data, but remain a suitable, if not ideal set of data. No age dependent differences between controls and age matched controls were noted. A recent

study has suggested that raised CRP in bronchiectasis may be associated with the presence of bacterial colonization even during periods of quiescent disease (Ergan Arsava and Coplu, 2011). These results may indicate that patients with MBL genotypes associated with low levels of MBL have increased systemic inflammation that may be related to pulmonary pathology. The long term effects of such systemic manifestations of bronchiectasis are not clear and would require longitudinal study.

The relationship between inflammatory markers in the setting of bronchiectasis was examined next. CRP, a sensitive marker of inflammation correlated weakly with α 1 antitrypsin levels (Figure 6.14, page 250). A weak correlation is to be expected as CRP is generally a more sensitive marker of inflammation (Amezcu-Guerra et al., 2011). MBL levels did not correlate well with transformed CRP (for skewness) or α 1 antitrypsin (Figure 6.15, page 251). Even when AA haplotypes were considered alone no correlation was observed (Figure 6.16a, page 251). However examination of YA/YA haplotypes did reveal a weak correlation with CRP (Figure 6.16b). Previous studies have shown that the presence of an O haplotype or the presence of the X promoter may reduce the ability to mount an MBL acute phase response (Herpers et al., 2009a).

These results suggest that MBL genotype and levels are not strongly associated with the presence of bronchiectasis. However, CRP levels are higher in patients with deficiency genotypes. Therefore, data from the clinical cohort was interrogated for evidence of an association between MBL genotype and levels and indices of severity of bronchiectasis. MBL levels were assessed at a number of different discriminatory points, due to the controversy that exists regarding what constitutes normality. Absolute deficiency of MBL was associated with increased production of purulent sputum only (Table 6.3, page 252). No other associations were noted. Examination of individual haplotypes suggested that patients with the LYQA haplotype

were less likely to have a hospital admission or to produce daily purulent sputum (Table 6.4, page 253). Individual MBL2 haplotypes have been identified as modifying genes in the past. LYQA has been associated with an increased risk of post operative myocardial infarction (Collard et al., 2007). Other A haplotypes have been associated with Behcets disease and ankylosing spondylitis (Park et al., 2005). However, in this study it is not clear why such a protective effect of LYQA is specific to that haplotype. Perhaps, of more concern is the inconsistency of the clinical association which stands in sharp contrast to the CD32A H131R clinical results presented previously. In particular no association with protection against sputum culture positivity or infective exacerbation was noted. Furthermore, no association with CT scores was noted (Table 6.5 and Figure 6.17, page 254).

These results suggest that MBL2 genotype and serum levels are not strongly associated with the presence of bronchiectasis. They add to the previous observations of Kilpatrick et al and stand in contrast to the emerging story of a possible association of MBL with COPD (Lin et al., 2011b, Yang et al., 2003, Kilpatrick et al., 2009). MBL2 genotypes associated with low serum levels were associated with higher levels of CRP. However, the consequences of this are unclear. While other studies have found an association between high CRP levels and bacterial colonization, no evidence of this was noted in the MBL deficient AO group (Ergan Arsava and Coplu, 2011). Very low levels of MBL (<0.2) in the serum were associated with daily sputum production, but no other associations were identified. Examination of haplotypes suggested that the presence of the LYQA variant was protective against hospital admission over 5 years and daily sputum production. This result may be questioned due to the small study size, the lack of consistency with other clinical results and the lack of a clear mechanistic explanation as to why the LYQA haplotype should be protective in isolation.

6.5 Conclusion

In general these results do not suggest that MBL plays a major role in the pathogenesis or severity of bronchiectasis in adults. As a defect in the innate immune system MBL deficiency may prove of more clinical importance in children, and may yet be shown to play a role in paediatric bronchiectasis. Furthermore, it may be noted that the association of MBL with disease is an area with much conflicting evidence. This may indicate that MBL, by itself, is having little direct effect but may have an effect in association with other variables. Very large studies would be required to derive meaningful data to answer this question. At this stage the routine measurement of serum MBL or MBL2 genotype cannot be recommended in the setting of idiopathic bronchiectasis in adults.

Chapter 7. Overall conclusions

This study represents a significant addition to the literature on bronchiectasis. We recruited a well characterized cohort of patients with bronchiectasis. Findings in this Irish population echoed those of similar studies from developed countries which suggest that bronchiectasis affects patients of late middle age and beyond. Patients suffer considerable problems both in terms of comorbid respiratory disease and infection related illness, with just under half of patients reporting at least four infective exacerbations per year, and at least one recent hospital admission. These results suggest that bronchiectasis represents both a burden to individual patients and to Irish healthcare providers. In this setting it is important that possible causes and modifying factors are explored.

This study used a candidate gene approach to examine possible associations of immune system polymorphisms with idiopathic bronchiectasis. A significantly higher proportion of patients with the CD32A RR131 genotype were identified in the bronchiectatic patient cohort than in a control population. Furthermore the heterozygote CD32A HR131 variants were under represented in bronchiectasis, a pattern that has been observed in other infection models (Yuan et al., 2008, Yuan et al., 2003). These findings suggested that CD32A genotypic variation could play a role in bronchiectasis. This observation adds to a number of other studies in this area that have identified genetic associations with bronchiectasis (Bienvenu et al., 2010, Boyton et al., 2008, Boyton et al., 2006b, Stankovic et al., 2009). The cohort in this study is small but is comparable to the majority of studies on bronchiectasis. Additionally, while patients were excluded for identifiable causes, we did not limit the selection of bronchiectasis patients based on HRCT involvement patterns as others have (Boyton et al., 2008, Boyton et al., 2006b). The cohort presented here may be more heterogeneous than other cohorts, a possible disadvantage. However, our cohort may represent a more 'real world' cohort.

This study has also demonstrated an association between variation in CD32A H131R genotype and a number of clinical parameters. Patients with the CD32A RR131 genotype were more likely to produce purulent sputum on a daily basis and to have cultured a potentially pathogenic organism. These findings echo previous studies that suggest purulent sputum is associated with bacterial colonization (King et al., 2007, Murray et al., 2009). The association with positive sputum culture was marked for both *Haemophilus influenzae* and *Streptococcus pneumoniae*. No association with *Pseudomonas aeruginosa* sputum culture was noted and in fact colonization with this organism was lower than in some other studies. This may reflect a less severe disease phenotype in our cohort.

Patients with the RR131 genotype reported more infective exacerbations and were more likely to have been admitted to hospital over a five year time window. Again, these findings contrast with CD32A heterozygosity which appeared to offer protection. CD32A H131R also appeared to be associated with less severe impairment of lung function, while the RR131 genotype was, in a subgroup, associated with more significant limitation of gas transfer. The RR131 genotype based on these observations appears to predispose to more severe lung disease. The reduced transfer factor may indicate alveolar inflammation secondary to bronchiectasis complicated by ongoing infection.

Finally the RR131 genotype is associated with worse HRCT scan scores than the heterozygote state. The clinical significance of the small, yet statistically significant difference observed, is open to debate. The observation does add further objective weight to the clinical and physiological data.

Taken together these data provide robust evidence that CD32A H131R is not only associated with idiopathic bronchiectasis, but also modifies the severity of the disease. This intriguing observation is complicated by the fact that all of the polymorphic variants of CD32A are common

in the population and compatible with perfect health. Obviously, this gene is not acting alone; other genetic and environmental factors are at play. One additional observation with possible relevance to this is a weak association of smoking with worse lung function scores in the CD32A RR131 group alone. In this small cohort the association reaches borderline clinical significance only, however, it clearly warrants further examination. The role of smoking as a primary cause of bronchiectasis remains controversial, however, a link between smoking and bronchiectasis in patients with rheumatoid arthritis has been identified (Kaushik et al., 2004). In addition other studies have observed a link between a smoking history and mortality in bronchiectasis (Finklea et al., 2010). If this finding is replicated in further studies, it could represent an important gene-environment interaction of importance in bronchiectasis and other respiratory disorders.

In the light of the robust clinicopathological data, the role of CD32A H131R variation in phagocytosis was examined in a blinded fashion. There has been interest in this area for several years (Flesch et al., 1998, Jansen et al., 1999, Rodriguez et al., 1999). Using a modification of a published flow cytometric assay we were able to demonstrate in a clear and reproducible fashion differences in the neutrophil phagocytosis of pneumococcal polysaccharide coated beads and whole bacteria depending on CD32A RR131 genotype (Martinez et al., 2006). A significantly lower proportion of RR131 bearing cells phagocytosed targets than other groups. HH131 phagocytosed the most, while the heterozygous state demonstrated intermediate phagocytosis. Genotype also had an impact on efficiency of phagocytosis, with a lower percentage of RR131 bearing cells phagocytosing multiple of beads. While other authors have shown similar findings using different methods we extended these observations under more physiological conditions and using pathogenic targets that have potential relevance in bronchiectasis. The differences in phagocytosis were also echoed by differences in the oxidative burst generated on stimulation of neutrophils by opsonised beads. Furthermore, similar differences in the release of elastase and myeloperoxidase from neutrophil granules depending

on genotype were also observed. These data demonstrate that variation at CD32A has an important impact on the *in vitro* phagocytosis of pneumococcal targets by neutrophils.

These findings were further extended by the development of a whole blood phagocytosis assay. Using this assay many of the neutrophil findings were replicated in whole blood using serotype 9V beads and both serotype 14 and 9v bacteria. This is strong evidence that the differential phagocytosis related to variation in CD32A is not specific to any one serotype of pneumococci. In addition cells of the monocyte macrophage lineage were also examined. These experiments demonstrated a similar pattern of reduced phagocytosis in RR131 bearing CD14 mononuclear cells exposed to opsonised pneumococcal targets. RR131 neutrophils and CD14 mononuclear cells both produced reduced oxidative burst. In neither the isolated neutrophil nor the whole blood assay were we able to identify genotype dependent differences in cytokine expression. However, caution should be exerted in interpreting these results because of the limited time points used. One interesting and unexpected observation from the whole blood assay was that at the 4 hour time point no genotype dependent differences in phagocytosis were observed. It may be that differences in phagocytosis caused by variation in the CD32A receptor are particularly important in the early phase of the response to bacterial challenge. The relevance of this 'speed of response' to clinical susceptibility to colonization or infection remains to be elucidated, but is likely to be of critical relevance.

The various strands of evidence presented here can be drawn together to form a compelling paradigm for the involvement of CD32A H131R variation in bronchiectasis. This data implies that CD32A RR131 is associated with a relative phagocytic defect in both neutrophils and CD14 mononuclear cells. Reduced phagocytosis by RR131 bearing cells is accompanied by the generation of reduced oxidative burst, and in neutrophils, reduced release of cytotoxic granular products. These abnormalities may favour the persistence of infection at mucosal surfaces. Failure to deal with infective insults in a timely manner may result in the propagation of overt

clinical infection and over long periods of time tissue damage resulting in bronchiectasis, and in the presence of bronchiectasis an increased susceptibility to infection with encapsulated bacteria. This is in keeping with the vicious cycle of infection and inflammation thought to be central to bronchiectasis (Cole, 1986). In addition, the failure of RR131 cells to phagocytose bacterial targets and to produce a large oxidative burst may prevent an important brake on inflammation in the lung (Marriott et al., 2008). This reduced oxidative burst may facilitate inflammatory cell recruitment in the lung allowing susceptible subjects to enter a vicious cycle of persistent infection and poorly regulated inflammation. The RR131 represents therefore a partial immune system defect and a double edged inflammatory sword. It is interesting to speculate, based on results presented here that HR131 represents an ideal, with efficient phagocytosis and adequate regulation of NADPH mediated inflammation, resulting in protection from both tissue damage and local infection.

The strength of the CD32A data is contrasted with the lack of a consistent story for MBL in bronchiectasis. No association between low MBL levels, deficiency genotypes and bronchiectasis was observed. Very low levels of serum MBL did appear to be associated with the production of purulent sputum. In addition the MBL sufficient genotype LYQA was associated with fewer admissions and less sputum production. These retrospective clinical observations were not supported by objective microbiological, physiological or radiological data. MBL deficiency genotypes were associated with higher levels of CRP, but, despite this we did not observe an association for higher CRP levels with bacterial colonization reported previously. The MBL data suggests that MBL is not a modifying gene in idiopathic bronchiectasis in adults.

This is a novel study which makes an important contribution to our understanding of bronchiectasis. However, as with all scientific observations, this study has a number of weaknesses. The method of patient recruitment, whilst efficient, is open to recruitment bias. In

addition, the method of data interrogation and collection is associated with all the criticisms common to retrospective studies. While the cohort shared many characteristics with other bronchiectasis populations from developed countries, the radiological disease presented was quite mild. This finding is supported by the relatively low frequency of pseudomonas colonization. Therefore the very heterogeneity of bronchiectasis that curses all studies in this area may limit the generalisability of these findings. As with all candidate gene studies, duplication in other populations will be necessary to determine the long term importance of these observations.

The functional aspects of the study also have limitations. Oposonised pneumococcal beads and bacteria were chosen as targets. However, although pneumococci are important pathogens in bronchiectasis other bacteria are involved more commonly in established bronchiectasis in adults (Pasteur et al., 2010). Extension of these observations to other relevant encapsulated target organisms such as *Haemophilus influenzae* would be useful to support this data. Nevertheless, it must be appreciated that pneumococcus, as an important and common pathogen, may be important at the initiation phase of bronchiectasis, about which we know very little. The use of peripheral blood CD14 mononuclear cells as a surrogate model for alveolar macrophage has obvious drawbacks. Despite this, the flow cytometry model developed here was easy to perform and generated interesting results. Furthermore, other possible approaches, such as using monocyte derived macrophages, or cultured bronchoalveolar lavage alveolar macrophages have inherent criticisms also. Validation of the phagocytosis findings in this study using these other techniques would bolster the hypothesis that CD32A polymorphic variation impacts on alveolar macrophage phagocytic function. Finally, it should be noted that the highly artificial *in vitro* experiments carried out here probably do not reflect the mix of pathogens, opsonins, cells and mediators in lung tissue. Studies in animals have the potential to address some of these criticisms.

Against these criticisms is the strength and consistency of clinical and laboratory correlations especially in the CD32A portion of the study.

Medicine is moving towards a practice that is individualized for the patient. In the setting of bronchiectasis, this study could lead to tests which identify patients at risk of the disease in potentially susceptible groups, or identify those with the disease who may benefit from more aggressive treatment. It seems likely that early identification and intervention in susceptible individuals, such as those with the CD32A RR131 genotype could improve outcome. The development of convenient salivary genotyping and the rapidly falling costs of genotype assessment, may mean that such tests could be available to clinicians on a routine basis in the near future (van Schie and Wilson, 2000). It is clear that in bronchiectasis CD32A is not acting alone. It is likely that a concert of genes, in addition to environmental factors, is at play.

Candidate gene studies are not the best way to approach this issue (Yang et al., 2008). Idiopathic bronchiectasis is a complex condition that is ripe for investigation using newly developed and newly affordable genomic and exomic sequencing strategies. Central to such strategies is the recruitment of very large, multicentre, well characterized cohorts. These strategies could help identify multiple genes of interest and allow the development of a true appreciation of the relative importance of immune system variation in bronchiectasis. For this to happen, clinicians and scientists with an interest in idiopathic bronchiectasis must strive to release it from the shackles of the 'orphan disease' label. The enthusiasm for idiopathic bronchiectasis at recent international meetings suggests that the next decade could see new interest, new collaboration and significant advances in this area.

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