

**RECOVERY FROM
COMMUNITY ACQUIRED PNEUMONIA**

THESIS SUBMITTED IN ACCORDANCE WITH THE
REQUIREMENTS OF THE UNIVERSITY OF LIVERPOOL FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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DECLARATION

The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification. This thesis is the result of my own work except where below.

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ABSTRACT.

RECOVERY FROM COMMUNITY ACQUIRED PNEUMONIA.

A PhD Thesis by Daniel Gower Wootton

Aims

To measure symptomatic recovery over a year among an adult cohort recruited from hospital with community acquired pneumonia (CAP). To measure the host recovery mechanism efferocytosis and the diversity of the bacterial microbiota in sputum and relate these to individual characteristics of subjects in the cohort.

Methods

Patients with CAP were recruited from two hospitals in Liverpool, (UK) and were followed-up for one year. The CAP-sym questionnaire was completed at multiple time-points in order to create a statistical model of symptomatic recovery. DNA was extracted from acute sputum samples and 16S rRNA sequencing revealed the diversity of bacteria in sputum. At one month into recovery subjects volunteered for bronchoalveolar lavage and rates of efferocytosis were measured by co-culturing ex-vivo alveolar macrophages with apoptotic autologous neutrophils.

Results

The 169 subjects recruited with CAP were found to have high levels of socio-economic deprivation, smoking and COPD and the median age was 64 years. A non-linear, longitudinal, statistical model of symptoms found that smoking impaired recovery but people tended to describe better recovery as they got older. Efferocytosis was impaired by smoking but improved by statins and these effects were modified by body mass index. Those with prior pulmonary disease had lower bacterial diversity in their sputum and in this cohort a species from the genus *Haemophilus* was dominant.

Conclusion

This work proves the principal that modelling CAP-sym scores can be used to investigate factors associated with differential recovery from CAP. It highlights the detrimental effects of smoking on both recovery and efferocytosis. This is the first study to show that the bacterial diversity of CAP sputum is influenced by prior lung disease. The translational outcomes are the potential for trials of statins as pro-recovery agents and to study modified empirical antibiotics for those with CAP and prior-lung disease.

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Defining Community Acquired Pneumonia

1.1.1 What is Pneumonia?

The term pneumonia is old. In the 5th Century BC Hippocrates is said to have referred to it as ‘a disease of the ancients’.[1] Hippocrates was using the term in a clinical context and defined its features:-

“... if fever be acute, and if there be pains on either side, or in both, and...if cough be present, and the sputa expectorated be of a blond or livid colour...” [1]

For practicing clinicians today, pneumonia remains a syndromic, clinical diagnosis based upon a combination of signs, symptoms, and where available, radiological findings. It describes a patient with an acute lower respiratory tract infection, positive examination findings and associated systemic features such as fever. Post-mortem examination of lungs from patients who have died with this syndrome reveals the distal airways and lung parenchyma filled with an inflammatory infiltrate that is predominantly neutrophilic.[2] This has led to the expansion of the term pneumonia to include the pathological finding of distal airway and parenchymal inflammation in general, including non-infective inflammation - for example ‘eosinophilic pneumonia’, ‘cryptogenic organising pneumonia’ and ‘non-specific interstitial pneumonia’.[3] The pneumonic infiltrate can sometimes be seen on a plain chest x-ray (CXR) or computed tomography (CT) scan where it is associated with a new set of terminology based on its extent and anatomical distribution.[4,5]

1.1.2 What is Community acquired pneumonia?

A patient with pneumonia who has not had a recent hospital admission is described as having community acquired pneumonia (CAP). The site of acquisition is relevant as it distinguishes this syndrome from others such as hospital acquired pneumonia (HAP) and ventilator acquired pneumonia (VAP). CAP includes those who have aspirated a large volume of oropharyngeal contents or vomitus.[6] This phenomenon is thought to be common in the elderly and in those with neurological deficit.[7] Most studies of CAP exclude patients where aspiration pneumonia is strongly suspected but in routine clinical practice it is difficult to verify when pneumonia has developed as a consequence of aspiration. In the United States of America another entity, Health Care Associated Pneumonia (HCAP), is recognised.[8] This is pneumonia which develops in a patient who resides in a nursing home or other healthcare institution where the use of antibiotics is common. Neither the UK British Thoracic Society (BTS) guidelines nor the recent National Institute of Clinical Excellence (NICE) pneumonia guidelines recognise HCAP as a separate phenomenon as the evidence for a different spectrum of organisms and or outcome is weak; these patients are treated as CAP. [9,10] Defining the various pneumonia syndromes above enables the clinician to empirically target therapy towards a particular spectrum of aetiological agents that are associated with each. Definitions of CAP differ across national guidelines and within individual guidelines there may be several definitions applicable to different clinical settings – for example primary and secondary care. For the purposes of this thesis the ‘in hospital’ definition of CAP found in the BTS Guidelines for the management of CAP will be used and are reproduced in figure 1.1.[6]

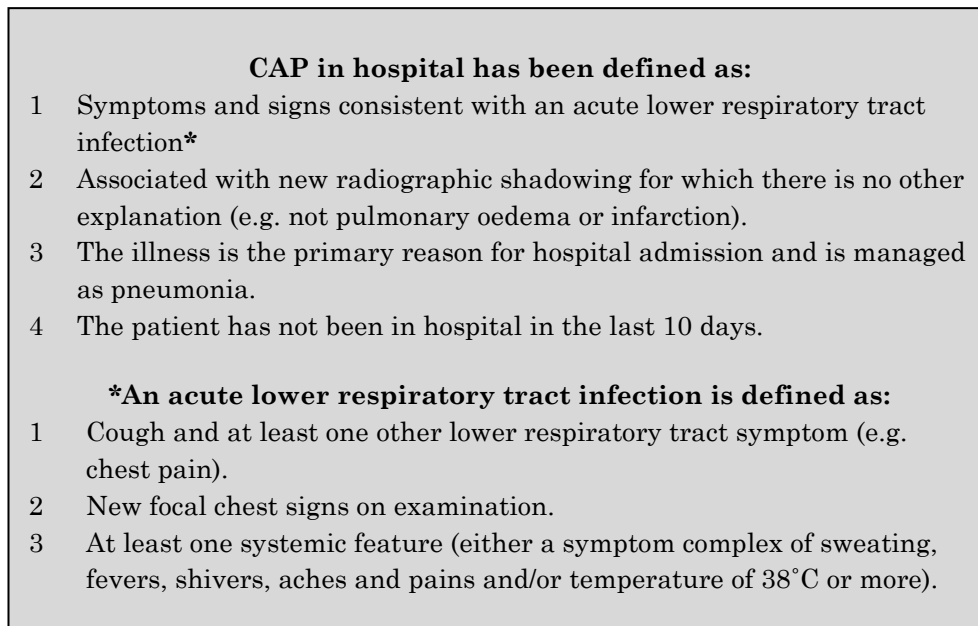


Figure 1.1 BTS in-hospital definition of CAP

1.1.3 Imprecision associated with the definition of CAP

The BTS definition of CAP requires the identification of a new infiltrate on a CXR but it is recognised that levels of inter-user agreement in the interpretation of CXRs can be low and that this reduces the precision of the diagnosis of CAP (see figures 1.2, 1.3, 1.4 and 1.5).[11] Moreover, although the term CAP has become widely adopted into clinical practice it has not yet been recognised by The World Health Organisation (WHO) International Classification of Diseases (ICD). The most up to date iteration of this document, WHO ICD-10, includes 18 major codes which are further sub-divided into 42 sub-codes for infective pneumonia.[12] Community acquired pneumonia does not easily map to any single one of these ICD-10 codes and this can cause inconsistencies when institutions and researchers try to assess the incidence of CAP.

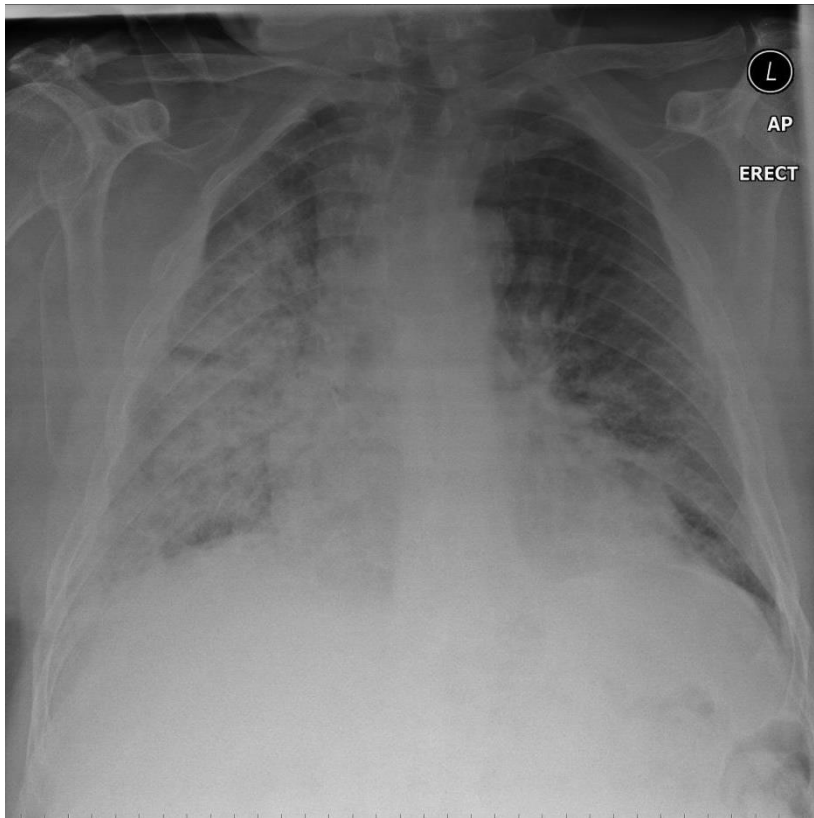


Figure 1.2 PASS subject admission CXR

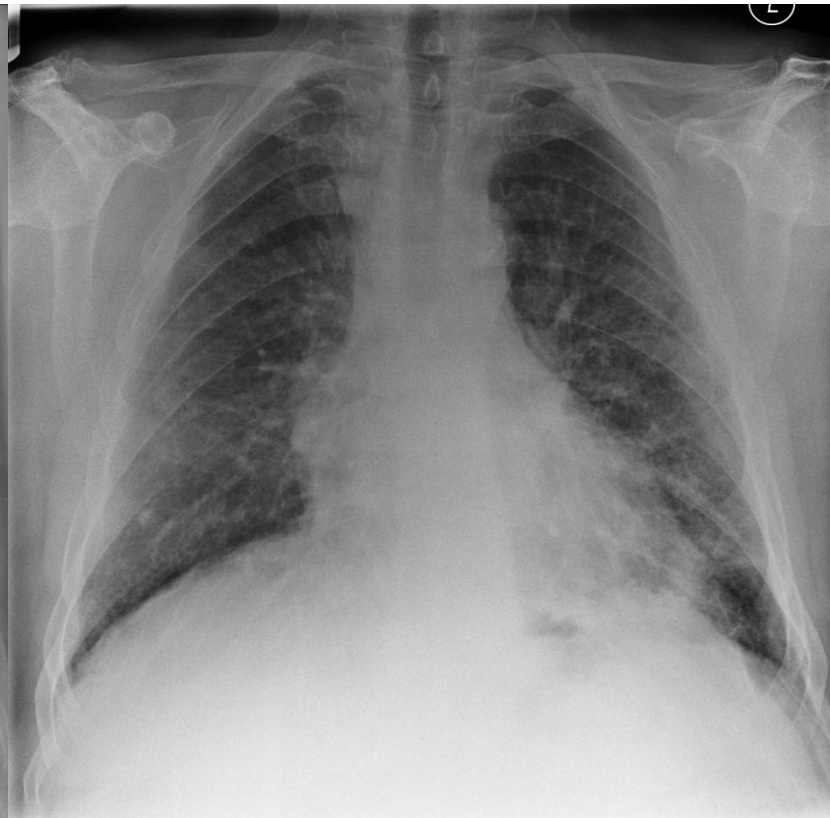


Figure 1.3 Repeat CXR at follow up one month later

These are examples of relatively straightforward chest x-rays in the context of a patient with a clinical syndrome consistent with CAP. The acute film on the left shows diffuse opacification throughout the right lung field. The recovery film at one month reveals good resolution. These films would likely produce high levels of agreement among reporting clinicians.

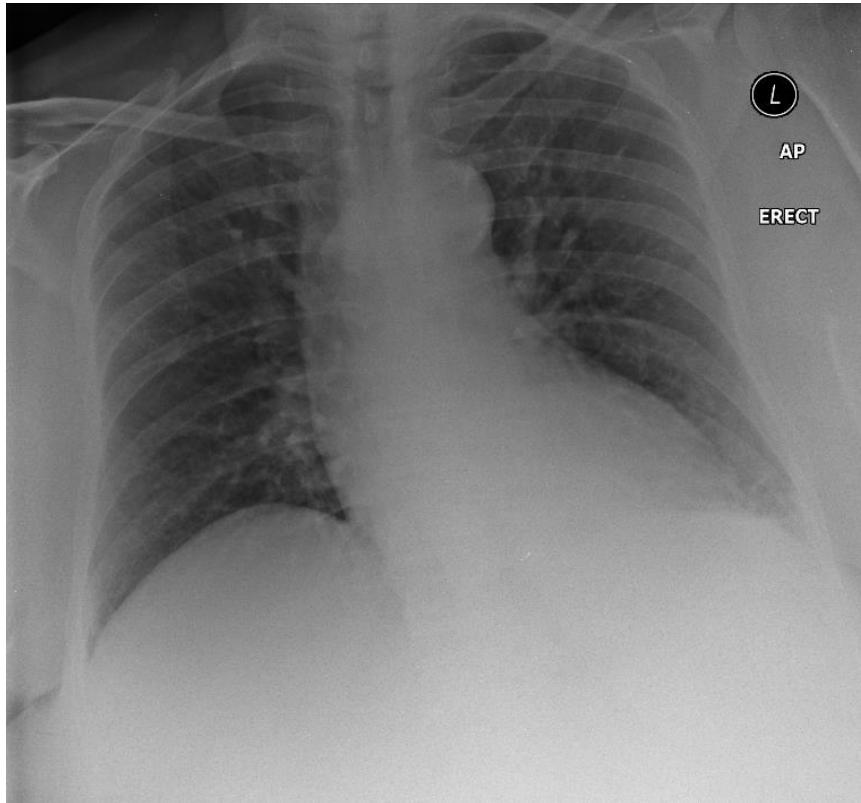


Figure 1.4 Patient screened for PASS



Figure 1.5 CT slice through the thorax of the same patient

The film on the left is more ambiguous than figure 1.2. This patient had a clinical syndrome compatible with CAP but pleuritic chest pain and hypoxia were prominent. A CT pulmonary angiogram excluded pulmonary embolus and revealed left basal consolidation behind the heart, in the x-rays 'blind spot'.

1.2 Epidemiology of Hospitalised CAP in the UK

1.2.1 Incidence

In the UK, between 5 and 11 of every 1000 adults develop community acquired pneumonia (CAP) each year.[6] In 1992/3 the National Health Service (NHS) treated 16.3 million episodes of lower respiratory tract infection (LRTI) and classified 261000 (1.6%) as CAP of which 32% was admitted hospital.[13] Between 1997/8 and 2004/5 the annual, age-standardised incidence of admission to hospital with CAP increased by 34% from 1.48 to 1.98 per 1000 of the UK population.[14]

1.2.2 Seasonality

Pneumonia is a disease of the cold winter months and the UK peak is normally around the weeks surrounding Christmas / New Year.[15] This trend is not restricted to the UK and a strong relationship with climate and in particular ambient air temperature has been demonstrated. In Japan, for every 1°C fall in temperature the rates of CAP increased by 0.03%.[16]

1.2.3 Age

CAP is a disease of the extremes of age. It is common in children under the age of 5 and incidence increases exponentially in adults for every decile above 55 years.[17,18] In the UK whilst the incidence of CAP hospitalisation has increased across all adult age groups the greatest increase (39%) has been in the most elderly (>85 years).[14] In the year 2009/10 the mean age of adults admitted to UK hospitals with CAP was 71 years.[19]

1.2.4 Comorbidity

Pneumonia is an illness associated phenomenon. It is strongly associated with a wide range of comorbidities and in most cases a pre-existing chronic condition or recently acquired comorbidity (such as viral respiratory tract infection) can be identified.[20] As might be expected pneumonia rates are high among those with inherited or acquired immune deficiency.[21] However, in addition to this a range of other chronic conditions predispose to pneumonia in particular pre-existing chronic lung disease and smoking.[22]

Patients recovering from CAP are at a significantly increased risk of death for at least the next year when compared to control patients.[23] This risk seems to be greatest in the elderly and is associated with comorbidity, [24-26] in particular there is a widely reported increased risk of cardiovascular morbidity and mortality following CAP.[27]

1.2.5 Aetiology and antimicrobial treatment

Current evidence suggests that, in adults, CAP is most frequently caused by bacteria and because of this patients are given antibiotics.[6] This is clearly an effective strategy as CAP mortality rates following the development of penicillin fell dramatically.[28] To be most effective antibiotics need to be given as soon as possible and this does not leave enough time to determine which bacteria are causing the pneumonia. Traditional diagnostic tests require culturing a clinical specimen, isolating the causative species and then determining which antibiotic it is susceptible to. This process currently takes 2-3 days. Therefore, at the time of commencing treatment the antibiotic choice is 'empirical', that is a best guess based on the most likely causative

organism. To help clinicians choose the most suitable empirical antibiotic, guidelines are drawn-up with various options for particular patient groups and circumstances. These guidelines are based on studies that have described the incidence of bacteria cultured from large numbers of people with CAP (see Table 1.1). Large prospective studies of CAP aetiology in the UK have demonstrated the importance of *Streptococcus pneumoniae* as the commonest causative organism of CAP. However, these studies only achieve a confirmed microbiological diagnosis in up to 50% of patients.[29] In routine clinical practice the causative organism of CAP is determined in less than 15% of hospital cases.[29,30] There are a number of reasons why detection rates are so low in CAP. Cultures of bacteria may be impaired if patients have recently taken antibiotics. Some bacteria are technically more difficult to culture and therefore we are only able to grow 1% of bacterial species that can be detected by molecular testing.[31,32]. As a consequence of these factors we have an incomplete understanding of the range of bacteria that cause CAP. The resulting empirical use of combination broad-spectrum antibiotics is associated with complications that are avoidable with more targeted therapy.[33] In the last 10 years antigen detection tests have become widely available for detecting *Streptococcus pneumoniae* and *Legionella pneumophila* in urine. These tests are very specific. The *Legionella* antigen test is much more sensitive than culture alone and the pneumococcal antigen test, when combined with culture of blood and sputum detects some additional cases of pneumococcal disease.[34] In the UK these antigen tests are now recommended for all severe CAP cases.

Recently developed multiplex quantitative polymerase chain reaction (qPCR) tests have the potential to markedly improve the

speed and precision of microbiological diagnosis in CAP.[35] Several of these platforms include a range of bacterial and viral qPCR based techniques.[36] If these techniques could be made more rapid then pre-treatment aetiological diagnosis would allow the use of targeted antibiotic therapy resulting in decreased antibiotic resistance and reduced antibiotic-related complications.[37,38] However, these qPCR platforms are limited to the detection of specific agents that are believed, a priori to be potential pathogens and do not help determine the full range of potential pathogens in an individual. There is a need for studies to look again at the aetiological agents in CAP using the comprehensive tools used in microbiome studies to determine what may account for the large number of culture negative cases in order to validate current treatment guidelines (see 1.6).

Aetiological Agent	Mean % (95% CI)
<i>Streptococcus pneumoniae</i>	39 (36.1 to 41.8)
<i>Haemophilus influenzae</i>	5.2 (4.0 to 6.6)
<i>Legionella</i> spp.	3.6 (2.6 to 4.9)
<i>Staphylococcus aureus</i>	1.9 (1.2 to 2.9)
<i>Moraxella catarrhalis</i>	1.9 (0.6 to 4.3)
Gram-negative enteric bacteria	1.0 (0.5 to 1.7)
<i>Mycoplasma pneumoniae</i>	10.8 (9.0 to 12.6)
<i>Chlamydophila pneumoniae</i>	13.1 (9.1 to 17.2)
<i>Chlamydophila psittaci</i>	2.6 (1.7 to 3.6)
<i>Coxiella burnetii</i>	1.2 (0.7 to 2.1)
All viruses	12.8 (10.8 to 14.7)
Influenza A and B	10.7 (8.9 to 12.5)
Mixed	14.2 (12.2 to 16.3)
Other	2 (1.3 to 3)
None	30.8 (28.1 to 33.5)

Table 1.1 BTS Analysis of aetiological agents of UK CAP in hospitalised patients

The above is based on data presented in the BTS CAP guidelines [6] and comprises data from 5 UK studies and a total of 1137 patients.

1.3 Outcome following CAP

1.3.1 Choice of outcome measure matters

Following an episode of CAP a range of outcomes can be measured against which we can prognosticate or assess the efficacy of interventions. Each has its own inherent strengths, weaknesses and applicability. Various studies have sought to link patient characteristics to a range of outcomes. However few of the traditional outcome measures used have been rigorously validated in CAP. Barlow et al. argued strongly for the inclusion of patient based outcome measures in studies of CAP and for more rigour when matching outcome measures to the design of studies.[39]

1.3.2 Clinical cure

Therapeutic trials of antimicrobials have commonly used clinical cure as an end-point. Clinical cure is determined by a clinician. It has no consistent definition. It generally implies that the clinician felt there was no longer a requirement for antibiotic. It is highly likely to be subject to significant inter and intra-clinician variation and it is not clear exactly which domains it is measuring.[40] As an example of its application, a recent antibiotic randomised controlled trial used clinical cure as its primary outcome measure of efficacy.[41] Clinical cure was not defined in the publication, but the protocol which is available as an online supplement, defines clinical cure as “*Total resolution of all signs and symptoms of pneumonia, or improvement to such an extent that further antimicrobial therapy is not necessary*”.[42] This definition of cure is ambiguous and open to a range of interpretations.

1.3.3 Clinical stability

Several authors have proposed formal physiological criteria for clinical stability that include factors such as resolution of pyrexia and hypoxia.[43] Clinical stability has then been used to make practical clinical decisions such as switching from intra-venous to oral antibiotics and the duration of antibiotics.[43,44] Clinical stability as a concept has been shown to be safe and the use of this endpoint is now incorporated into guidelines although criteria vary depending on the application.[10]

1.3.4 Mortality

CAP mortality rates in the UK are amongst the highest in Europe and each year 34,000 patients die.[6] The 2009/10 British Thoracic Society (BTS) audit of CAP demonstrated an in-patient mortality of 21% for CAP managed in UK hospitals.[19] Of the 7% admitted to intensive care over 50% die.[6,19] However, these results confirm that most patients hospitalised for CAP survive their initial infective insult to be discharged back to the community. Moreover mortality in the 70% of CAP treated in the community is <1%.[6] Therefore mortality may be a useful outcome in certain populations where death rates are high – for example the very elderly or those admitted to intensive care – but for less severe CAP in hospital and for those managed in the community the event rate is low and using mortality as the primary outcome measure would provide no information as to the clinical course of those who survived.

1.3.5 Health Economics of UK CAP

In 1992/3 the annual direct healthcare costs of CAP were estimated at £440 million and this figure will have increased with the increase in CAP hospital admission rates.[13,14] Although only 32% of patients with CAP are admitted to hospital they account for 96% of the total NHS CAP expenditure.[13] The health and economic consequences of CAP extend beyond the period of acute management. Many patients will not return to work for days or weeks following a period of hospitalisation with CAP. Others will have been carers and will be too frail to return to caring necessitating periods of respite for their dependents. Few studies have taken into account these post-discharge costs.

1.3.6 Length of stay

Length of stay is an important measure for hospital managers and commissioners since a night in a hospital bed is so expensive. However, length of stay is influenced by many factors that are not directly related to the severity or aetiology of the CAP or to the quality of care received.[45] Most CAP occurs in the winter when the NHS invariably has a hospital bed crisis and clinicians face a huge pressure to discharge patients as soon as possible. Moreover, due to regional differences in demographics and socioeconomic factors some hospitals will be more affected than others by discharge delays due to lack of availability of residential and nursing home places.

1.3.7 Readmissions

A significant number of patients are re-admitted to hospital following an episode of pneumonia. A Spanish study found that 2.5% of patients were re-admitted within 30 days with pneumonia related problems and 4.6% were readmitted with non-pneumonia related problems.[46]. In a study specifically designed to capture medium and long-term morbidity associated with re-admission, Johstone et al. found 2% of their cohort were re-admitted with pneumonia within 30 days of hospital discharge and a further 9% by one year.[24] In addition to these re-admissions there were a significant number of non-pneumonia re-admissions. Adamuz and colleagues looked not only at re-admissions to hospital but contact with healthcare in general following a CAP discharge.[47] They found that within 30 days of discharge 17% of patients presented to primary care with ongoing symptoms. 10% of patients presented to emergency departments with pneumonia related symptoms, 9% with non-pneumonia symptoms and 2% with a combination of pneumonia and non-pneumonia symptoms.

3% of patients were re-admitted due to pneumonia, 4% due to non-pneumonia illnesses and 1.6% with a combination of pneumonia and non-pneumonia problems. Rates of re-admission are difficult to compare across countries with different healthcare systems and even vary within regions of the UK due to the availability of out-of-hours primary care provision. However these studies demonstrate that despite being well enough to be discharged, for some patients, CAP is associated with substantial ongoing morbidity.

1.3.8 Patient based CAP outcomes

It is now widely accepted that a key component of healthcare delivery is the involvement of patients in decision making and management of their conditions.[48] Patients' experiences and expectations are now understood to be vital metrics of quality care.[49] Patients receiving a diagnosis of community acquired pneumonia (CAP) often ask "how long will it take to get better?" The literature reveals that answering this question is more difficult than it might seem. The British Lung Foundation (BLF) advice leaflet on pneumonia suggests patients should not expect to feel back to normal for several weeks or months following CAP – but does not suggest who might take weeks or who might take months.[50] Bruns et al. assessed recovery at 10 and 28 days following an episode of CAP of moderate severity. Their measures of recovery were chest x-ray (CXR) resolution (assessed by a radiologist), clinical recovery (assessed by a doctor) and patient reported recovery (assessed by using a symptom score).[51] They found discrepancies between all measures of recovery. Doctors declared the patients 'recovered' long before the patients' symptom score demonstrated they actually felt better. CXR resolution lagged behind doctor defined recovery but CXR

abnormalities were deemed resolved at a stage when many patients had yet to recover their symptom scores. Each of these modalities, clinical assessment, radiology and symptom evaluation has a role to play in the assessment of patients following pneumonia. Chest x-rays (CXRs) are performed with the aim of screening for an underlying pathology, such as lung cancer, or for identifying pneumonia associated complications such as empyema. Clinical assessment, supported by bio-markers can be used to inform acute management decisions such as duration of antibiotic therapy or day of safe discharge.[52] But only symptom scoring has been formally validated as an outcome measure in community acquired pneumonia (CAP).[39]

1.4 Symptomatic recovery from CAP

1.4.1 Validity of symptom questionnaires

A number of studies have used patient based techniques to assess symptomatic recovery from CAP. In a prospective study of LRTI in the community 35% of patients took longer than 7 days to return to normal activity.[53] Other groups suggest thirty percent of patients have not regained normal function after four weeks.[54,55] The validity of a healthcare outcome is determined by the application of the techniques of psychometrics to healthcare – sometimes referred to as clinimetrics.[56] This involves testing an outcome measure for its responsiveness (can it detect change in a patient's condition), reliability (e.g. same result from two different observers) and validity (does the outcome measure what it purports to measure). In the studies that have investigated symptomatic recovery from CAP most have used scoring systems that have not been validated.[39] However there are two scoring systems which measure symptomatic recovery from CAP that have been psychometrically validated: the 'CAP

SCORE' developed by a group from the Netherlands and the 'CAP-sym questionnaire' developed in the UK.[57,58].

1.4.2 The CAP SCORE

The CAP SCORE is divided into two sections and can be decomposed to give an overall CAP SCORE and two sub-scores for respiratory symptoms and well-being. The respiratory section enquires about 3 symptoms – shortness of breath, cough and sputum production. The well-being section has two questions – 'fitness' and 'general state of health'. The questionnaire uses mixed methods – the fitness question is a visual Likert scale and the other questions require the patient to choose one of several possible answers. When the numerical values for each question are summed the lower the CAP SCORE the worse a patient is feeling. During the validation study of this score the authors noted that the well-being scores took longer to improve than the respiratory component.[57] They also noted a lack of correlation between CAP SCORE and either the severity of CXR abnormality or speed of resolution of CXR findings. A follow-on study showed that resolution of respiratory symptoms in a cohort with CAP preceded the resolution of general well-being. The mean value of respiratory symptoms for the group had returned to baseline by 14 days where as the well-being mean took six months to normalise.[59] No statistically significant associations were found between patient characteristics and recovery defined as >80% of the pre-morbid score. One major criticism of this study of recovery is that patients who deteriorated in the first 3 days of treatment were excluded meaning this is a study of patient pre-destined to do well.

1.4.3 The CAP-sym questionnaire

The community acquired pneumonia (CAP) symptom (sym) questionnaire stands out among all other pneumonia symptom tools for the rigor applied during its design and validation.[60] A long-list of component questions was created by trained interviewers formally consulting 33 patients, drawn from the US and France, at a number of stages during pneumonia treatment. The resulting list of symptoms was then forwards and backwards translated by linguistic experts into 13 languages (English plus 12 others). The 18 question CAP-sym questionnaire was then tested within a randomised controlled pneumonia treatment trial among 556 patients from 13 countries. Validation used gold standard psychometric techniques. The final validated score consists of 18 questions which are asked by the study team member and it can be completed in about 2 minutes. A shorter version including just 12 of the 18 questions has very similar performance characteristics to the full version. Of note, during the validation of the CAP-sym questionnaire it was compared to a generic health questionnaire (SF-36) and was found to be more responsive to changes in the clinical state of patients with pneumonia.

1.5 The Pathophysiology of CAP

1.5.1 Host pathogen interactions

The factors associated with differential recovery from CAP are not clear however it is likely both host and pathogen factors play a part.[61,62] On the host side the resolution of the acute inflammation associated with CAP is essential for good recovery. Kruger et al. demonstrated that peak levels of several inflammatory and stress related biomarkers predicted mortality at 28 days.[63] Yende et al. showed that the acute phase cytokine IL-6 is elevated in many patients at the time of discharge

following CAP and that there was a correlation between IL-6 level and adverse events in the subsequent 3 months.[64] From the pathogen side it is clear that the pneumonia syndrome a patient experiences is influenced by the causative organism. For example the CAPNETZ cohort produced a number of papers describing in detail the similarities and differences between clinical characteristics and outcome among a large number of CAP cases of defined aetiology. They found that CAP caused by *Mycoplasma pneumoniae* was more likely to occur in younger patients, was a much less severe condition (lower severity scores, commonly out-patients) and the mean C-reactive protein level was a third of that in CAP caused by other confirmed pathogens.[65] In contrast, CAP caused by *Legionella pneumophila* was indistinguishable from CAP of other causes.[66] CAP associated with *Streptococcus pneumoniae* was more severe, more frequently treated in hospital and associated with higher rates of complications such as pleural effusions than the syndrome associated with other proven pathogens.[67]

1.5.2 Quiescent immune homeostasis in the healthy lung

(see figure 1.6) The distal regions of healthy lungs contain very few neutrophils and each alveolus contains on average one resident alveolar macrophage.[68] Alveolar macrophages are the principle phagocyte in the distal airway but also play a vital role as the interface between the innate and adaptive immune system.[69] In particular, via direct contact with the alveolar epithelium, they are the effectors of epithelial regulation of immune activation state in the lung.[70] When the alveolar epithelium is in an unthreatened homeostatic resting state, alveolar macrophages are subject to tonic inhibition in comparison to those in other anatomical sites.[71] This is desirable as the

lung is constantly challenged by antigen. There are on average 190,000 airborne bacteria per cubic metre of the low earth atmospheric air that we breathe, although this concentration varies in response to climatic, topographical and human geographical factors between 10^4 and $10^7/\text{m}^3$. [72] Our lower airway is in unbroken mucosal continuity with the mouth and nasopharynx which harbour very high concentrations of bacteria. Gleeson et al. showed that if you infuse radio-labelled technetium into the nose of normal people during normal sleep, the next morning a gamma camera will demonstrate isotope throughout the lung parenchyma of at least 50% of subjects. [73] Bacteria therefore continuously reach the lung via the air we breathe, by direct outgrowth across mucosal surfaces and by low-volume aspiration of nasopharyngeal secretions. If all of these bacterial challenges were to elicit a full blown immune response then the resulting continuous inflammation would make gas-exchange impossible. Anatomical features such as airway bifurcations and mechanical features such as the mucociliary escalator reduce the total burden of bacteria that reach the lung. Moreover, bacteria that do reach the airway find it an inhospitable environment. The lumen of the airway is rendered nutritionally barren by the action of various host scavenger molecules – a defence strategy referred to as nutritional immunity. [74] The mucosal lining fluid is suffused with multiple soluble antimicrobial compounds which are either directly toxic or inhibit bacterial growth; examples include lactoferrin [75], lysozyme [76], phospholipase A2 [77], defensins and cathelicidins [78] collectins and surfactant proteins [79]. Having run the gauntlet of these defences, bacteria that reach the lung encounter alveolar macrophages, which although subject to T-regulatory cell suppression and inhibition by the epithelium, remain active phagocytes. [80]

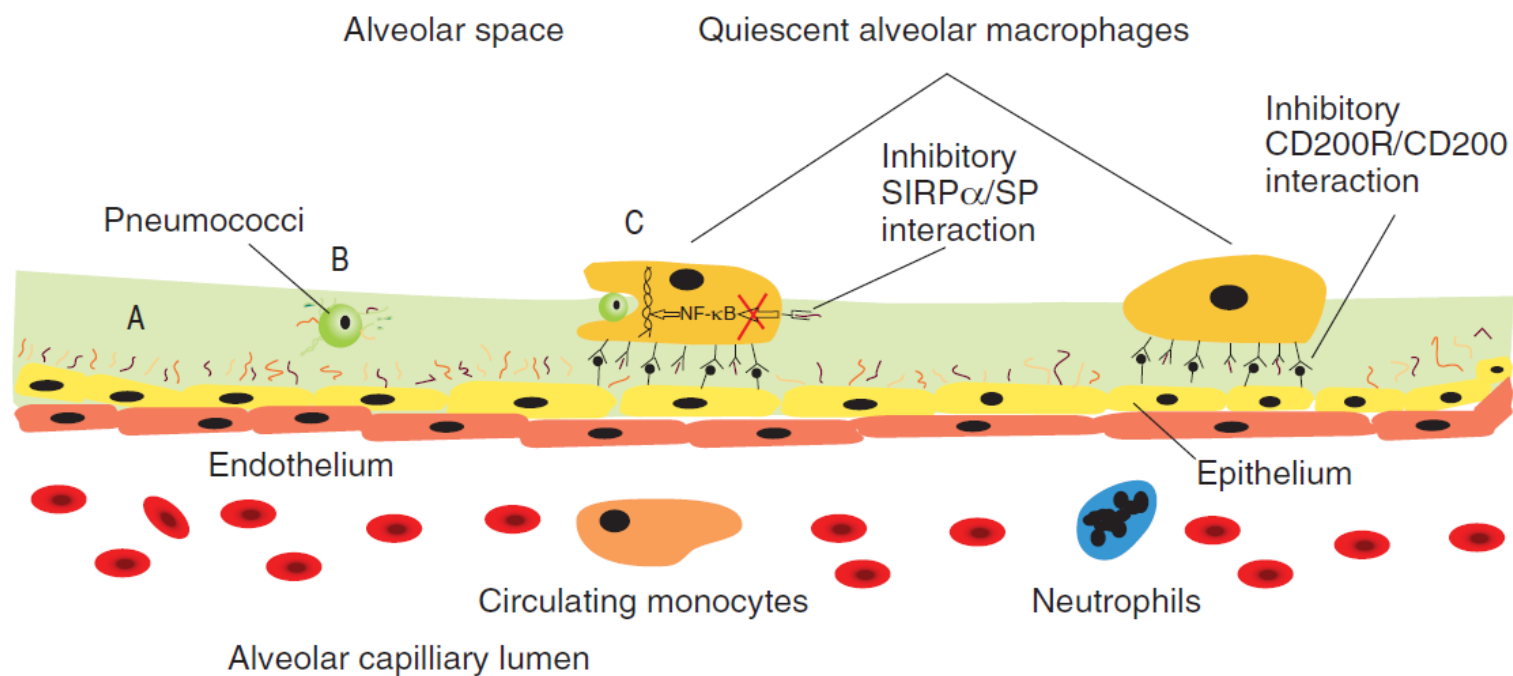


Figure 1.6
Maintenance of
quiescent immune
homeostasis in the
lower airway

A healthy alveolar epithelium is vital to the maintenance of innate immune homeostasis in the lung. **A**: Alveolar lining fluid is nutritionally barren and replete with antimicrobial compounds. **B**: Bacteria are lysed by secreted innate factors such as lysozyme, phospholipase-A2 and surfactant proteins (SP) A and D. **C**: Induction of an anti-inflammatory phenotype in alveolar macrophages. Phagocytic functions are maintained but the ability to present antigen and secrete pro-inflammatory cytokines is suppressed by surfactant proteins, granulocyte-macrophage colony-stimulating factor, interleukin-10 and transforming growth factor- β , and the CD200 and signal regulatory protein α (SIRP α) interactions.

Wootton DG et al. The pathophysiology of pneumococcal pneumonia. *In*: Chalmers JD, Aliberti S, Pletz M, eds. European Respiratory Society Monograph: Community-Acquired Pneumonia. 63rd Edn. European Respiratory Society, 2014; 42-63 **Reproduced with permission of the European Respiratory Society** ©

1.5.3 Macrophage / epithelial interactions in the early development of CAP

Most of the time, for most people, the innate-host defence defeats the constant tide of bacterial intruders into the airway. The earliest events surrounding the switch from quietly dealing with bacteria to the development of the inflammatory ‘total war’ seen in pneumonia are unclear. Most of the bacteria that cause pneumonia are frequent visitors to the lung and why they should be tolerated most of the time but cause potentially fatal disease on other occasions is debated.[81] Bacterial density seems to matter. Many bacteria have the ability to phenotypically switch to a more virulent form when they quorum sense their population is thriving.[82] Virulence often means tissue damage as bacteria attempt to gain access to nutrients by lysing host cells.[83] Tissue damage is a potent inducer of innate immune activation via the release of damage-associated molecular pattern (DAMPs) molecules.[84] Early in the development of pneumonia DAMPs are spilled by damaged alveolar epithelium.[85] These soluble DAMPs are macrophage activators. Moreover macrophages, previously suppressed by CD200 interactions whilst anchored to intact epithelium, become highly susceptible to activation when released from damaged cells.[86]

1.5.4 Macrophage neutrophil interactions in CAP

(see figure 1.7) With the removal of epithelial restraints, alveolar macrophages become activated in the presence of bacteria and rapidly escalate the production of pro-inflammatory cytokines.[87] In particular they are responsible for the early burst of chemokines, such as CXCL8, which lead to the ingress of neutrophils from alveolar capillaries.[88] Once neutrophils numbers build, paracrine effects result in positive feedback loops

which lead luminal neutrophils to become the major producers of cytokine and chemokine in the lung. Alongside the recruitment of neutrophils from the circulation, this chemokine production leads to the influx of tissue macrophages from the lung parenchyma.[89] In addition to their key role in the initiation of the pneumonic inflammatory response, alveolar macrophages have an equally vital role in tempering and eventually resolving inflammation. In particular they act as a check on neutrophil numbers and activation state. [90]

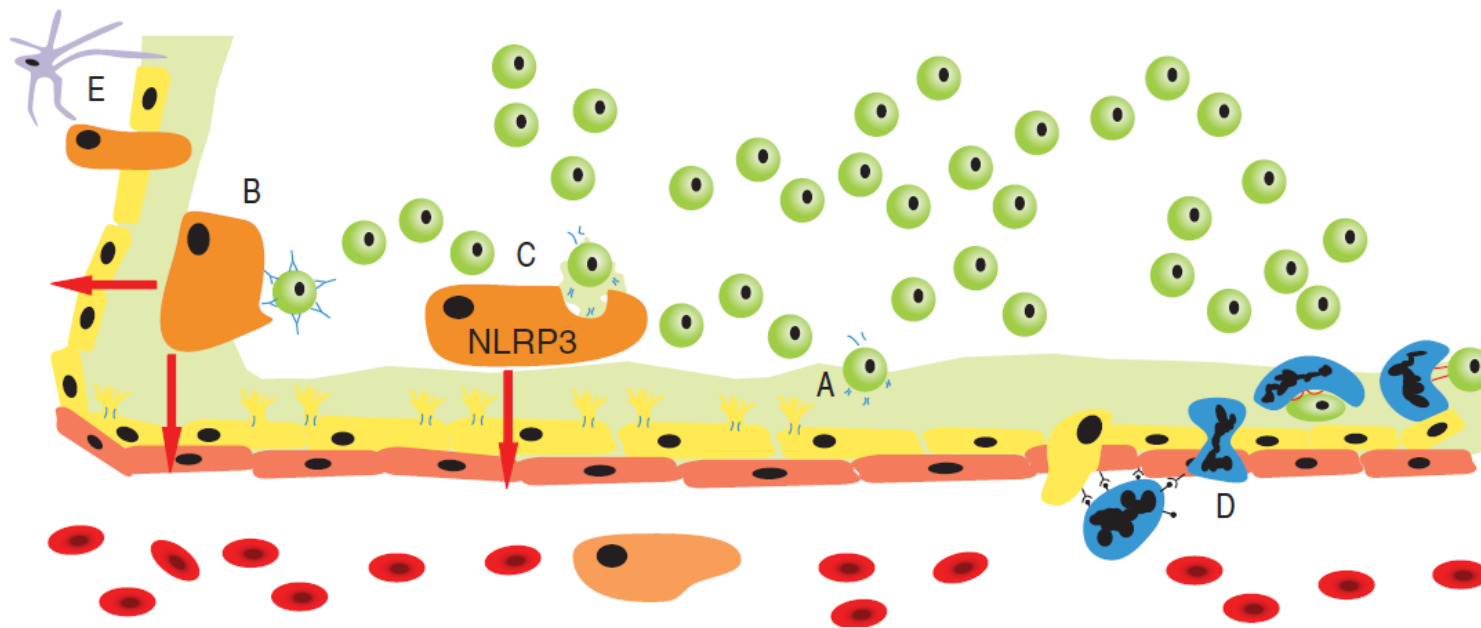


Figure 1.7 The evolution of an acute inflammatory response that results in the syndrome of pneumonia.

Pneumococcal pneumonia is the result of overwhelming numbers of pneumococci provoking an inflammatory response orchestrated by alveolar macrophages that have been unrestrained by a damaged, activated epithelium. **A:** Pneumolysin breaches the cell walls releasing damage-associated molecular patterns. **B:** Macrophages recognise opsonised pneumococci and non-opsonised pneumococci via Toll-like receptor-2 and platelet activating factor receptor interactions with the pneumococcal cell wall constituents. **C:** Pneumolysin recognition leads to activation of the NLRP3 inflammasome. **D:** Activated neutrophils translocate across the endothelium and epithelium into the alveolar lumen. **E:** Macrophages present antigen to dendritic cells and migrate to regional lymph nodes. The red arrows represent inflammatory cytokine and chemokine (e.g. CXCL8) release by activated macrophages and epithelium.

Wootton DG et al. The pathophysiology of pneumococcal pneumonia. *In:* Chalmers JD, Aliberti S, Pletz M, eds. European Respiratory Society Monograph: Community-Acquired Pneumonia. 63rd Edn. European Respiratory Society, 2014; 42-63 **Reproduced with permission of the European Respiratory Society** ©

1.5.5 Efferocytosis

If bacterial replication and metabolism are arrested by antibiotics then neutrophils can reduce the numbers of bacteria to safe levels. Neutrophils are terminally differentiated and they eventually enter a process of programmed cell death called apoptosis.[91] As a consequence, during pneumonia the lung is filled with cellular debris such as hyaluronan and dead and dying neutrophils.[92] Clearing this dead 'self' material is the job of alveolar macrophages and is called efferocytosis.[93] As they apoptose neutrophils release DAMPs such as ADP which act as macrophage chemo-attractants.[94] Macrophages recognise apoptotic cells and material destined for efferocytosis via a range of receptors. Many apoptotic ligands and phagocyte efferocytotic receptors have been described.[91] CD44 is a receptor for degradation fragments of hyaluronan which is the main component of human extra-cellular matrix. [92] CD44 is expressed at uniquely high levels on macrophages and is crucial in the recognition of apoptotic neutrophils in mice. [95] Phosphatidylserine (PS) is a constituent of cell walls that is usually hidden on the inner surface. During apoptosis the enzyme phospholipid translocase stops functioning and PS is exposed on the outer wall leaflet where it can be recognised by macrophage scavenger receptors.[91]

1.5.6 Efferocytosis is defective in chronic inflammatory lung conditions

Efferocytosis is vital in the resolution of inflammation in the lung.[96] Not only does efferocytosis reduce the numbers of potentially damaging neutrophils but the act of efferocytosis induces an anti-inflammatory phenotype on the macrophage itself.[97] This results in increased levels of several anti-

inflammatory factors including transforming growth factor- β (TGF- β).[98] Therefore following an episode of pneumonia the effectiveness of efferocytosis may be associated with the rate and extent of symptom resolution. Efferocytosis can be measured *ex vivo* by culturing alveolar macrophages with a labelled apoptotic challenge then observing either microscopically or by flow cytometry the proportion of macrophages that contain the apoptotic cells.[99] In several chronic inflammatory lung conditions efferocytosis has been shown to be defective and therapeutic intervention to improve efferocytosis has been suggested.[100] Azithromycin has been shown to improve efferocytosis of apoptosed bronchial epithelial cells by alveolar macrophages taken from patients with chronic obstructive pulmonary disease (COPD).[101] Statins have been shown to improve the efferocytosis of apoptotic neutrophils by alveolar macrophages from patients with COPD.[102] If rates of efferocytosis were demonstrated to vary among patients recovering from CAP, and if these differences could be linked to symptomatic recovery, then this would raise the possibility that pro-resolution treatment aimed at enhancing efferocytosis could be trialled.

1.6 The lung microbiome and its relationship with CAP

1.6.1 Sequencing the 16S rRNA gene to identify bacteria

As discussed above a defining feature of community acquired pneumonia (CAP) is its infectious aetiology. Despite this, even the most rigorous contemporary attempts to describe the microbiological epidemiology of CAP achieve positive pathogen identification in only 50% of cases. The identification of bacteria in a sample using culture is hampered by our limited ability to

culture the majority of bacterial species.[103] An alternative to culture is to identify the presence of bacteria in a sample by detecting 'bacteria only' genes. The three main branches of life, Eukarya (which includes among others humans and fungi) Archaea, and Bacteria can be separated by differences in the gene that encodes for ribosomal RNA.[104] Ribosomes are split into two sub-units and each contains a length of RNA. Ribosomal RNA (rRNA) is measured by the Svedberg unit (S) which is a measure of sedimentation rate. The rRNA found in the bacterial small ribosomal subunit is 16 Svedbergs (16S).[105] Ribosomal RNA plays a fundamental role and the gene that encodes bacterial rRNA, the 16S rRNA gene, is highly conserved. However within the 16S rRNA gene are regions which are variable and sequence differences in these variable regions are approximately species specific.[106] If universal primers, which cover the variable region of the 16S rRNA gene, are used in a PCR reaction and the products sequenced, then by referencing online sequence data-bases the bacteria in a sample can be identified.

1.6.2 Microbiome and microbiota

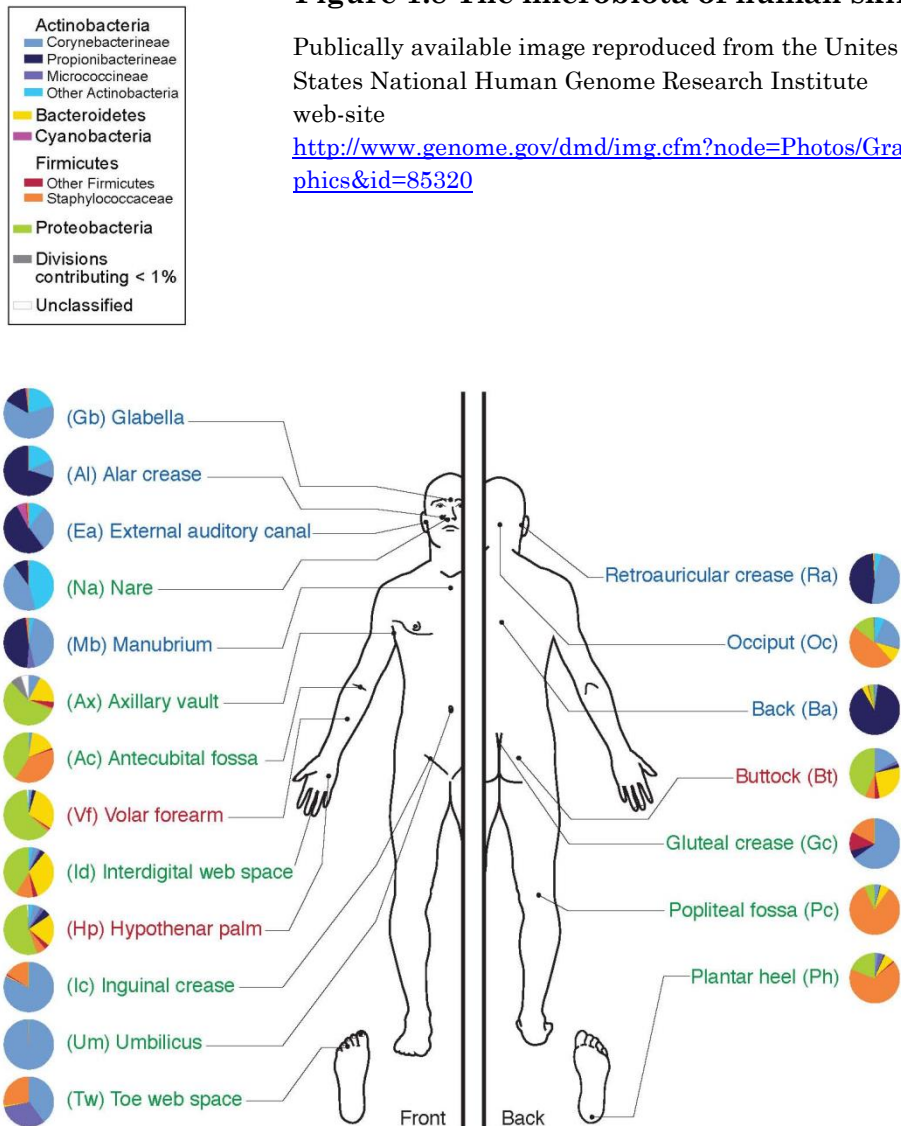
The methodology above has been used to describe the bacteria present in a wide range of environmental niches.[107] Related molecular techniques can identify fungi and viruses and the complete range of microorganisms in a sample is referred to as the microbiota. The relationships between the constituents of a microbiota, how they react and adapt to specific characteristics, is largely a function of their genes and the totality of genes in a sample has been termed the "microbiome". This nomenclature is not universally adhered to and many general articles use the terms microbiota and microbiome interchangeably. Since 2008 the Human Microbiome Project (HMP) has sought to describe the

range and interactions of bacteria from selected anatomical sites such as the skin, gut and female reproductive tract of healthy individuals.[108] Perturbations in the microbiome between health and select disease states (e.g. type II diabetes and inflammatory bowel disease) have also been studied by the HMP. Importantly, at the time the Human Microbiome Project was conceived the lower respiratory tract was not included as a site for investigation largely due to controversy surrounding the paradigm of the healthy lung's bacterial sterility and the invasive methods required for obtaining samples. As an example of the output of the Human Microbiome Project figure 1.8 demonstrates the current understanding of the bacterial ecology of human skin.

Figure 1.8 The microbiota of human skin

Publically available image reproduced from the Unites States National Human Genome Research Institute web-site

<http://www.genome.gov/dmd/img.cfm?node=Photos/Graphics&id=85320>



In the above schematic diagram it can be seen that different bacterial phyla thrive in different anatomical niches. It should be noted that differences at the taxonomic level of phylum are profound and bacteria of the phylum *firmicutes* are as different from bacteria of the phylum *actinobacteria*, as a sponge (kingdom *Animalia*, phylum *porifera*) is from an elephant (kingdom *Animalia*, phylum *chordata*).

1.6.3 The microbiota of the healthy lung

As mentioned above, the lung is constantly challenged by bacteria. Bowers et al. described the range of bacteria recovered from the air of US Mid-Western Cities.[109] They filtered the air and using bead beating and methods optimised for soil samples they extracted the DNA and sequenced variable regions of the 16S rRNA gene using the Roche 454 FLX platform (described in detail in chapter 5). They found a strong seasonal signal with winter bacterial concentrations being on average 52% lower than in the Summer. Summer samples were dominated by soil and plant derived bacteria. Samples from the winter months, when the soil is less moist and plants have fewer leaves, were dominated by bacteria derived from dog faeces! Since we breathe 6 or more litres of this air a minute the fact that some of these bacteria reach the lung is not controversial. However, determining whether any of the bacteria that reach the lung by inhalation or aspiration take up residence there, establishing a community adapted to the lung as a niche, is more difficult to prove. Charlson et al. used an elaborate and rigorous methodology to sample the length of the healthy airway whilst minimising the impact of oral contamination of the samples.[110] By analysing bronchoalveolar lavage (BAL) samples they consistently found bacteria in the lung. However, the bacteria detected in BAL were numerically smaller in quantity (3 orders of magnitude lower) and compositionally indistinguishable from mouth samples. Moreover within subject comparisons of paired samples (e.g. oral, upper-airway and BAL) were always more closely related than was sample type (e.g. BAL) compared between subjects. The summary conclusions were that a) there are bacteria in the lung in health, b) they are derived from the upper respiratory tract and mouth c) they are not established residents but transients. This last

observation is important and is based on the long established environmental observation that community composition is exquisitely sensitive to even the smallest differences between niches suggesting that, since the lung is physiochemically different from the mouth, any established lung microbiome would be clearly distinguishable from a mouth microbiome in the same individual.

1.6.4 The microbiota of chronic stable lung disease

Hilty et al. compared the bacterial microbiota of BAL obtained from patients with COPD, asthma and healthy controls.[111] The healthy samples contained a similar pattern of bacteria to the Charlson study described above with the bacterial phyla Firmicutes (including the genera *Staphylococcus* and *Streptococcus*) and Bacteroides (in particular the genus *Prevotella*) dominating. However there were very distinct differences in the patients with airways disease. Both asthma and COPD were characterised by a comparative increase in the abundance of Proteobacteria (including the genera *Haemophilus* and *Neisseria*). Importantly, this paper also demonstrated that in health oral samples and lung samples were similar but in disease the lung samples diverged from the oral samples. This suggests that environmental changes in the lung in the context of lung disease are associated with the emergence of a distinct, resident, colonising bacterial microbiota.

Erb-Downward et al. conducted a similar study to investigate the bacterial microbiota of healthy non-smokers, 'healthy' smokers and patients with spirometrically proven COPD.[112] They used quantitative PCR of the 16S rRNA gene to compare levels of bacteria across conditions and found no statistically significant

differences. However, when they investigated the range of bacteria their findings were similar to the two studies previously mentioned. BAL from healthy smokers diverged from the oral samples suggesting that even before the establishment of COPD smoking had altered the lower airway environment in such a way as to promote the establishment of a distinct, adapted microbiota. BAL from patients with COPD had reduced bacterial diversity which was associated with the dominance of a particular genus – most commonly *Pseudomonas* or *Haemophilus*.

1.6.5 Microbiota of respiratory samples in acute lung disease

Huang et al. analysed the bacterial microbiota in tracheal aspirates from 8 patients who had been intubated and ventilated for “exacerbation of COPD”.[113] They found a range of bacteria in these samples but the patients had been intubated for an average of 10 days at the time of sampling and had received on average 18 days of antibiotic. Therefore it is not possible to draw any inferences as to the causal relationship between the microbiota and the exacerbation from this study. It is interesting to note however that despite large quantities of antibiotic bacteria remained detectable in culture negative lung samples. Fodor et al. conducted a longitudinal analysis of self-expectorated sputum from 23 patients with cystic fibrosis.[114] The samples were taken at exacerbation (pre-antibiotic) during treatment and then during stability. They found that CF sputum was dominated by *Pseudomonas* and *Burkholderia* but changed very little before during or after antibiotic. They also showed that mouthwash samples were nearly identical to sputum samples highlighting again that the lower respiratory tract is likely challenged by, and in disease states colonised by, oral flora.

1.7 What are the gaps in the literature?

1.7.1 Recovery from CAP

Only two studies have used validated, pneumonia specific, patient based tools to describe symptom patterns in CAP. Each study used a different tool meaning each has been tested only once. Given the current drive to personalise medicine and involve patients more closely in the optimisation of processes of care it would seem vital to re-use these tools in a contemporary cohort. Moreover, it is likely that symptoms, particularly when systematically quantified, will detect more subtle perturbations in a patient's condition than blunt outcomes such as mortality or length of stay.

1.7.2 Efferocytosis and CAP

To date there are no studies exploring the relationship between patient factors, efferocytosis and symptom recovery following pneumonia. Yet since efferocytosis plays such a vital role in returning the lung to its neutrophil free homeostatic state, it seems highly likely that differential rates of efferocytosis will affect levels of inflammation in the lung and therefore respiratory and systemic symptoms. This is a cellular mechanism that has already been shown to be amenable to therapeutic manipulation and this raises the possibility that, if it is found to be deficient in some patients recovering from CAP, treatment trials could be designed.

1.7.3 Sputum microbiota of CAP

The aetiological cause of a significant proportion of CAP is unknown. 16S rRNA sequencing is a powerful tool for the detection of bacteria in clinical specimens and has less inherent

bias than platforms which target specific bacteria. This technique has begun to reveal the patterns of microbiota in the lung and how they vary with disease state. The bacterial microbiota in sputum from patients with CAP is unknown and may provide a deeper insight into the bacterial aetiology of CAP.

1.8 **Thesis aim**

To explore how efferocytosis and sputum microbiota vary depending on the clinical characteristics of patients with CAP and to relate these to symptomatic recovery.

2 PASS METHODS

2.1 Introduction

The Pneumonia Aetiology and Severity Study (PASS) was funded by a Doctoral Research Fellowship from the National Institute of Health Research (NIHR) awarded to the author. This chapter is derived from the PASS protocol and describes how the study was carried out. The protocol incorporated elements of the ethics submission which was made via the Integrated Research Ethics Service (IRAS). Sections of the protocol pertaining to background and rationale have been incorporated into chapter 1. Chapter 3 will describe the clinical results of PASS and chapters 4, 5, 6 will describe the methodology and results for each of the experimental components. Study documents referred to below such as information sheets, case record folders, consent forms and the CAP-sym questionnaire appear in **appendix 1**. PASS was approved by the North Wales Research Ethics Committee (Central and East) (NHS REC Number 10/WNo03/40). It was adopted to the National Institute of Health Research, Local Clinical Research Network (NIHR LCRN) portfolio and sponsored by the Research and Development department of Aintree University Hospital NHS Foundation Trust.

2.2 Objectives

PASS aimed to prospectively recruit a representative cohort of hospitalised adult patients with CAP in order to describe recovery over one year and investigate host and pathogen factors that may affect recovery.

2.3 Study design

2.3.1 Hospitals

The patients who volunteered for the PASS study were recruited from two acute hospitals in Liverpool. Aintree University Hospital NHS Trust (AUH) and the Royal Liverpool University Hospital (RLUH) provide acute secondary care services to the Liverpool Local Authority. Patients requiring acute care are taken or directed to the hospital that is geographically nearest their location at the time of illness, with AUH serving predominantly the North of the City (Sefton and Knowsley) and RLUH the South (Liverpool). Both hospitals see a large number of CAP cases each year with AUH recording 1572 cases of pneumonia (predominantly CAP) in the year 2013.[115]. The hospitals are operationally very similar. Both have an accident and emergency department (A+E), medical admissions unit (MAU) and Intensive care unit (ITU). One difference between the hospitals is that the Royal Liverpool has an Infectious Diseases department that, following initial stabilisation and assessment on MAU, provides ongoing in-patient care for a small proportion of CAP admitted to the RLUH. CAP patients who fall under the care of the Infectious Diseases department tend to be younger with less co-morbidity or have a history of recent travel or immune suppression.

2.3.2 Liverpool

Liverpool is a large city in the North West of England and has one of the most populous metropolitan areas in the UK [116] with a population that grew from 445,200 to 469,700 during the period of recruitment. Based on the UK Government Index of Multiple Deprivation (IMD statistics (2004 and 2010 reports), at the time this study recruited, Liverpool Local Authority was the most

deprived in England.[117] A quarter of the 100 most deprived wards in England were in Liverpool. 17.5% of Liverpool's wards were in the bottom 1% for the IMD domain "health deprivation and disability" with 61.9% being in the bottom 10%.[117] The North West of England has the highest rates of lower respiratory tract infection and the third highest rates of CAP in the UK.[118] This is in part explained by the association between rates of CAP and the IMD. Rates of CAP in the UK are 70% higher in the highest (most deprived) quintile of IMD compared to the lowest.[118]

2.3.3 Rationale for the study design

In order to study how patients recover from CAP it was important to have assessments at a number of time-points. Recovery was measured against a perceived baseline level of symptoms. Since it was not possible to identify patients who would develop pneumonia in advance the earliest we could recruit them was on presentation to hospital. At that time-point the best approximation of patients' pre-pneumonia (in health) symptoms was derived from patient recall and was best quantified using a structured, pneumonia specific questionnaire such as the CAP-sym score (see 1.4.3). Previous studies have reported the validity of this approach.[57] Since the time to full recovery is variable we extended follow-up to a point where a large proportion of patients were expected to have recovered. Not all patients would have been fully back to their pre-pneumonia level of symptoms by six months and therefore follow-up was for one year. In order to study the mechanisms behind delayed recovery it was important to determine what was happening in the lung. The cells in the lung are not easily accessible and only small numbers are obtained from sputum samples. Surgical biopsies of lung tissue

would have been scientifically valid specimens but unacceptable ethically due to the risk and discomfort involved. Bronchoscopically obtained specimens therefore represented the best compromise of risk, discomfort and validity. See figure 2.1 for a schematic representation of the study design.

2.4 CAP case-definition

Radiological evidence of a new pulmonary infiltrate compatible with the presence of acute pneumonia (where the author was the final arbiter with regards to ambiguous x-rays), **plus** TWO of the following symptoms, signs or investigations consistent with a diagnosis of CAP **and** no alternative clinical explanation e.g. pulmonary oedema, pulmonary embolism:

Symptoms

- Cough.
- Production of purulent or mucopurulent sputum.
- Dyspnoea or tachypnoea.
- Pleuritic chest pain

Signs

- Pyrexia (within 24 h before recruitment), defined as temperature $\geq 38^{\circ}\text{C}$ OR hypothermia, defined as a temperature $< 35^{\circ}\text{C}$.
- Respiratory examination findings suggestive of pulmonary consolidation (dullness to percussion, crepitations, or bronchial breath sounds).

Investigations

- White blood cell (WBC) count $> 10,000/\text{mm}^3$ OR $> 15\%$ immature neutrophils (bands) OR leukopaenia with a total WBC count $< 4500/\text{mm}^3$.
- Hypoxaemia ($\text{PaO}_2 < 8\text{kPa}$ or oxygen saturation $< 90\%$ while the subject was breathing room air).

2.5 Inclusion criteria

- Unselected adolescents (16-18 years) and adults (>18 years) with CAP (as defined in 2.4 above) as their primary diagnosis after medical admission.
- Recruitment possible within 24 hours of the first dose of antibiotic.

2.6 Exclusion criteria

- Previous admission to hospital within the last 14 days*.
- Non-pneumonic exacerbations of COPD (no new radiological change).
- Dementia of a level preventing CAP-sym completion.
- Primary lung cancer or any malignancy metastatic to the lungs.
- Advanced malignancy under ongoing care.
- Known bronchiectasis or cystic fibrosis.
- Immunocompromised patients:
 - Immunosuppressive therapy including cancer chemotherapy and long-term corticosteroid treatment at an equivalent daily dose of prednisolone 40mg or greater.
 - Solid organ, bone marrow or stem cell transplant recipients.
 - Known infection with human immunodeficiency virus.
- Concurrent haemodialysis, haemofiltration, peritoneal dialysis, or plasmapheresis.
- Requires invasive ventilation.
- Requires acute renal replacement therapy.

*BTS guidelines suggest 10 days excludes hospital acquired pneumonia. This is based on weak evidence and some hospital contacts are ambiguous (out-patient and A+E visits). We extended to 14 days to ensure all cases would be regarded as community acquired.

2.7 Study Procedures

2.7.1 Identification of potential study subjects

The study team was in regular communication with the bed managers and the nursing and medical coordinators in A+E and in the Medical Admissions Unit. Via regular education and dissemination events and through day to day interaction with the study team, these coordinators were fully aware of the research study, its aims, objectives and processes. As a result of their unique overview of the patients admitted to the hospitals these individuals were best placed to generate a list of potential subjects for the study. This list was discussed with the study team at pre-arranged time-points throughout a recruiting day or by phoning or bleeping a study team member to alert them to a potential recruit. The assistance and feedback of these coordinating members of the clinical team was essential to the study and they were included in the 'User Group' who will meet at regular intervals throughout the study to discuss its running, feedback and improvements.

2.7.2 Initial approach to potential subjects

The first approach to a patient identified as a possible subject occurred as soon as the study team become aware of a possible diagnosis of CAP. Practically this meant after the patient had seen the admitting doctor, had a chest x-ray (CXR) and a decision had been made to manage community acquired pneumonia. The latest a patient was approached regarding the study was 22 hours after administration of the first dose of in-hospital antibiotic for CAP. The first approach to a patient regarding the study was by a doctor or nurse from the study team. The medical notes, blood tests and CXR were reviewed by the study team to assess the suitability of the patient against the inclusion and exclusion

criteria. The study representative then introduced themselves. It was essential at this point for the study representative to gauge the clinical stability of the patient, form an opinion about capacity and assess the appropriateness of the environment for the initial interview. The study was explained and the patient information leaflet (PIL) reviewed with the patient. At the end of this process, and once any questions had been answered either consent was obtained or the patient declined to participate or an agreed length of time was provided for the patient to consider and discuss the study with their advocates.

2.7.3 Consent

The same member of the study team who carried out the initial approach returned to the patient to answer any further questions and written informed consent was obtained, assuming the patient had capacity. We sought to obtain assent on behalf of those subjects deemed not to have capacity and the process for doing so is explained below. All those involved in obtaining consent were GCP trained and had experience of the consent process in both clinical and research settings. Moreover, from prior clinical experience all study members involved in consent were accustomed to making decisions about patient capacity.

The process of consent had the following pattern:

- An introductory conversation took place between the study team member and the potential study subject. During this conversation the team member began to judge the degree of capacity the patient had and the best style of language, spoken volume and vocabulary to use in the description of the study.

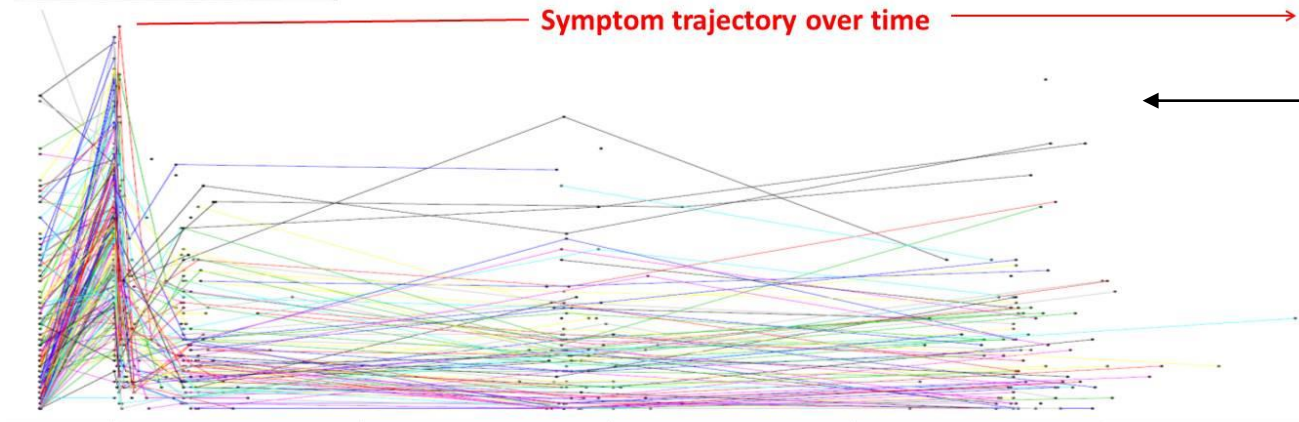
- A description of the study occurred with frequent summaries and requests for recall and understanding of the information. Particular focus was placed on the potential risks and benefits of the study. Emphasis on the implications of not participating was given and recall of this was sought – in particular that non-participation would have in no way altered the standard, duration or personnel involved in the care that would otherwise be provided by the NHS then or in the future.
- Questions were invited.
- The study information leaflet was provided and its contents demonstrated.
- Patients were encouraged to consider whether the presence of a confidant – friend, carer, relative or member of the clinical team – would have helped them consider the decision making and consent process. Where support from such a person was sought care was taken for the explanation of the study to be repeated in this individual's presence. The study team was mindful of the need to ensure that these individuals merely helped facilitate a decision rather than directly influencing or coercing a decision from the patient.
- An agreed period of reflection (normally in the order of 1-2 hours) was offered before the study team member returned.
- Upon return the study team member invited questions and gently tested recall of important information relevant to consent.

Figure 2.1 The Pneumonia Aetiology and Severity Study Design

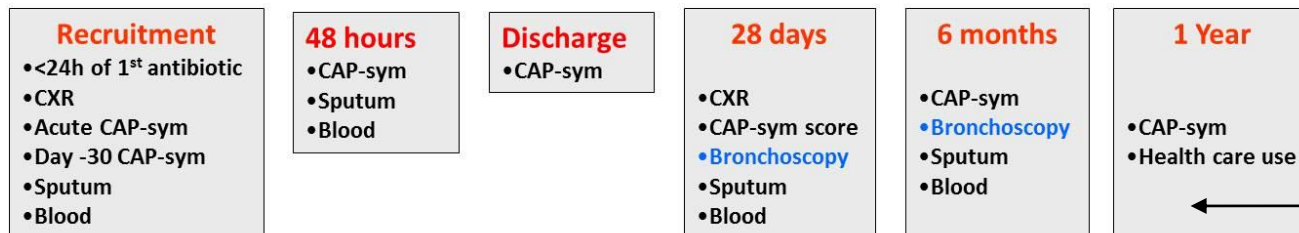


Study design

NHS
National Institute for
Health Research



The backbone of the study was a longitudinal record of subjects' symptom burden. This was measured and recorded using the CAP-sym questionnaire. This enabled the level of symptoms to be plotted for each subject at each timepoint in order to infer different patterns of recovery and explore clinical associations.



At each visit, from admission to one year's follow-up a range of procedures and measures were taken.

2.7.4 Consent in the context of lack of capacity

We sought to include patients who lacked capacity and in doing so adhered to the procedures set out in the National Patient Safety Agency document, “Information and Consent Forms Guidance for Researchers and Reviewers” with particular reference to sections 8.0 and 37.[119]

Advice regarding the patient’s wishes were obtained from a suitable advocate in the following hierarchy:

- Direct discussion with accompanying next of kin
- Telephone conversation with next of kin.
- Direct conversation with accompanying adult.
- Telephone conversation with relative, friend or GP.

If following a single phone call we were unable to contact anyone able to provide advice regarding the patient’s wishes the patient was not recruited. Persons able and willing to provide advice were asked to sign the ‘Consultee Declaration Form’. If the patient regained capacity during the study we sought their consent and asked them to sign a consent form.

2.7.5 Initial study visit and procedures

- a. Following consent the patient was allocated a study identifier code. These non-sequential 4 figure codes were created prior to the onset of recruitment by random number generating software. A case record folder (CRF) was created for the subject into which study related data and notes were hand written.
- b. Clinical Review. Following consent a study team member completed clinical review which took the form of a structured history and examination. The history included a review of the patients symptoms and duration, their past medical history,

prior medication use and social history. The examination focussed on the respiratory system and the primary presenting complaint. The aim of this clinical review was to obtain patient related information that was comparable between subjects and could be used to test for associations with recovery.

- c. CAP-sym. Next the CAP-sym questionnaire was completed. Once the CAP-sym questionnaire had been completed to reflect the patient's acute symptoms it was repeated to reflect how the patient felt 30 days prior to admission. This pre-pneumonia CAP-sym score was used as a baseline with which to judge recovery.
- d. Blood. Verbal consent was obtained for a venous blood sample. Where possible this venous sample was combined with the initial routine clinical blood sampling. However in some instances these routine bloods may have already been drawn and a second blood draw was necessary. Blood was used for a range of diagnostics related to pneumonia including blood cultures to determine the causal organism. Spun serum was stored for serological diagnosis of influenza infection and measurement of pro-calcitonin.
- e. Sputum. We then asked all patients for expectorated sputum samples. The sputum specimen was placed on wet water ice and transferred to the -80 freezer for storage prior to subsequent batched DNA extraction. In Many cases this resulted in two sputum samples being taken; a sample collected by the study team as above and another being sent to the hospital microbiology lab for routine identification of causal organisms at the discretion of the clinical team..
- f. Communication

Following the conclusion of the initial visit outlined above we discussed methods for contacting the study team prior to their next visit. Attention was drawn to the telephone numbers and email addresses in the study information leaflet and the ability to contact the team via subject's clinical team. We placed a sticker into the clinical and nursing notes to identify the patient as a study subject. This sticker included contact details for the study team. All samples were labelled to identify the patient as a study subject and this facilitated study standard operating procedures (SOPs) to be enacted in the laboratory. Any laboratory results that become available that had clinical relevance to the subject were communicated to the subject's clinical team in a timely fashion.

The study did not influence or participate in the in-patient care. This remained at the discretion of and the responsibility of the named clinician and their team. No further study procedures were performed on patients who triggered exclusion criteria between recruitment and subsequent time-points (e.g. through admission to intensive care unit for invasive ventilation). However, specimens and data obtained to that point will be used for analysis unless consent for its use was withdrawn.

2.7.6 Second study visit (48 hours post recruitment)

At 48 hours following recruitment another study visit occurred. Verbal consent was obtained for blood to be drawn. A structured clinical review was undertaken and the CAP-sym score was repeated. The subject had the opportunity to ask any questions and information about any results obtained from initial tests was provided.

2.7.7 Third (pre-hospital-discharge) study visit

Having been informed of discharge planning the subjects were seen immediately prior to discharge. If a subject's clinical team planned to discharge the subject before 48 hours had elapsed following enrolment then the clinical team contacted the study team to enable a study visit to take place prior to discharge. Subjects had a structured clinical review and a CAP-sym score at this visit. Subjects were not routinely seen by the study team again until follow up. Therefore at this visit we spent time ensuring the subjects were aware of the procedures for contacting the study team and the arrangements for the follow-up clinic one month post-admission.

We discussed research bronchoscopy with the subject. If a subject volunteered for the research bronchoscopy this took place shortly after the one month follow-up clinic. The rationale and practicalities of the bronchoscopy were explained and a separate study information leaflet was provided. The subject was asked to provide written consent to the bronchoscopy. The subject had a follow up visit scheduled prior to the bronchoscopy and this provided an opportunity for further questions to be asked and for the subject to change their mind.

2.7.8 Loss to follow-up

Subjects were asked if they were happy to receive a phone call as a reminder prior to a study follow-up visit. They were also asked for permission to try to contact them or their GP to clarify reasons for failure to attend a follow-up visit.

2.7.9 Fourth study visit (one month post recruitment)

During the in-patient stay the study subject's clinical care was the responsibility of the admitting clinical team. However, all subjects were then followed up as out-patients by the study team who provided the dual function of clinical care and study investigators. Subjects were seen by a study doctor in a dedicated pneumonia clinic. Prior to seeing the study doctor a number of procedures occurred:

a. Chest X-ray

All study subjects had a chest x-ray to assess pneumonia resolution. This was part of routine clinical practice.

b. Clinical samples

Subjects were asked to provide expectorated sputum and a sample of venous blood.

c. Clinical Review

A structured clinical review took place and a CAP-sym questionnaire was completed.

d. Bronchoscopy consent check

As is clinically routine when assessing patients post pneumonia, particular focus was placed on detecting symptoms or signs suggestive of underlying health problems – most notably lung malignancy. It was likely that a proportion of the study subjects would have been told that a bronchoscopy and further tests were clinically advised due to their signs and symptoms. It was made clear to the subject which tests were being suggested clinically and which were study tests not required as part of routine clinical assessment. Those subjects who required a bronchoscopy on clinical grounds were asked to provide consent for a lavage to be taken if the initial bronchoscopic visualisation of the airways

was normal. If the airways were not normal or if procedures other than inspection were needed on clinical grounds then the lavage was not performed. All subjects were then asked to return for a final study visit at six months post enrolment.

2.7.10 Fifth study visit - bronchoscopy

All bronchoscopies were carried out at Aintree University Hospital in the Elective Care Centre which has a dedicated bronchoscopy suite. They occurred as soon as was practically possible following the one month follow-up visit. PASS bronchoscopies occurred on Tuesday mornings between 8 and 9am prior to the routine clinical bronchoscopy list. Subjects were asked to fast for 4 hours before hand and not to drink fluids for 2 hours of the procedure. Subjects were asked to arrive for the test 30 minutes before the scheduled procedure time. They were met by a study nurse and endoscopy nurse and went through standard procedures and checks used for all bronchoscopies performed in the Aintree University Hospital endoscopy suite. Following the procedure the subjects were recovered into the endoscopy recovery room and were monitored as per standard procedures. It was anticipated most subjects would be able to leave after two hours of recovery. In the event that a subject was unwell and required admission this would have followed standard procedures and the subject would be have been looked after by the relevant admitting team (i.e. not the study team). However, fortunately no PASS subjects required admission. The pre-bronchoscopy checks, a copy of the bronchoscopy report and recovery details were filed in the hospital notes. During the bronchoscopy visit subjects also had a venesection to provide blood for the extraction of neutrophils for the efferocytosis assay (see table in section 7). We phoned all subjects 48 hours after their bronchoscopy for feedback.

2.7.11 Sixth study visit (six months post recruitment)

The sixth study visit at 6 months was identical to the forth. However, another x-ray was only requested when this was clinically indicated. Extra x-rays, over and above routine clinical care, were not requested.

2.7.12 One year phone call and / or visit

Subjects were asked if they would accept a phone call to their General Practitioner and themselves one year after their original admission (if the GP call identified that that the subject had died since their last visit the subject's home was not contacted). During the call to the patient they were asked to confirm if they were still happy to attend for a last visit. The purpose of this visit was to complete a final CAP-sym score and to ask about health events that had occurred since the 6 month follow up visit. At the end of this final contact all subjects were reminded about how to contact the study team and were advised about plans for dissemination of study results.

2.8 Laboratory Testing

2.8.1 Biochemistry, haematology and microbiology samples

All routine samples were processed by the hospital laboratories. Study subject samples were labelled with an extra sticker to identify them as belonging to a study and this triggered the study SOPs for sample handling. This SOP directed excess sample to be labelled with a study number and frozen for storage. Samples were stored for potential future testing in ethically approved research relevant to the study aims. Since the laboratory staff had no idea of the recovery status of study subjects there was no opportunity for bias at this stage in the study.

2.8.2 Efferocytosis assay

Bronchoalveolar lavage samples were labelled with a study code and used immediately in the efferocytosis assay. This involved the safe transfer of samples to the laboratories at the Clinical Sciences Unit of Aintree University Hospital.

2.8.3 16S rRNA analysis

Sputum samples were deep frozen very soon after receipt. Samples were subsequently defrosted in batches, the DNA extracted and purified and transported to The National Heart Lung Institute, Imperial College London. Here the author, under the supervision of Imperial scientists, conducted the 16S rRNA PCRs prior to sending the samples for sequencing.

Table 2.1 Summary of PASS Subject Sampling

Blood specimens	Enrolment	48 hours	Discharge	28 days	Bronchoscopy (1 month)	Six months	Bronchoscopy (6 months)	1 year
Full blood count (3 ml)	X			X		X		
Biochemistry and pro-calcitonin (5 ml)	X	X		X		X		
Clotting profile (6 ml)	X	X		X		X		
Serum glucose (4 ml)	X							
Blood culture (20 ml x 2)	X							
HIV and influenza serology (5 ml)	X			X		X		
Whole blood for DNA extraction (10 ml)	X							
Whole blood for RNA expression (5 ml)	X			X		X		
Whole blood for neutrophil extraction 36 ml					X		X	
Maximum total blood volumes (ml)	78	11		24	36 ml	24	36 ml	
Volume extra to routine clinical care(ml)	15	6		24	36 ml	24	36 ml	
Respiratory tract specimens								
BAL fluid (200 ml instilled)					X		X	
Sputum – Microbiology and 16S rRNA sequencing	X			X		X		
CXR								
	X			X		X*		
CAP-SYM score								
*Only if clinically indicated	X	X	X	X		X		X

2.9 Safety considerations

2.9.1 Venesection

The study protocol required a total blood donation over one year of 94 mls in excess to that which was clinically indicated with 10 mls extra being drawn on admission. This volume was within safe limits. For individual participants, the required blood donation was minimised by the use of results of investigations performed on samples obtained by the clinical team.

2.9.2 Research bronchoscopy with BAL

Flexible bronchoscopy is a safe procedure with a published complication rate of 0.12-0.5% and a minor complication rate of 0.8%.[120] Major complications of flexible bronchoscopy include respiratory depression, pneumonia, pneumothorax, bronchospasm, cardio-respiratory arrest, arrhythmias and pulmonary oedema. Minor complications include discomfort, vasovagal reactions, fever, nausea and vomiting.

Bronchoalveolar lavage (BAL) involved the instillation and removal by gentle hand suction of 200 ml of warm sterile saline. BAL can induce a post-BAL acute phase response which peaks at 24 hours. This can be measured in up to 30% of individuals but is often asymptomatic and seems to be related to volume of fluid and number of lobes lavaged. [121,122]

Efferocytosis has been shown to be influenced by factors such as smoking and COPD and these comorbidities affect all regions of the lung. It was therefore postulated that rates of efferocytosis would be relatively uniform throughout the lung and that differences in efferocytosis between individuals would be greater than differences between lung lobes. Local practice was to

perform research bronchoscopy in the “sat up, face to face” position in which the middle lobe bronchus was positioned in a favourable way for aspiration of lavage fluid.[123] For these reasons we lavaged the middle lobe, rather than targeting the recently pneumonic lobe, as good sample yield was key to the success of the experiment and for patient comfort and safety.

2.10 Ethical considerations

2.10.1 Purpose and design

The biological plausibility and prior evidence to support the scientific rationale behind this study were considered in chapter 1 of this thesis. Since the key cell involved in resolution of inflammation caused by pneumonia is the alveolar macrophage the best way to obtain these is by bronchoalveolar lavage (BAL). The British Thoracic Society describes bronchoscopy as safe and our group has published a large series of research bronchoscopies with extremely low rates of adverse events.[123,124]

2.10.2 Recruitment

It was important that this study recruited a representative group of patients so that its results had the widest possible applicability. As described in 2.3.1, 2.3.2. and 2.4.1 the hospitals, region and study processes employed by PASS were chosen to maximise recruitment. Since one of the study aims was to identify the organisms responsible for CAP it was important to obtain samples from as many patients as possible before antibiotics have had time to influence the results. For this reason we attempted to complete the consent process before twenty four hours had elapsed following the first dose of antibiotic. This put pressure on the study team, but not patients who were offered time to read

the study information in private and to discuss joining the study with family, friends or other confidants.

2.10.3 Inclusion / exclusion

The diagnosis of CAP is inherently subjective (see 1.2) but we took particular care to ensure that this cohort would be considered representative of CAP and comparable with similar studies. Whilst it was recognised that children represent an important group of patients at risk of pneumonia and that many children suffer significant health problems following pneumonia, the CAP-sym (Community Acquired Pneumonia – Symptom) score has not been validated for use with children and so they were excluded. CAP is important in the elderly and hence we had no upper age limit to recruitment. Due to underlying chronic illness some subjects were not able to participate in the bronchoscopy studies but age per-se was not an exclusion criterion. It would have been ideal to include primary care in this study since 70% of CAP cases are managed in the community but for logistical and financial purposes we restricted this initial study to hospital patients. It was our intention to subsequently include a primary care study in a larger programme grant to validate PASS findings in that population.

2.10.4 Consent

The consent process was carefully thought out and adhered to as in 2.7.3. A common clinical feature of community acquired pneumonia is the development of confusion. Confusion occurs across all age groups but is particularly common in the elderly in whom community acquired pneumonia is itself more common. We therefore included patients whose presentation included acute confusion and this will enable the results to be extrapolated into

normal clinical practice. As a consequence this study was reviewed and approved by a research ethics committee convened specifically to consider studies involving lack of capacity.

2.10.5 Conflict of interest

There was no financial conflict of interest in this study. The author who was also the lead investigator received a salary from the Fellowship awarded to support the study and no other financial remuneration. Subjects received small sums of money as reimbursement and to compensate for their time. These are laid out in the patient information leaflet (PIL) (appendix 1) and were approved by the research ethics committee.

2.10.6 Use of tissue samples in future research

We stored excess samples for future work relating to this topic. This was made clear to the patients prior to consent and was articulated clearly in the PIL. If subjects objected to their samples being stored for use in the future, they were destroyed and this in no way precluded them from ongoing participation in the study.

2.11 Confidentiality and data management

The NHS Code of Confidentiality was followed at all times:

Case record folder (CRF)

Information contained within the source documents was transcribed into Case Record Folders (CRFs). CRFs contained no patient identifiable information and were identified by the pre-generated study codes only. The CRFs remain at the study sites and will be kept there for 3 years after the study closure.

On-site computer data base

Data from the CRFs was entered into a study computer data-base. The data-base is only accessible via University networked computers. The data base is password protected. To access a subject's data the study identifier code is required.

2.12 Data handling

All clinical and laboratory data was transcribed from the CRFs and source documents by the author and entered into excel spreadsheets. Data in individual spreadsheets was linked across to other spreadsheets by the four digit study number. The data for the CAP-sym scores – which amounted to over 1100 rows of data was double entered by the author and a research admin assistant.

2.13 Statistical analysis

All statistical analysis was performed by the author in the R computing environment using R studio over R versions 2.13.2 through to 3.1.3. Statistical supervision and tutorage was supplied by Professor Peter Diggle initially at Lancaster University and latterly at University of Liverpool. Individual analysis plans are articulated in each of the results chapters 3-6. In chapter 5 I am particularly grateful for the additional input of Dr Graeme Hickey (Clinical Lecturer in Statistics University of Liverpool) for his thoughts and advice on compositional analysis. All subjects who were recruited were included in the analyses in the manner of an intention to treat group. Where patients dropped out prior to completion of the study their data to the point of drop-out remained in the analysis.

3 PASS CLINICAL RESULTS

3.1 Introduction

The Pneumonia Aetiology and Severity Study (PASS) opened for recruitment at Aintree University Hospital (AUH) on the 7th February 2011 followed by the Royal Liverpool University Hospital (RLUH) on 10th December 2012. Both sites closed on March 29th 2013. During this period 169 subjects from across Liverpool consented to join the study (figure 3.1.) The PASS cohort was used to address the experimental questions posed by this thesis and this chapter describes its salient characteristics.

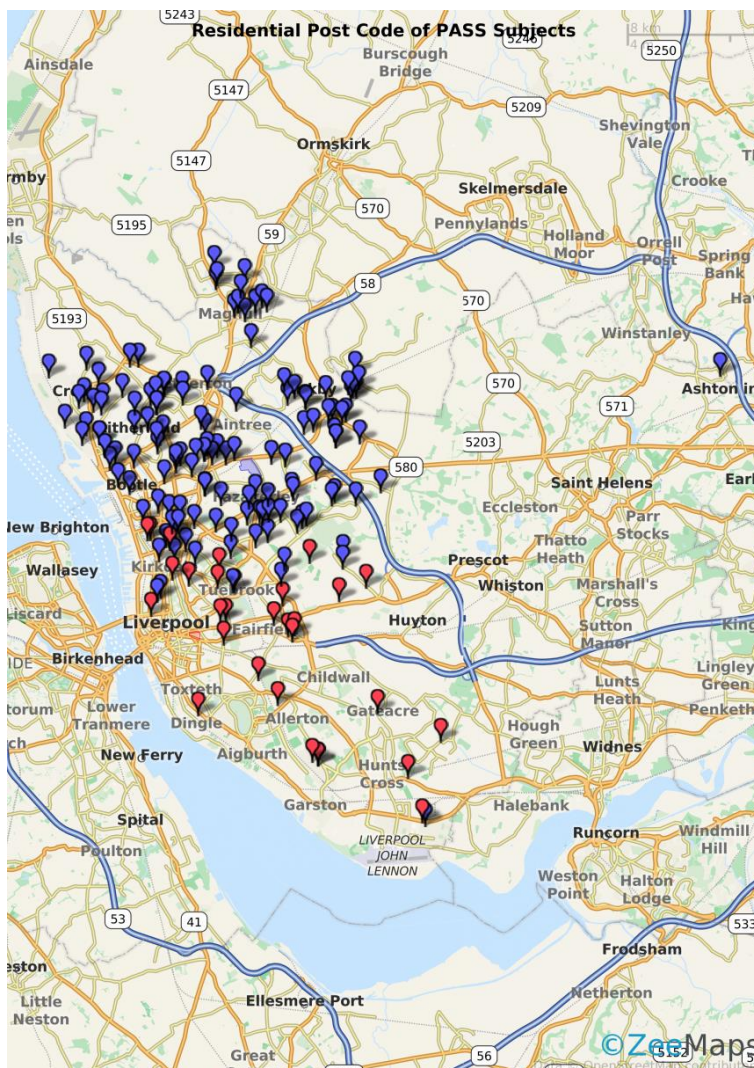


Figure 3.1
PASS subject
map

The geographical distribution of residential post codes for PASS cases are shown. Blue flags are those subjects recruited at AUH and red flags are those recruited at RLUH. One subject resident in Wales is not shown

3.2 Results

3.2.1 Screening and Recruitment

4739 patients were screened for eligibility for PASS. 76% of screened cases had an alternative primary diagnosis (figure 3.2). 25% of those identified by the study as being treated for CAP were deemed eligible and the majority of eligible patients volunteered to join PASS. Key reasons for being in-eligible despite having CAP included:-

- more than 24 hours had elapsed since hospital antibiotics commenced.
- advanced dementia such that the CAP-sym questionnaire would not be available at any time point.
- CAP that was so severe either mechanical ventilation had been or was expected to be instigated or the patient was being managed palliatively.

As a result of this screening process 169 subjects were recruited to PASS and their subsequent rates of retention within the study can be seen in the study flowchart figure 3.3.

3.2.2 Seasonality

Weekly patterns of recruitment can be seen in figure 3.4. Winter recruitment peaks were observed and these were associated with peaks in national notifications of 'Influenza Like Illness (ILI)' to the Health Protection Agency (now Public Health England (PHE)).

Figure 3.2 PASS screening flowchart

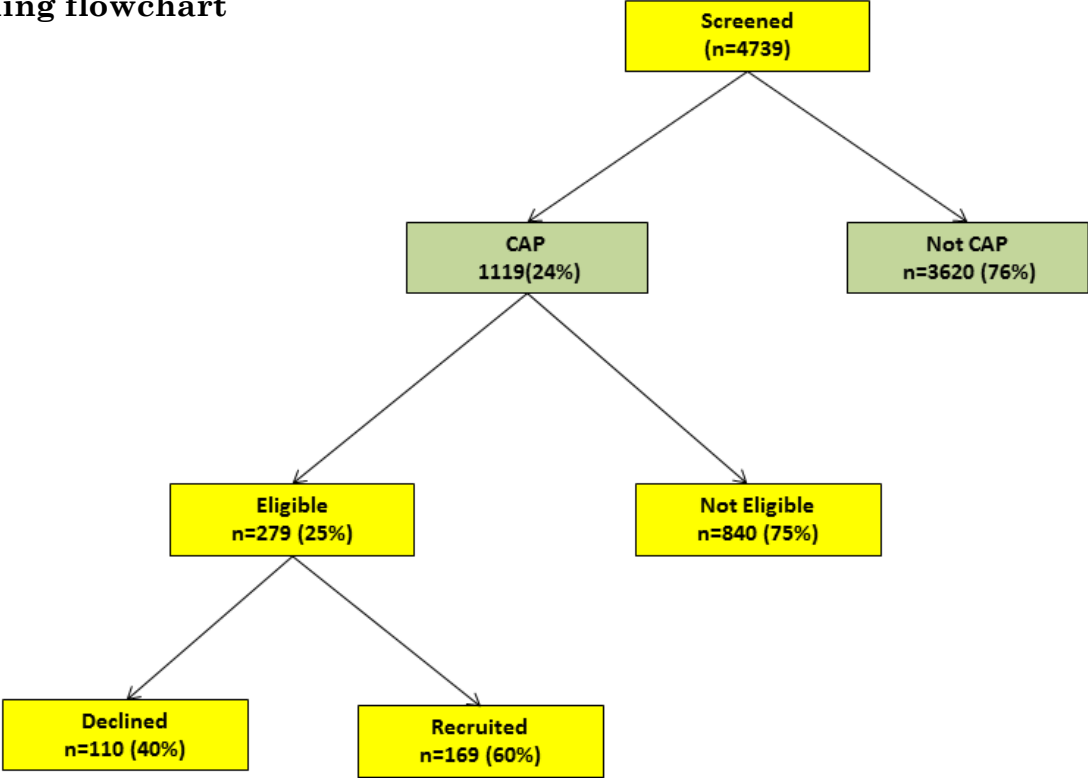
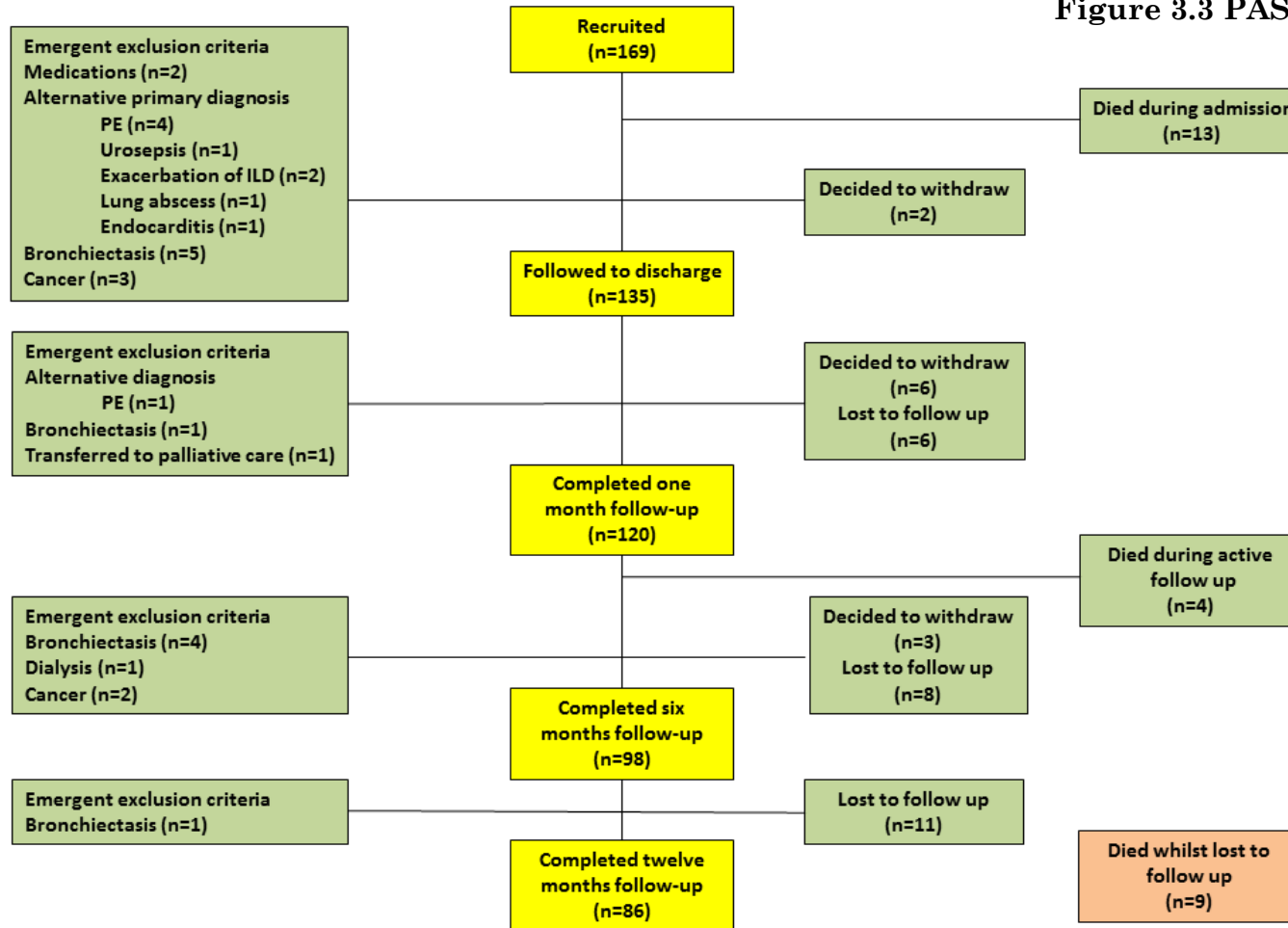


Figure 3.3 PASS flowchart



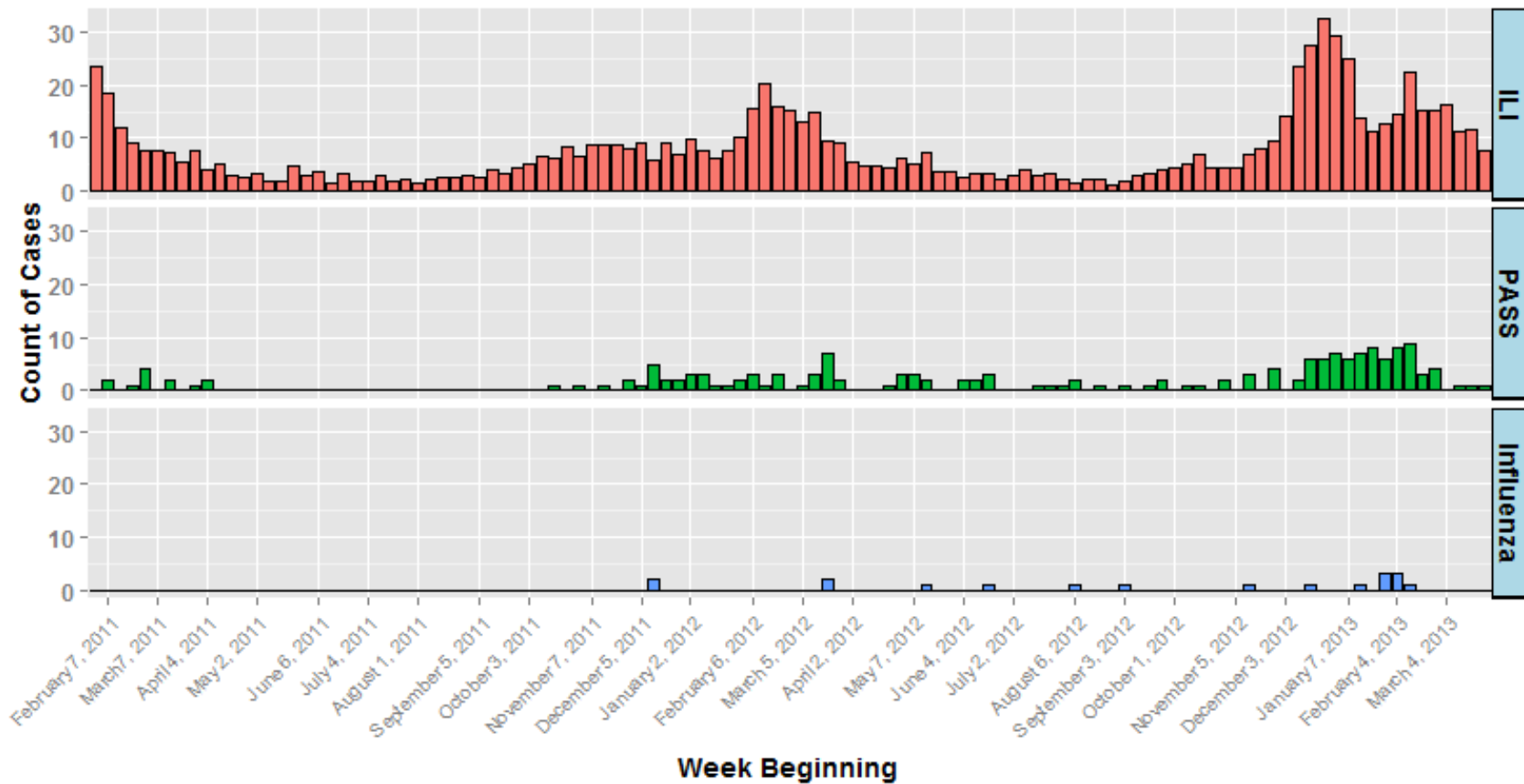


Figure 3.4 PASS weekly recruitment, flu rates and national ILI rates

Peaks in recruitment of PASS subjects corresponded with winter peaks in influenza like illness (ILI) peaks. The ILI counts represent counts per 100000 population. ILI data courtesy of Public Health England.

3.2.3 Demographic details

The demographics of the PASS cohort are presented in table 3.1. The age and sex distribution of the PASS cohort were similar to the contemporary British Thoracic Society Audit of UK Community Acquired Pneumonia (CAP).[125] The age distribution of PASS subjects was also compared to the largest reported cohort of patients hospitalised with CAP, the CAPNETZ cohort (see figure 3.5).[18] In both PASS and CAPNETZ each successive age centile contributes a greater proportion of CAP patients followed by a decline at the upper extreme of the age range. In PASS the decline occurs in the decile 80-90 years which is younger than in CAPNETZ where the proportion of cases increased until 90-100 years. The Ethnicity of PASS subjects was almost entirely white British; one Polish, one Brazilian and one Nigerian born subject were also recruited.

Table 3.1 PASS demographics

Demographics		PASS n=169	BTS 2010/11 n=3570
Age,			
Mean, range, (SD)		64, 16-98, (18)	68, 16-102, (20)
Male, n (%)			
		88, (52.0)	1758, (49.2)
Ethnicity	White British	166 (98.2)	NA
n (%)	White other	2 (1.2)	
	Black African	1 (0.6)	

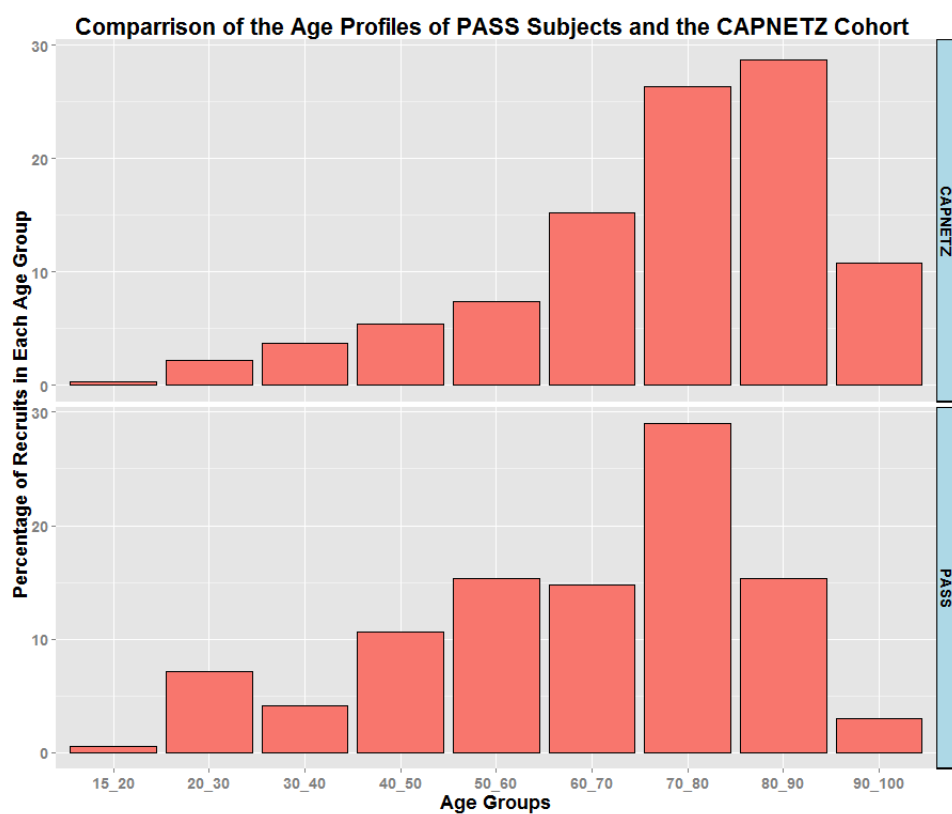


Figure 3.5 Age profile of PASS subjects compared to a CAPNETZ cohort

3.2.4 Socioeconomic status

The IMD is a summary measure of socioeconomic status that is used in epidemiological studies and incorporates individual metrics of deprivation relating to seven sub-domains:- health, education, housing, income, crime, employment and living environment. IMD scores are available for 32,482 Lower Super Output Areas (LSOAs) in the UK.[126] Each LSOA is a small geographical area containing 400 houses (an estimated average of 1500 individuals). All UK post-codes map to a LSOA. Each UK resident can therefore be assigned an IMD score by cross-referencing their post-code with LSOAs and the associated IMD. Two individuals' IMD can be compared either by the raw value where low IMD indicates *less deprived* and high IMD *more deprived* or the position of the IMD score in the rank order of all IMD scores for the 32,482 LSOAs in the UK. If rank is considered then 1st is most deprived and 32482nd is least deprived. Using residential postcodes, we were able to reference the Index IMD score for each subject in PASS. One PASS subject was not assigned an IMD score as they were resident in Wales which has a different method of calculating IMD. Figure 3.6 shows the proportion of PASS subjects that were drawn from each centile of the national rankings. Proportions of PASS subjects in each group are compared to the proportion of LSOAs in Liverpool that fall into each centile. The distributions were very similar.

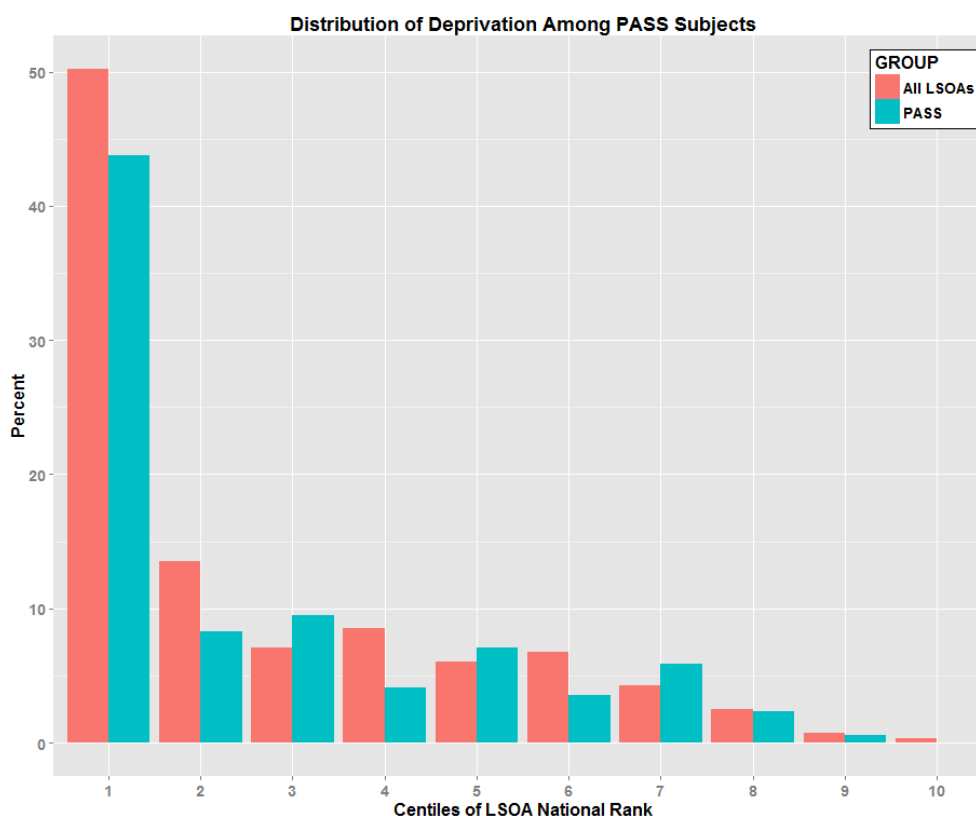


Figure 3.6 Distribution of social deprivation among PASS subjects

The 32,482 UK Lower Super Output Areas (LSOAs) were ranked from 1 (most deprived) to 32,482 (least deprived). The ranks were then divided into centiles. The plot displays the percentage of PASS subjects that fell into each centile (blue bars) compared to the percentage of all Liverpool LSOAs that fall into each centile. PASS recruited subjects from all but the least deprived 10% of the UK population but was dominated by subjects from the most deprived 10%.

3.2.5 Comorbidities

Subjects in PASS had high rates of co-morbidity (see table 3.2). In particular 39% of PASS subjects were active smokers and a further 20% were ex-smokers having abstained for at least 12 months. 53.4% of subjects had some form of prior-pulmonary disease (41% COPD and 12.4% other lung disease).

Table 3.2 PASS comorbidities

Individual comorbidities	PASS n=169
COPD n (%)	70 (41.0)
Chronic lung disease other than COPD n (%)	21 (12.4)
Congestive cardiac failure n (%)	23 (13.6)
Dementia n (%)	2 (1.2)
Diabetes* n (%)	28 (16.7)
BMI, median(IQR) *	26 (22-30)
Hepatic disease n (%)	5 (3.0)
Renal disease n (%)	14 (8.3)
Lived in nursing/residential care, n (%)	8 (4.7)
Smoking status* n (%)	
Active smoker	63 (39.0)
Ex-smoker	66 (41.0)
Never smoker	32 (20.0)
Influenza infection* n (%)	18(16.8)
Infection Markers	
Pyrexial, n (%)	90 (53.0)
Neutrophil count, median (IQR)	9.9 (7.1-14.8)
CRP (mg/ml), median (IQR)	145 (61-248)
Pro-calcitonin* (ng/ml) median (IQR)	0.70 (0.1-3.9)
>0.25 n (%)	98 (64.5)
>0.5 n (%)	83 (54.6)

* incomplete data available for these variables

Diabetes n= 168

BMI n=126

Smoking status n=161

Pro-calcitonin n=152

3.2.6 Clinical Characteristics at Presentation

(Table 3.2) Half (53%) of PASS subjects had a temperature $>37.5^{\circ}\text{C}$ recorded during the first 24 hours of their admission to hospital. Self-expectorated sputum was sent to the microbiology lab from 67/169 (40%) subjects. Median values of blood neutrophil counts and C-reactive protein were above the normal range and 140/169 (82.8%) of subjects had one of either pyrexia, raised neutrophils or raised pro-calcitonin demonstrating that the majority of the cohort had evidence of a systemic inflammatory response. The Confusion, Urea, Respiratory Rate, Blood pressure and age >65 (CURB65) score is a risk stratification index which predicts mortality at 30 days following CAP.[127] The distribution of CURB65 scores for PASS subjects can be seen in table 3.3. The pattern was very similar to that reported by the BTS audit of CAP in the UK. However, within the 3-5 group there were notable differences between PASS and the distribution of severity seen in UK hospitals. None of the PASS subjects had a CURB score of 5 and only 4 (2.4%) subjects had a CURB65 score of 4.

Table 3.3 PASS CURB65 Scores

CURB65 Score	PASS n=169	BTS 2010/11 n=3570
0-1	79 (46.7)	1655 (46.4)
2	50 (29.6)	952 (26.7)
3-5	40 (23.7)	934 (26.2)

3.2.7 Antibiotics and vaccination

18/169 (11%) of subjects had received an at least one dose of antibiotic in the community before presenting to hospital. Information was provided by general practitioners about the vaccination status of 119/169 (70.4%) of PASS subjects. 92% of those for whom we had information were, according to NHS vaccination guidelines, “at risk” from influenza. 55% of those had been vaccinated in the season during which they had been admitted with CAP and 85% had been vaccinated against influenza at some point in their lives. 76% of those for whom we had information were eligible (at risk group) for pneumococcal vaccination and 82% of those had received the vaccine.

3.2.8 Bacterial Aetiology of CAP

155/169 (92%) of PASS subjects had blood cultured in the NHS microbiology laboratories to investigate for a bacterial cause for their pneumonia. 67/169 (39.6%) of PASS subjects had a sputum sample cultured. Overall 156/169 (92%) of subjects had at least one of either a sputum sample or blood culture sent (table 3.4). 1/28 patients tested was diagnosed with legionella pneumonia on the basis of a positive urinary antigen test. Using these three diagnostic modalities 28/156 (18%) had potentially pathogenic bacteria reported from their samples. Yeasts and upper respiratory tract flora, and “mixed coliforms” where reported in sputum, were not regarded as the aetiological cause of CAP.

3.2.9 Influenza infection

107/169 (63%) of subjects had paired acute and convalescent serum samples available for measurement of antibody responses to circulating influenza viruses. 18 of these 107 subjects (16.8%) had met the Public Health England diagnostic criteria of a four-

fold rise in influenza specific IgG titres, suggestive of acute influenza infection at the time of presentation with CAP and recruitment to PASS.

Table 3.4 PASS blood and sputum cultures

	Blood Cultures n (%)	Sputum Cultures n (%)
Negative	139 (89.7)	47 (70.1)
<i>Streptococcus pneumoniae</i>	8 (5.2)	0
<i>Escherishia coli</i>	2 (1.3)	0
<i>Klebsiella pneumoniae</i>	2 (1.3)	1 (1.5)
<i>Streptococcus dysgalactiae equisimilis</i>	1 (0.6)	0
<i>Staphylococcus aureus</i>	1 (0.6)	0
<i>Lactococcus lactis</i>	1 (0.6)	0
<i>Diphtheroid bacilli</i>	1 (0.6)	0
<i>Haemophilus influenzae</i>	0	6 (9.0)
Coliforms	0	3 (4.5)
Yeasts	0	3 (4.5)
Upper Respiratory tract flora	0	3 (4.5)
<i>Pseudomonas spp.</i>	0	4 (6.0)
Total	155 (99.9)	67 (100.1)

3.2.10 Mixed infection

Two PASS subjects grew *Streptococcus pneumoniae* in the blood and were serologically positive for influenza virus. One grew *Haemophilus influenzae* in the sputum and was serologically positive for influenza virus.

3.2.11 Clinical outcomes

(Table 3.5) On average PASS subjects spent one day longer in hospital (6 days) than patients in the 2010/11 BTS pneumonia audit of UK CAP (5 days). PASS subjects and BTS audit patients were equally likely to be re-admitted to hospital within 30 days (9%). In-patient mortality was three times lower in PASS than in the BTS audit (7.7% vs 20.4%) and only one PASS subject (0.6%) died in the 30 days following discharge compared to 3.8 % in the BTS audit. In addition to the 13 in-patient deaths and the one early discharge death, a further 12 PASS subjects had died by 1 year from admission. In-patient mortality among the PASS cohort increased with age and followed the same pattern as in previous reported studies including the CAPNETZ cohort (Figure 3.7).

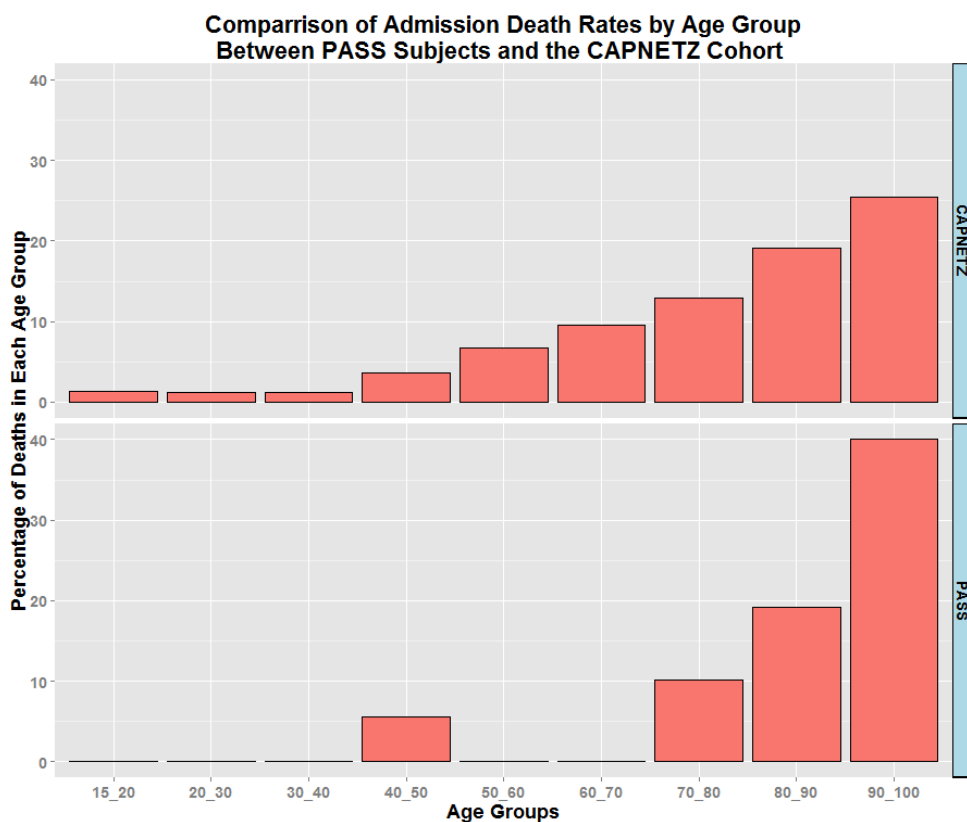


Figure 3.7 PASS mortality by age compared to a CAPNETZ cohort

Table 3.5 PASS outcomes

Outcome	PASS n=169	BTS 2010/11 n=3570
Length of stay		
Median, range (SD)	6, 0-58, (7.8)	5, 0-62, (9.0)
Re-admission within 30 days of discharge, n (%)	16 (9.0)	322 (9.0)
In hospital mortality, n (%)	13 (7.7)	730 (20.4)
Death within 30 days of discharge, n (%)	1 (0.6)	108 (3.8)
Death post discharge n (%)	13/156 (8.0)	NA
Total one year mortality, n (%)	26(15.0)	NA
Cause of in hospital death n (%)		
CAP	8 (62.0)	
Sepsis	2 (15.0)	
Myocardial infarction	1 (8.0)	
Respiratory failure	1 (8.0)	
Unknown	1 (8.0)	
Cause of death post discharge n (%)		
CAP	2 (15.0)	
HAP	1 (8.0)	
Gastric cancer	1 (8.0)	
Lung cancer	3 (23.0)	
Interstitial lung disease	1 (8.0)	
COPD	2 (15.0)	
Unknown	3 (23.0)	

3.2.12 Charlson co-morbidity scores in PASS

Patients' pre-existing chronic illnesses were used to calculate the revised version of the Charlson co-morbidity index.[128] This index can be used to estimate the magnitude of a patient's risk of death during a hospital admission that is attributable to comorbidity. Comorbidity is defined as pre-existing conditions other than the primary diagnosis. It is calculated by summing a weighted score for each of the 12 comorbidities that have been shown to be most closely associated with in-patient mortality (Figure 3.8). In patient mortality rates for PASS subjects in comparison to an Australian cohort are shown in table 3.6

Comorbidity	Weighted score
Chronic pulmonary disease	1
Rheumatologic disease	1
Renal disease	1
Diabetes with chronic complications	1
Congestive cardiac failure	2
Dementia	2
Hemiplegia or paraplegia	2
Any malignancy	2
Mild liver disease	2
Moderate or severe liver disease	4
Aids	4
Metastatic solid tumour	6
Maximum Charlson score	24

Figure 3.8 The Charlson comorbidity index

Table 3.6 Charlson comorbidity scores of PASS subjects

Charlson comorbidity index	PASS subjects per score n (%)	Rates of PASS in-patient mortality n (%)	Australian rates of mortality per score n (%)
0	56 (33.1)	2 (3.6)	15 (1.2)
1	69 (40.8)	4 (5.8)	38 (3.1)
2	18 (10.7)	4 (22.2)	84 (6.8)
3	17 (10.1)	3 (17.6)	115 (9.2)
4	6 (3.6)	0	168 (13.6)
5	2 (1.2)	0	226 (18.3)
≥6	1 (0.6)	0	247 (20)

Each of 12 comorbidities is assigned a weighted score and a subject's Charlson index is the sum of those scores. The risk of in-patient death rises as scores increase from 0 to a maximum of 24.

PASS mortality data is compared with the mortality rates of patients in an Australian cohort which was used in the validation of the revised Charlson index. The validation study used large cohorts from 6 developed nations and the Australian cohort has been chosen here as the Australian health care system is the closest match to the NHS.

The highest Charlson score in PASS was 6. The proportion of PASS subjects with scores 0-6 is shown along with the proportion of each group who died during admission.

The absence of PASS deaths in the Charlson categories 4,5 and 6 is almost certainly an artefact of low numbers of subjects in these groups.

3.3 Discussion

3.3.1 Summary of Results

The PASS study recruited a cohort of 169 patients with CAP. The cohort was predominantly composed of older, socially deprived, white, British patients who often had COPD. PASS subjects had many similarities with previously published CAP cohorts and, where differences existed, they could be explained by the study design, or reflected idiosyncrasies of the local population (see below).

3.3.2 What were the strengths of this work?

3.3.2.1 Strict case definition

The diagnosis of CAP is subjective. In particular determining the presence or absence of pneumonic consolidation on the CXR is prone to both intra and inter-user inconsistencies.[11] As this was a prospective study we were able to carefully assess each subject's eligibility and since the author acted as the final arbiter of eligibility for each subject, inter-user variability was reduced.

3.3.2.2 Wide screen

It was important to recruit a representative cohort and in order to do this we needed to ensure we screened the highest proportion of patients who presented with possible CAP. This generated an enormous amount of work, particularly in the summer months where cases were rare, but as a result we considered the majority of cases of CAP that fell within our recruiting window.

3.3.2.3 Comprehensive characterisation

The cohort was characterised in detail such that similarities and differences with other cohorts could be clearly elucidated. We

focussed on factors that have been or may be associated with outcome such as socioeconomic status, co-morbidity, markers of inflammation, severity scoring and risk-stratification. As a result we were able to demonstrate similarities with other work and explain differences where they existed.

3.3.2.4 Socioeconomic status

The inclusion of socioeconomic status as a clinical variable is novel among UK adult cohort studies of CAP and enabled us to exclude the possibility of any related bias that may have influenced outcomes. 43% of PASS subjects were drawn from areas which have the highest level (lowest centile) of social deprivation in the UK. The distribution of Index of Multiple Deprivation (IMD) scores for PASS patients was very similar to the distribution of IMD scores assigned to all the Liverpool Lower Super Output Areas (LSOAs) that would be served by our two recruiting hospitals. This suggests PASS recruits were representative of the local population and that there was no obvious bias in PASS towards recruiting from any particular socioeconomic group.

3.3.3 What were the limitations of this work?

3.3.3.1 Pause in recruitment

Active recruitment was suspended between May and September 2011 as there were inadequate numbers of staff to conduct the study effectively. During this period, only potential subjects identified by NHS colleagues were screened and, since there were very few pneumonia cases presenting to the hospital, this effectively meant recruitment stopped (see figure 3.1 central panel). Following the addition of a nurse to the study team, active

case finding resumed in October 2011. Due to the small number of cases recruited prior to this pause no formal statistical comparison was been made with the main body of subjects; however, no clear differences could be seen and the recruitment criteria were identical before and after.

3.3.3.2 In-eligible patients with CAP

Three quarters of patients being managed as CAP by the hospitals could not join the study as they failed to meet the PASS inclusion criteria. The most common reason for this was the identification of the patient after 24 hours had elapsed since their first dose of in-hospital antibiotics. ‘Late’ identification of patients was a manifestation of the complexity of hospital pathways, and in many instances a late clinical diagnosis. Late clinical diagnoses occur where a patient is not immediately recognised as having CAP – but occult infection is included in the differential diagnosis and antibiotics are administered. As test results arrive and more senior clinicians review the case, or opinions on the CXR change, the diagnosis becomes crystallised as CAP but by that point a study’s opportunity to recruit may have passed. Another frequent reason for a patient being unavailable for recruitment within 24 hours is admission at the weekend. Most of the time PASS was unable to recruit after 1600 on Friday evening and this meant all patients admitted from then to midway through Sunday would be ineligible. Another common reason for ineligibility was dementia of a level that meant the CAP-sym questionnaire could not be completed. A less frequent but important reason for ineligibility were patients who were expected to die shortly after admission or who were rapidly intubated and ventilated.

Ineligibility for the reasons listed above may have influenced our results. Although the time and day of presentation is unlikely to have affected the findings of the CAP-sym analysis, efferocytosis or microbiota studies – dementia and severe disease requiring ITU care may have done. Patients with dementia may have been more likely to aspirate and or have Gram negative pneumonias. Patients with severe disease may have had a higher incidence of pneumococcal disease.

3.3.3.3 Duration of illness and prior treatment

This was, as far as possible, a pragmatic cohort designed to be representative of CAP in an acute UK hospital. As such a proportion of subjects were several days into their disease episode at the time of recruitment. Some patient's route to hospital was to see the GP, be diagnosed with lower respiratory tract infection, attend for an outpatient CXR then at GP follow-up they were referred to hospital due to ongoing symptoms. The initial CAP-sym score may therefore not always have captured the initial trajectory of symptoms. Since some patients had been pre-treated with antibiotics in the community prior to enrolment this will undoubtedly have affected the microbiological identification of the casual organism by culture and will also have had an impact on the microbiota identified. However, all patients met the case definition of CAP at recruitment and were prescribed in-hospital antibiotic suggesting they had ongoing symptoms of acute infection. This is reinforced by the high levels of CRP and procalcitonin measured in this group suggesting that a large proportion of the cohort were still in an acute inflammatory state at enrolment.

3.3.3.4 Ethnicity

A striking feature of the PASS cohort was its ethnic uniformity with 98% of subjects being white British. The ethnic distribution in PASS is however a very close reflection of the local population. In Liverpool local health authority 84.8% of people report being white British, 2.6% as white other and 1.8% as Black African. In Knowsley local health authority, which is directly in the catchment of University Hospital Aintree, 96.1% of people are white British, 0.2% are Black African and 0.7% white Other.[129] This distribution of ethnicity is very different to many areas of the UK including central Manchester which is only 30 miles away. Ethnicity may impact on the incidence, severity, recovery and mortality from pneumonia via cultural associations and mechanistic effects. For example dietary differences can significantly affect the gut microbiota which in turn affects immunity at distant sites such as the lung.[130] Differences in Vitamin D levels have had been linked to incidence and outcomes in pneumonia and patients from different ethnic backgrounds can have profound differences in vitamin D metabolism due to dietary, skin pigmentation and microbiome effects.[131]

3.3.3.5 Socioeconomic status

The socioeconomic profile of the PASS cohort was heavily biased towards the lowest end of the socioeconomic spectrum of England. Since the incidence of CAP is 70% higher among the most deprived quintile of England's population than among the least deprived, patterns of admission for CAP may be different in other areas.[118] Since low socioeconomic status is associated with higher comorbidity and worse outcomes in many diseases this may reduce the generalisability of these findings to more affluent

populations. Future multi-site studies could investigate this by clustering sites based on socioeconomic data.

3.3.4 Comparison with other published work

3.3.4.1 Comorbidities

Smoking is known to be a major risk factor for invasive pneumococcal disease and CAP.[22] Despite this, recent UK cohort studies of CAP have not reported rates of smoking.[132] 80% of PASS subjects were either active smokers or ex-smokers. This compares with 64.3% in a similar Spanish CAP cohort.[133] The high rate of smoking in the PASS cohort reflects the local population where Liverpool Local Health Authority has a smoking prevalence of 29% and Knowsley 32% both of which are significantly higher than the UK average of 20%.[134]

COPD was very common among PASS Subjects with 41% reporting this condition. This compares to a UK rate of 19% in the 2010/2011 BTS CAP audit and 9.41% in the German CAPNETZ cohort. The high prevalence in PASS is likely a reflection of the very high rates in the local population. The UK prevalence of COPD in men over 16 years has been estimated to be 4.49% and in women is 2.82%.[135] Of 323 UK Local Health Authorities Knowsley has a predicted COPD rate of 7.0% in Men and 4.85% in Women which are the third and second highest rates in the UK respectively. Liverpool and Sefton Local Health Authorities are also both in the top 5% of all UK health authorities for both male and female COPD rates.

The revised Charlson co-morbidity index has been validated using data from 6 developed nation databases. The index estimates the mortality risk attributable to diagnoses other than the primary

reason for admission. 72% of PASS subjects had Charlson scores of 0,1 or 2 - the lower end of the spectrum of mortality risk associated with comorbidity. PASS subjects followed the predictable trend towards increasing mortality as the index increased through 0, 1 and 2. There was however a higher mortality in these four groups compared to the Australian comparator cohort and this is probably explained by the high mortality associated with CAP.

3.3.4.2 Aetiological diagnosis

Rates of aetiological diagnosis in CAP vary widely in the literature. This is likely to be due to variation in the samples taken, the range of assays used and to variation in the inclusion criteria between cohorts. Moreover, some studies are designed with aetiological diagnosis as the primary aim whereas others, like ours, are pragmatic and simply report the results of clinical microbiological testing. The 2009 BTS guidelines summarise 5 UK studies which focussed on aetiological diagnosis of CAP and report rate of pathogen identification between 66.5%-71.9%.[6] A recent systematic review of European studies investigating the aetiological causes of CAP revealed rates of pathogen identification of between 73.3 and 12.7%.[136] PASS relied on routine microbiological testing undertaken by the hospitals on behalf of the patients in this study. The hospitals perform sputum and blood cultures on the basis of local guidelines and in addition they offered urine antigen testing for *Legionella* spp. in more severe CAP cases. Of note neither hospital offers urine pneumococcal antigen testing. In addition to the routine clinical samples PASS aimed to take a blood culture and send serum for flu serology on all patients. Using these 4 modalities bacteria

were identified as the sole cause in 25 patients, flu in 15 patients, and 3 had mixed flu and bacterial infections. Overall 43/169 (25.4%) had a positive aetiological diagnosis. This places PASS at the lower end of aetiological confirmation studies and at the higher end of pragmatic studies and this is not surprising given its pragmatic design and the fact that molecular diagnostics were not used.

The top four pathogens identified in PASS were Influenza virus, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. This is different to the rank order of pathogens summarised in the BTS guidelines (table 1.1).[6] The top four in the BTS summary were *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* then Influenza virus. The prominence of *Haemophilus* spp. and *Pseudomonas* spp. in our cohort likely reflects the high rates of COPD and smoking.[137,138]

Rates of detection of Influenza in PASS were within the range reported in previous studies. However, influenza virus has a complex relationship with CAP and assertions regarding causality are difficult. Viral pneumonia has long been recognised as an entity and Influenza viruses are commonly felt to be the leading cause of “virus only” pneumonia.[139] However, Influenza virus is closely linked to the pathophysiology of CAP caused by bacteria, and sequential or mixed infections, as detected in three PASS patients, are common.[20] As part of the CAPNETZ programme of research a study was conducted to evaluate the impact of seasonal influenza vaccination on CAP.[140] That study used rt-PCR to detect Influenza virus in respiratory specimens and found that positivity rates fell in the winter in response to vaccination

and were higher in the summer between rounds of vaccination. Patients who developed winter CAP but had received seasonal Influenza vaccination fared better across a range of outcomes. However, their overall PCR detection rates were low at 3-6%. The investigators concluded that this low influenza detection rate was likely an under-estimate of the number of influenza related CAP cases. They suggested that influenza infection which led to secondary bacterial CAP was unlikely to be detectable by PCR at the time of the presentation with CAP. As molecular techniques improve it is clear that many other viruses are also capable of influencing the development and course CAP and when we factor in the recent shifts in our understanding of the bacteria present in the lung it becomes more difficult to clearly define viral from bacterial from mixed infections.

3.3.4.3 Outcomes

PASS aimed to describe outcomes over one year and mortality rates were remarkably similar to a comparable Spanish study that also sought to quantify and understand mortality also over one year. That study recorded an in-patient mortality of 8.6%, a post-discharge mortality of 7.2% and total one year mortality of 15.2%; equivalent figures for PASS were 7.7%, 8.3% and 15.3%.[133] Both PASS and the Spanish cohort excluded patients with immune-suppression but the Spanish cohort included most other comorbidities including patients with cancer. The 7.7% in-patient mortality in PASS was much lower than in either the recent UK based BTS audit of CAP (20.4%) or the German CAPNETZ cohort (17.43%). Both of those cohorts included “all comers” with CAP and in particular CAPNETZ demonstrated that the highest rates of in-patient mortality were among those who

had active malignancy (27.65%). PASS excluded those with active malignancy and also excluded those who were managed palliatively, ventilated acutely, or had advanced dementia; comorbidities that are associated with significantly increased mortality. Interestingly, the most recent prospective cohort study of CAP in the UK reported 30 day mortality of 7.5% which is similar to PASS at 8.3%.[132] That study had similar recruitment criteria to PASS in that it excluded thoracic malignancy (PASS excluded all active malignancy) and those who were not actively treated. The exclusion of these severely ill patients from PASS ought to be reflected in the CURB65 scores yet PASS CURB65 scores looked very similar to the BTS audit range. However, the BTS audit summaries collapse CURB65 scores of 3-5 into a single group. The rationale for that was that any score of 3 and above should be regarded as severe indicating the patient should be considered for level 2-3 care (high dependency or Intensive care). Grouping the scores however, masks important differences in likely outcome; CURB-65 scores of 3, 4 and 5 are associated with 30 day mortality rates of 17%, 41.5% and 57% respectively.[127] The lack of any score 5s will have reduced the overall in-patient and 30 day mortality in PASS.

3.3.5 Implications

The effort spent on clinical characterisation and the rich set of clinical samples obtained from the cohort allowed experimental hypotheses to be tested in the context of known clinical phenotypes. This cohort was a true reflection of hospital CAP in our area and the results will be relevant to regions with similar epidemiology and healthcare services.

4 WHICH FACTORS ARE ASSOCIATED WITH EFFEROCYTOSIS IN PATIENTS RECOVERING FROM CAP?

4.1 Introduction

4.1.1 Efferocytosis and inflammatory lung disease

Efferocytosis is the phagocytosis of autologous apoptotic cells (see 1.6.5). It is an essential mechanism for returning a site of inflammation to its homeostatic state. In the lung the key effector of efferocytosis is the alveolar macrophage. In health these are the most abundant leukocytes in the alveolar space and are (in addition to multiple other roles) professional phagocytes. The effectiveness of macrophage efferocytosis has been studied in patients with a number of inflammatory lung conditions. In patients with chronic obstructive pulmonary disease (COPD) efferocytosis has been shown to be impaired in comparison to healthy individuals. [100] In Cystic fibrosis (CF), non-CF bronchiectasis and Idiopathic Pulmonary Fibrosis (IPF), similar results have been observed.[141,142]

4.1.2 Efferocytosis and recovery from pneumonia

Pneumonia involves pathology in which efficient efferocytosis could be expected to be vital for recovery. The pathophysiology of pneumonia is characterised by the recruitment of large numbers of neutrophils into alveolar spaces.[20] This influx is necessary and beneficial in terms of pathogen removal by neutrophil phagocytosis. However, once the pathogen threat has been overcome their presence is a risk to the delicate architecture of the gas exchanging structures of the lung. Removal of neutrophils by alveolar macrophage efferocytosis is therefore a key process in the resolution of pneumonia. In addition to the

benefits of removing pro-inflammatory cells, the act of efferocytosis has the effect of inducing a pro-resolution phenotype on the macrophage with a reduction in the production of inflammatory cytokines and an increase in TGF- β production. [143] In mice, during resolution of pneumonia, the efferocytosis of neutrophils by alveolar macrophages leads to the secretion of hepatocyte growth factor that promotes resolution of inflammation. [144] Therefore in the process of understanding human recovery from pneumonia, determining if clinically significant differences in rates of efferocytosis exist, and identifying patient characteristics that are associated with impaired efferocytosis, are key steps towards testing possible pro-resolution, pro-recovery interventions. Rates of efferocytosis and factors that affect this in patients recovering from pneumonia have not been previously published.

4.2 Aim

To determine the association(s) between observed clinical characteristics and efferocytosis in patients recovering from community acquired pneumonia (CAP).

4.3 Hypothesis

Patients recovering from CAP will display different rates of alveolar macrophage efferocytosis that can be predicted by observed parameters.

In order to address this hypothesis alveolar macrophages were obtained from Pneumonia Aetiology and Severity Study (PASS) subjects who were recovering from CAP and we measured their efferocytosis of autologous apoptotic neutrophils using flow cytometry.

4.4 Methods

4.4.1 Subjects and bronchoscopy

Eligibility, consent, the bronchoscopy procedure and the initial processing of bronchoscopy samples are described in sections 2.4, 2.7.3 and 2.7.10 respectively. For standard operating procedures (SOPs) for the processing of bronchoalveolar lavage (BAL), culture of macrophages, separation of neutrophils and the ex-vivo efferocytosis experiment (see appendix 2). Briefly, eligible volunteers had bronchoscopy performed one month following enrolment into the study. BAL of the middle lobe was performed with 200 ml warm saline which was removed by gentle syringe suction.[123]

4.4.2 Alveolar macrophages

Alveolar macrophages were derived by centrifugation of fresh BAL samples and were confirmed as the dominant population by cytopspin. The macrophages were washed and suspended at 1×10^6 cells/ml in Iscove's Modified Dubecco's Medium (IMDM) supplemented with 10% pooled human AB serum (same lot used for all experiments) and antibiotics to prevent bacterial and fungal growth. This has previously been described as optimal for supporting uncontaminated macrophage growth.[145] 500 μ l (0.5×10^6 cells) of the macrophage suspension was placed in as many wells as possible, (avoiding the outside rows) of a 48 well cell culture plate and then incubated for 4 hours at which point the media was replaced with antibiotic free media. The culture plates used had a temperature sensitive coating which promoted macrophage adhesion at 37°C but caused macrophages to detach at 20°C (Nunc™, UpCell™ Thermo Scientific). These plates were

chosen as they facilitate maximum macrophage recovery with the least amount of cellular damage.[146]

4.4.3 Autologous neutrophils

After two hours recovery in the endoscopy unit (during which time the bronchoscopy specimens were processed) 36 ml whole blood was collected into EDTA bottles from subjects who had undergone bronchoscopy. The blood was placed over Polymorphprep™ density gradient and spun (see figure 4.1). This resulted in the separation of the whole blood into several layers containing different components. The granulocyte layer (predominantly neutrophils) was removed, added to Hepes buffered saline and spun to pellet the cells. Erythrocyte contamination of the granulocyte pellet was lysed and the cells washed, re-suspended in serum free IMDM and counted. A cytopsin was prepared to check that viable neutrophils were the dominant cell population. The neutrophil preparation was now divided into two centrifuge tubes, spun and both pellets re-suspended in 5 ml serum free IMDM. One neutrophil aliquot was stained green with CellTracker™ Green and the other aliquot was left unstained. The stained and unstained neutrophil preparations were made up to 1×10^6 cells/ml with IMDM +5% AB serum in cell culture flasks and the degree of apoptosis was assessed by flow cytometry (see below). Both culture flasks, containing stained and unstained neutrophils were incubated at 37°C in 5% CO₂. After 20 hours incubation the neutrophils were harvested from their flasks, washed and counted. The unstained and stained neutrophils were independently re-suspended at 5×10^6 cells/ml in IMDM + 10% AB serum. A cytopsin was made of the unstained neutrophils to visually confirm features of apoptosis and the

proportion of apoptotic neutrophils was measured using flow cytometry.

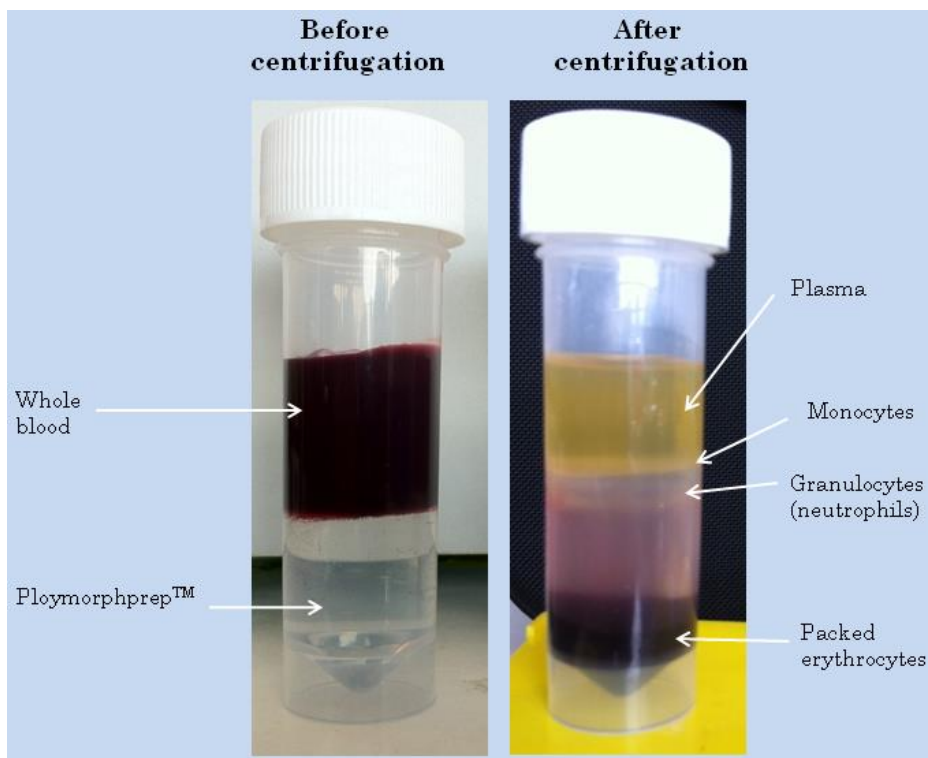


Figure 4.1 Separation of neutrophils from whole blood

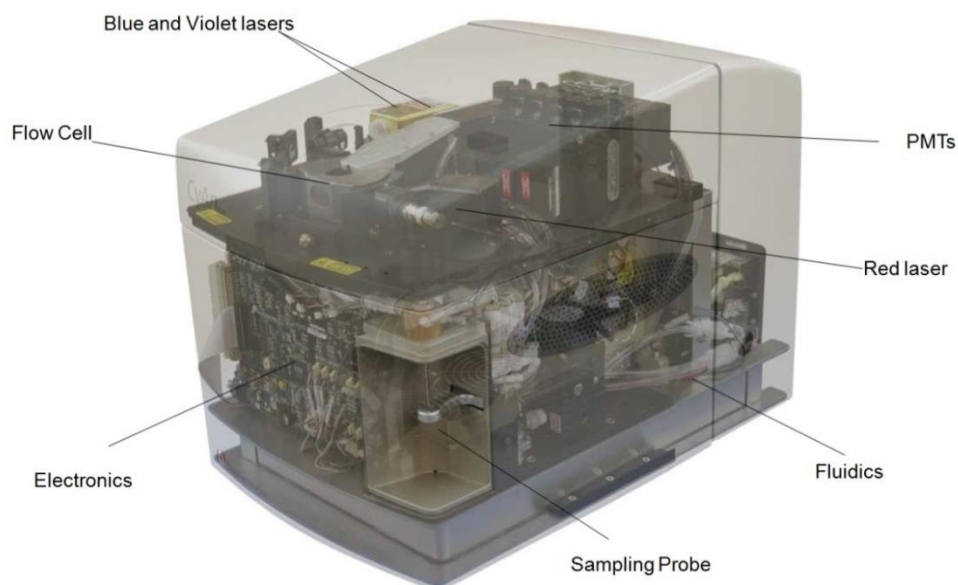


Figure 4.2 Internal configuration of the CyAn ADP benchtop flow-cytometer

PMT=photomultiplier tube. Note the version used for these experiments was set up with a blue and red laser only

4.4.4 Measuring neutrophil apoptosis using flow-cytometry

Four small neutrophil aliquots were washed and suspended in annexin binding buffer (a source of Ca^{2+}). One of the aliquots was then stained with annexin V – APC, another with 7-Aminoactinomycin D (7AAD) and a third with both. The last aliquot was left unstained. The cells were all acquired on a CyanAn-ADP™ benchtop flow cytometer (see figure 4.2 previous page) using Summit software version 4.3.02 (Beckman Coulter). Figure 4.4 describes the gating strategy for the flow-cytometric method for measuring apoptosis.

4.4.5 Efferocytosis assay

The media was removed from the macrophages and 500 μl of the appropriate challenge was added to each well i.e. unstained neutrophils, stained neutrophils or media alone. The macrophage plate was then placed back in the incubator. After 90 minutes, during which time efferocytosis took place, the media was removed and the macrophage monolayer washed gently to remove some of the remaining un-ingested neutrophils (see figure 4.3 for an image of the cells in co-culture). The macrophages from each well were then removed to individual centrifuge tubes. The macrophages were washed once, pelleted and re-suspended in 100 μl phosphate buffered saline (PBS). To quench extracellular autofluorescence (see figure 4.5) 100 μl crystal violet was added to each tube of macrophages and after 30 seconds 1 ml of PBS was added and the macrophages washed 3 times before being transferred to cytometry tubes and placed on ice.[147]

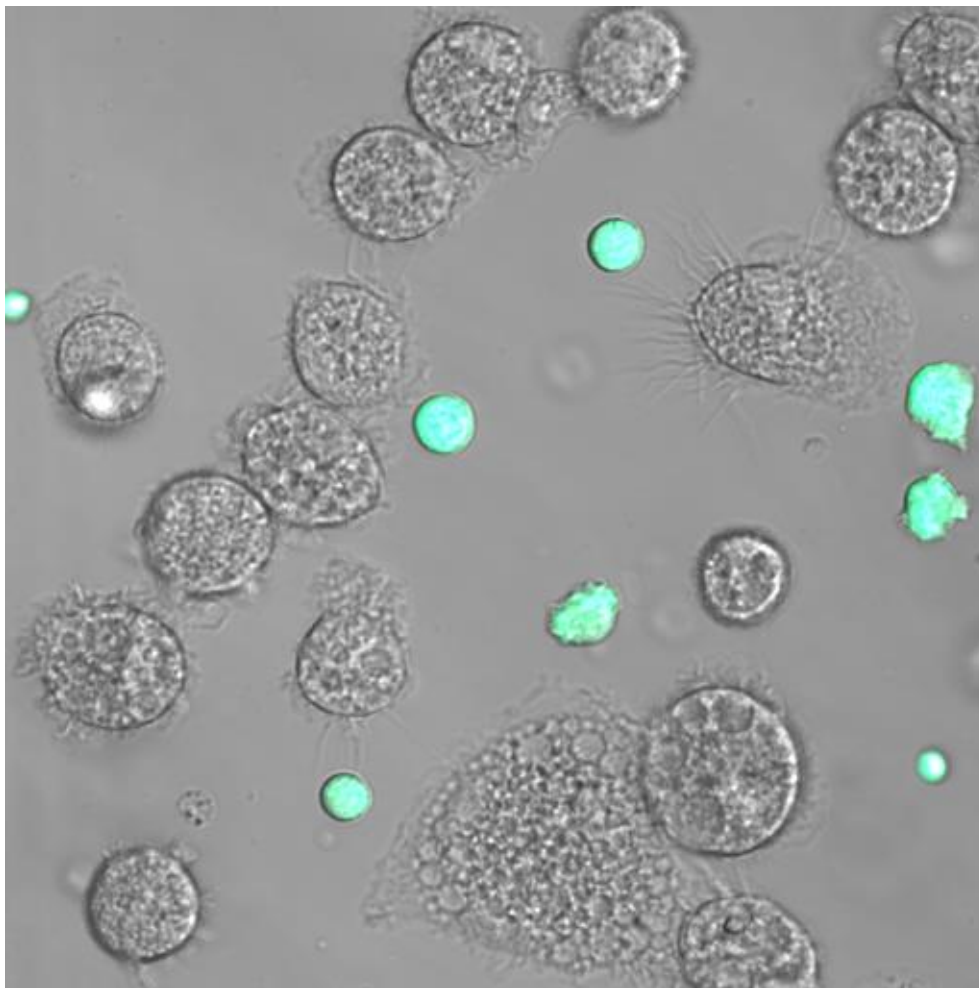


Figure 4.3 Confocal fluorescence microscopy image of alveolar macrophages in co-culture with apoptotic neutrophils

The image above was obtained at the cell imaging suite of the University of Liverpool using a Zeiss confocal microscope. Ex-vivo alveolar macrophages and autologous neutrophils are in co-culture in complete media in specialised cell imaging wells at 37°C in 5% CO₂. The image is a composite of two images – a standard bright field image is combined with a blue laser image. The bright green small cells (fluorescing green in blue laser light) are apoptotic neutrophils that have had their cytoplasm stained green (see 4.4.3). The larger cells are healthy alveolar macrophages. Macrophage podosomes can be seen reaching out to neutrophils.

4.4.6 Development of the flow cytometry acquisition protocol to determine efferocytosis

A literature search discovered only one publication of a flow-cytometric analysis of efferocytosis of neutrophils by macrophages.[148] The following is a modification of that method published by Michlewska et al. to suit our application where alveolar macrophages are used.[148] The light scatter characteristics of alveolar macrophages, in particular SCC, varied considerably between subjects and this was most pronounced comparing macrophages from smokers and non-smokers. Hence it was necessary to adapt the above settings using a number of different volunteers to create a protocol with acquisition settings that would capture all inter-patient variations in macrophage light scatter within the plot boundaries (see figure 4.6).

4.4.7 Analysis of efferocytosis

See figure 4.7 A sample of green neutrophil apoptotic target was acquired first and the light scatter observed on a bivariate plot of forward scatter / side scatter (FSC/SCC) using a linear scale. Gain and voltage settings were modified to capture the neutrophil population in the left hand corner (first log order) of the plot. Next a tube of macrophages (unchallenged by neutrophils) was acquired and visualised on the same light scatter plot and settings adjusted to ensure the whole of the macrophage population could be observed. A gate was drawn around the macrophages and this population observed on histogram of log FITC. The FITC voltage was adjusted to place the macrophage population in the first log order of this plot. Next the control experiment was acquired which included a mixed population of macrophages challenged by unstained neutrophils. Any macrophages that had ingested unstained neutrophils should

have an unaltered level of FITC fluorescence. By observing the statistics produced by the software a new gate was created that excludes all but the highest 0.1% by FITC expression of these control macrophages. Finally the efferocytosis experiment was acquired which was a mixed population of alveolar macrophages along with green stained neutrophils. Any of the gated macrophages that had a level of FITC fluorescence greater than the control macrophages must have ingested a green apoptotic neutrophil. The read-out of the experiment was therefore the proportion of macrophages falling in the high FITC expression gate.

4.4.8 Statistical Analysis

The analysis was performed using R (R core team, 2013). Associations with efferocytosis were analysed by multiple regression. For the purposes of this analysis it was assumed, a priori, that efferocytosis is a good thing i.e. a higher value is associated with improved outcome following community acquired pneumonia. An initial univariate analysis using the `lm` (linear model) function (core R package) was undertaken to determine which observed parameters to select as explanatory variables in subsequent multivariate modelling. For the univariate analysis, for each subject, the mean of efferocytosis experimental replicates was used.

A maximal linear mixed effects model using the LME (linear mixed effects) function of the `nlme` package (Jose Pinheiro, Douglas Bates, Saikat DebRoy, Deepayan Sarkar and the R Development Core Team (2013). `nlme`: Linear and Nonlinear Mixed Effects Models. R package version 3.1-111.) was constructed using efferocytosis as the response variable, all

correlated variables from the univariate analysis as the fixed effects and subject id as a random effect. The inclusion of the random effect “subject id” enabled the stochastic variation associated with the experimental technique to be separated from the stochastic variation between subjects, a process which was helped by including experimental replicates.

A backwards, stepwise, model simplification was performed to derive the minimum set of explanatory variables required to give a statistically acceptable fit. At each stage in the model simplification, parameters were removed if their association with efferocytosis was not statistically significant ($p > 0.05$). The contribution of individual parameters to the ‘fit’ of the model was assessed by likelihood (using ANOVA), equivalent to a likelihood ratio test between the model containing and the model without the parameter of interest. Once this model simplification was complete, interactions between the remaining explanatory variables were explored using ANOVA to compare a null model without interactions with a model including interactions. A residual analysis was then carried out to further assess the ‘fit’ of the model.

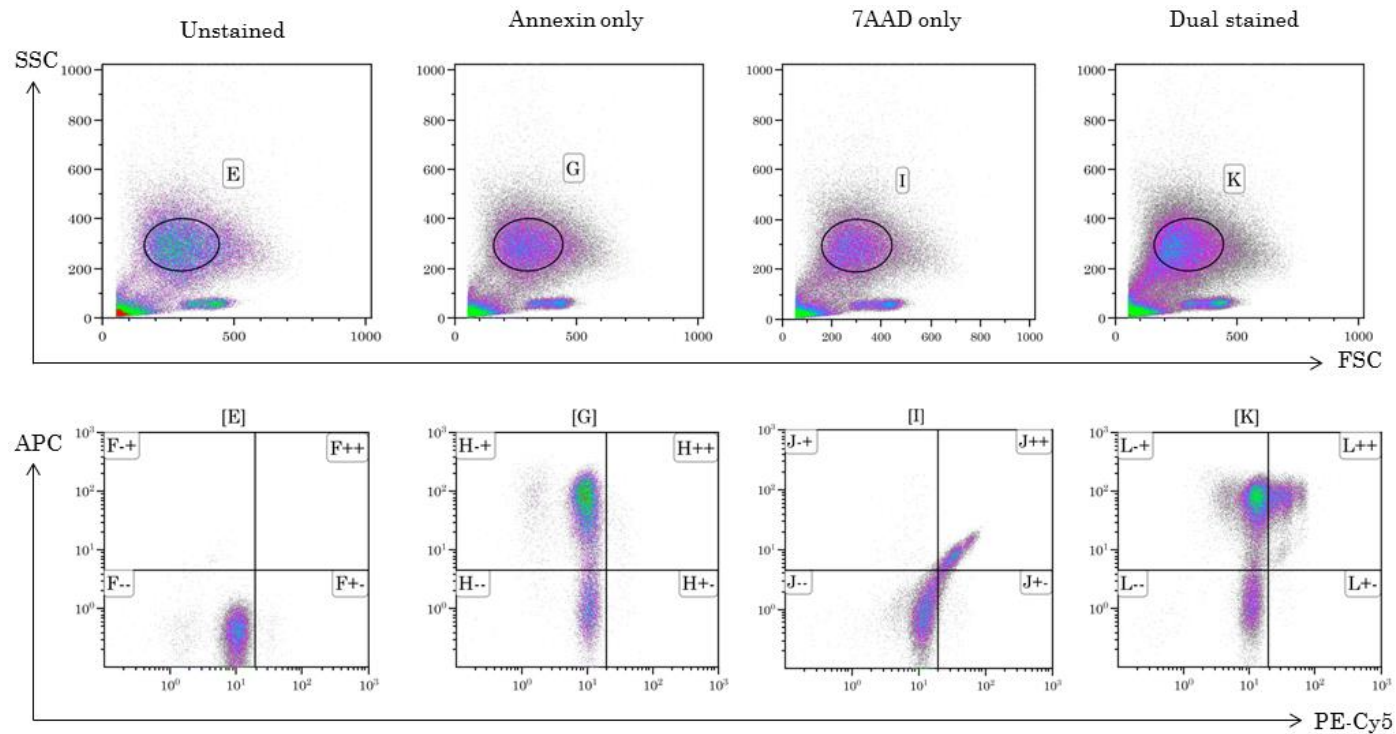


Figure 4.4 Flow cytometric measurement of neutrophil apoptosis

Top row – neutrophils from 4 tubes are identified by gates labelled with letters. Bottom row – displays neutrophils from the gates above and quadrants identify cells with different staining patterns. FSC=forward scatter SSC=side scatter.

[E] unstained (negative control) cells are placed in F--

[G] cells in early stage of apoptosis (annexin-APC only control) are seen in H-+

[I] late stage apoptotic cells (7AAD only control) are seen in J++

[k] Total apoptosis (dual stained) = sum of % cells that fall into L-+ and L++

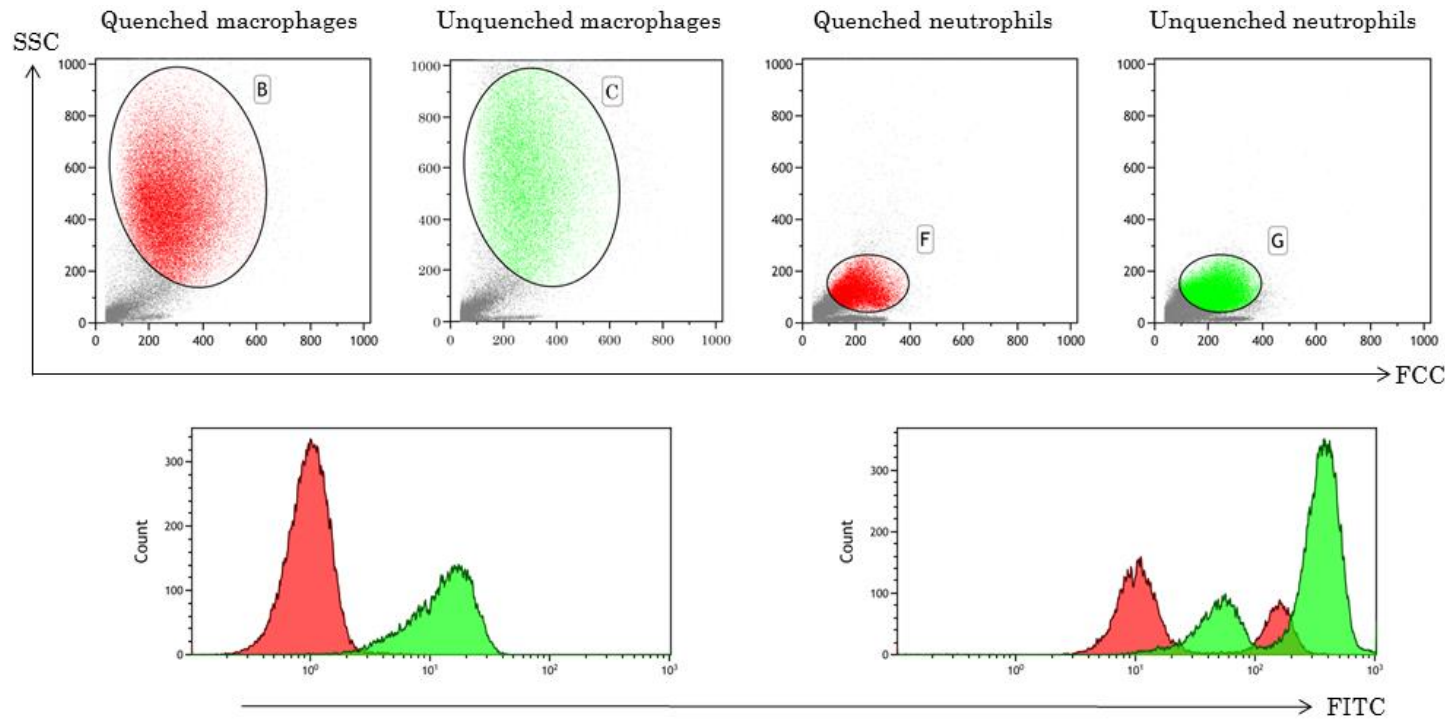


Figure 4.5 Effect of quenching on alveolar macrophages and neutrophils

Top left plots show the light scatter characteristics of two tubes alveolar macrophages from the same subject. The pair of plots top right show the light scatter characteristics of two tubes of green stained neutrophils from the same subject. The macrophages and neutrophils have been gated and those coloured red have been exposed to crystal violet and those coloured green have not. The plots on the bottom row display the FITC signal from (left plot) the gated macrophages and (right plot) the gated neutrophils. Exposure to crystal violet can be seen to reduce the FITC signal of the macrophages and neutrophils by approximately 1 log.

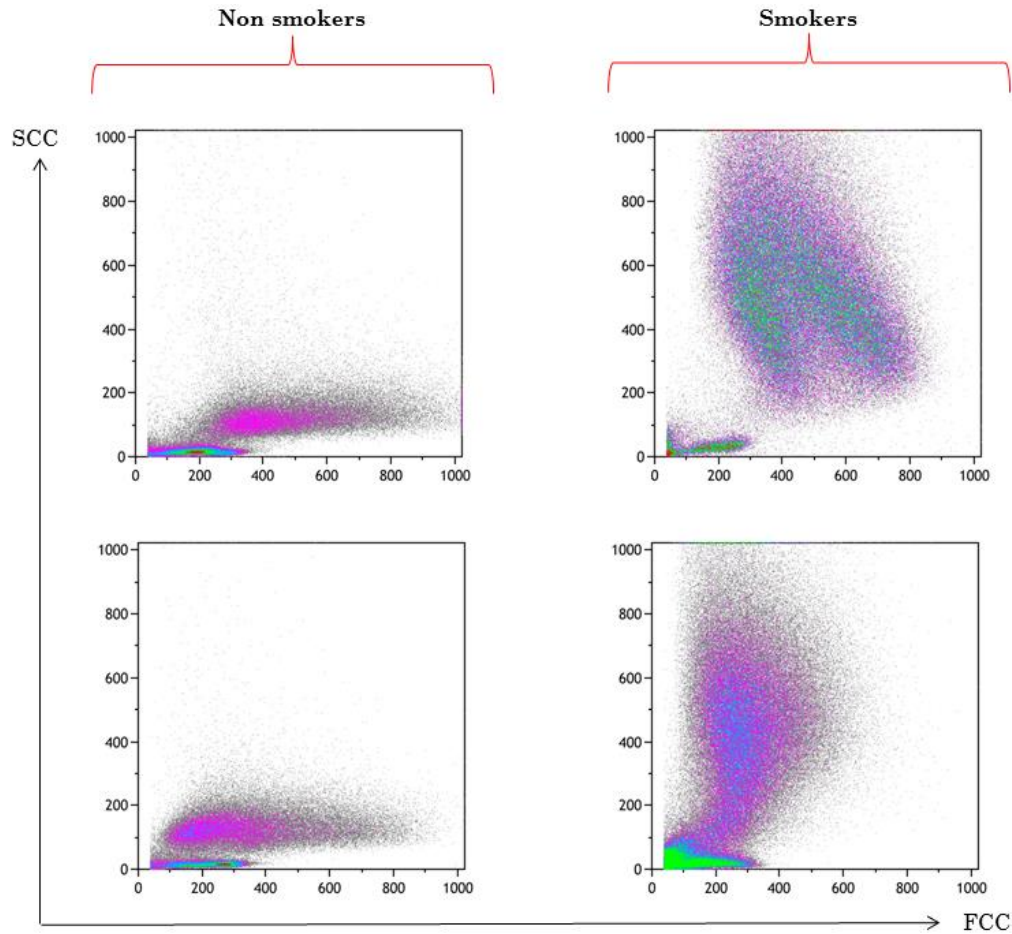


Figure 4.6 Comparative light-scatter properties of alveolar macrophages from smokers and non-smokers

Smokers tended to have significantly greater side scatter (SCC) than non-smokers. Laser light from the cytometer is side scattered by internal cellular components and is a surrogate for internal complexity. It is likely that particulates from smoking that have been taken up by phagocytosis lead to this increased SCC.

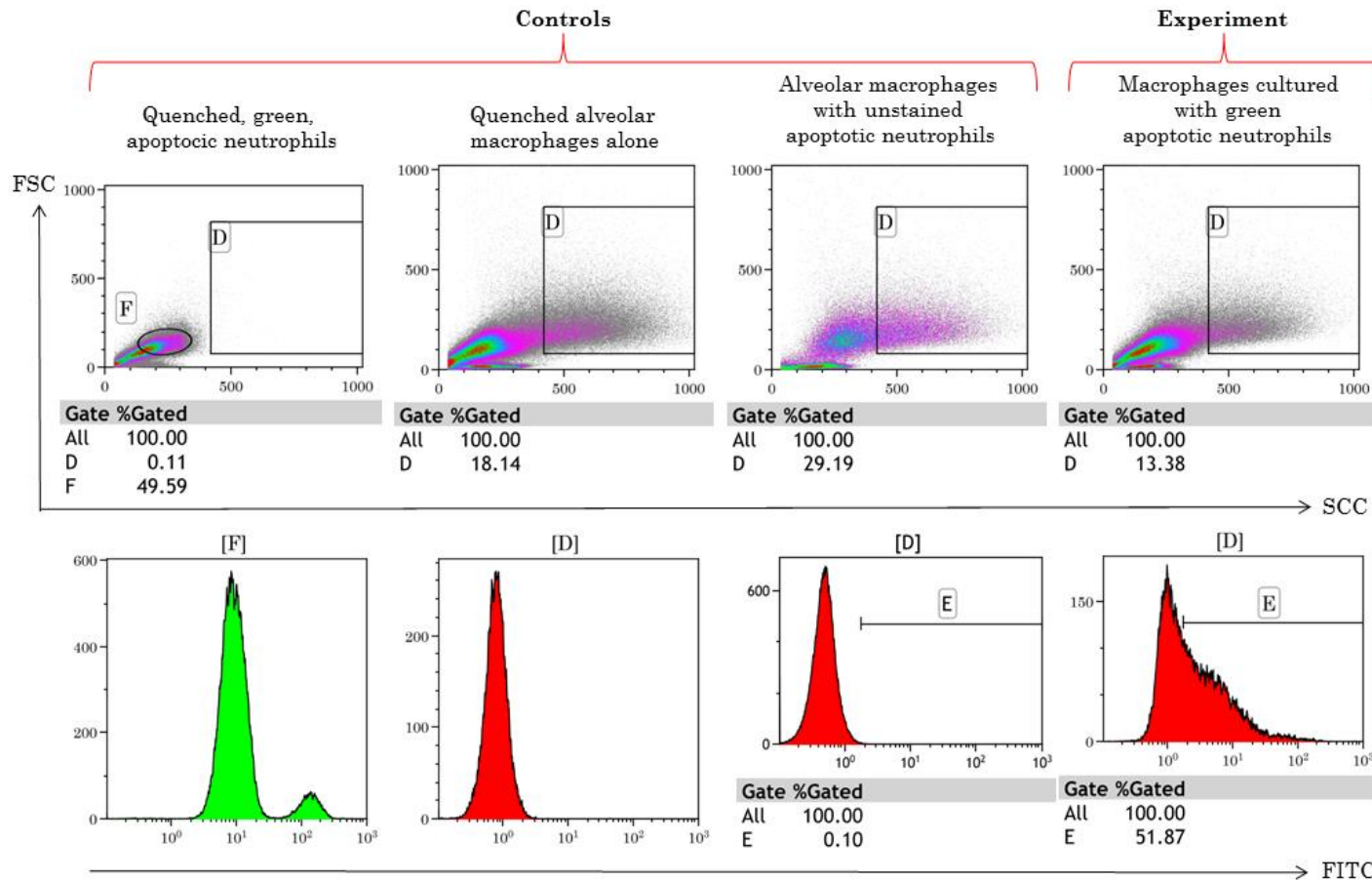


Figure 4.7 Flow cytometric measurement of efferocytosis

Cells in gate [F] are quenched, green stained neutrophils.

Gate [D] excludes all but 0.11% of neutrophils but includes alveolar macrophages.

Gate [E] excludes all but 0.1% of control macrophages.

51.87% of macrophages fall in gate [E] in the experimental cells. This increase in FITC signal (when compared to the control experiment) is attributed to the efferocytosis of green neutrophils.

4.5 Results

4.5.1 Subjects undergoing bronchoscopy

(See figure 4.8 flow chart). 112 of 169 subjects were seen at timepoint 4 (one month follow-up) and were assessed for their eligibility for bronchoscopy and bronchoalveolar lavage (BAL). 47 of those 112 assessed were eligible, of whom 15 declined the procedure. Of those who underwent bronchoscopy, most tolerated the procedure well and efferocytosis was analysed among alveolar macrophages derived from 22 subjects. The characteristics of those 22 subjects are compared to the complete PASS cohort in table 4.1. Compared to those who did not have a bronchoscopy the subjects contributing to this work were younger (median age 51 v 71 years $p < 0.0001$) and had a higher CRP (median CRP in bronchoscopy group 188 v 139 $p = 0.02$).

4.5.2 Efferocytosis

BAL produced a range of cell yields and some subjects generated enough alveolar macrophages for several replicates of the efferocytosis experiment: see table 4.2 for the raw data. Levels of apoptosis among neutrophils cultured for 20 hours varied between subjects. In order to control for the proportion and degree of apoptosis in each subject's neutrophils we quantified these parameters and included them as explanatory variables in the regression analysis. In both the univariate analysis and mixed effects modelling there was no association between the proportion of apoptotic neutrophils and rates of efferocytosis.

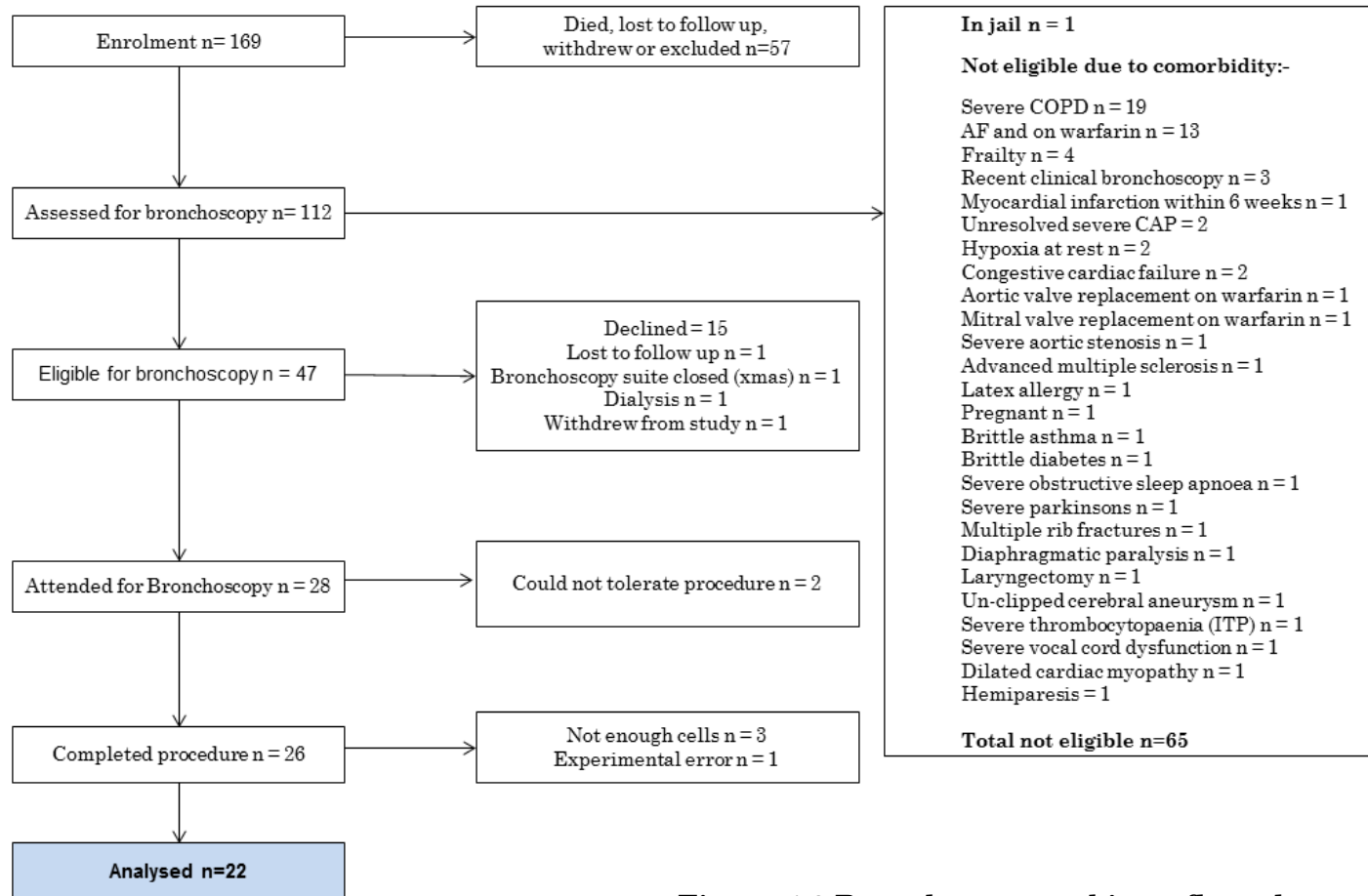


Figure 4.8 Bronchoscopy subjects flow-chart

Table 4.1 Clinical characteristics: bronchoscopy versus no bronchoscopy

Characteristic		Bronchoscopy Group n=22	No-Bronchoscopy Group n=147	<i>p</i>
Median Age, (IQR)		51(42-58)	71(56-79)	<0.0001 [#]
Male, n (%)		14(63.6)	74(50.3)	0.7*
Smoking, n (%)	Active	13(59.1)	50(36.0)	0.7*
	Quit	4(18.2)	62(44.6)	
	Never	5(22.7)	27(19.4)	
Modified Charlson comorbidity index	0	13(59.1)	43(29.3)	0.8*
	1	5(22.7)	64(43.5)	
	2	2(9.1)	16(10.9)	
	3	2(9.1)	15(10.2)	
	4	0	6(4.1)	
	5	0	2(1.4)	
	6	0	1(0.7)	
Prior statin use, n (%)		5(22.7)	52(35.6)	0.7*
COPD, n (%)		6(27.3)	67(45.6)	0.7*
CURB65, n (%)	0	14(63.6)	26(17.7)	0.7*
	1	2(9.1)	37(25.2)	
	2	5(22.7)	45(30.6)	
	3	1(4.5)	35(23.8)	
	4	0(0)	4(2.7)	
	5	0(0)	0(0)	
Presenting CRP, median (IQR)		188(135-316)	139(60-229)	0.02 [§]
BMI, median (IQR)		27(24-30)	24(22-31)	0.8 [§]
Flu, n (%)		5(23.8)	13(15.1)	0.7*
Pneumococcal bacteraemia n (%)		3(13.6)	5(3.6)	0.7*

[#] Wilcoxon rank sum test

* Chi squared test

[§] Welch's t test

Data was incomplete for the highlighted clinical variables, n for those groups is indicated below:-

Smoking status, no bronchoscopy group, n=139

CRP, no bronchoscopy group, n=144

BMI, no bronchoscopy group, n=109

Flu, bronchoscopy group, n=21; no bronchoscopy group, n=86

Pneumococcal bacteraemia, no bronchoscopy group, n=137

Prior statin use, no bronchoscopy group, n=146

Table 4.2 Raw efferocytosis assay data

Subject ID	Efferocytosis %	Subject ID	Efferocytosis %
42	39.8	4661	3.0
42	41.5	4661	2.5
1420	64.6	4690	18.9
1420	58.6	5048	45.7
1571	6.4	5048	44.0
2123	17.5	5432	9.7
2123	21.0	5432	7.9
2506	26.9	6391	58.6
2506	31.8	6391	58.3
2738	6.5	6391	70.9
2738	5.3	6391	61.8
2738	6.1	6391	54.8
2738	3.0	6391	53.2
2841	41.8	7094	31.7
2841	44.0	7201	36.2
3091	4.3	7201	40.6
3091	8.6	8902	7.8
3091	7.5	8902	4.7
3925	1.5	8902	7.6
3967	58.1	8902	7.7
3967	59.8	8914	11.6
3967	69.2	8914	13.6
4105	1.4	9961	6.8
4105	3.0	9961	8.2
4238	7.6		
4238	7.7		
4238	5.8		

Subjects are arranged by study id number in the shaded columns. Multiple efferocytosis values for a single id are experimental replicates. Highlighted values indicate outliers as identified in figure 3.16 and 3.17

4.5.3 Univariate analysis

Univariate analysis of the mean of replicates for each subject revealed four variables that were significantly ($P < 0.05$) correlated with efferocytosis: smoking status, prior statin use, BMI and gender (see table 4.3 and the figures 4.9 – 4.13). Three subjects presented at recruitment with consolidation which was reported to include the right middle lobe. Presence or absence of middle lobe consolidation was included as a variable in the univariate analysis of factors which may affect rates of efferocytosis and no statistically significant association was found (table 4.3).

4.5.4 Multi-variate analysis using a linear mixed effects model

Variables which were significantly associated with efferocytosis in univariate analysis were combined in a maximal linear mixed effects model. Those variables that were not significantly correlated with efferocytosis when combined were sequentially removed from the model. This backwards stepwise approach is explained further in table 4.4. The final model, containing only variables with statistically significant effects ($P < 0.05$), included the interaction of smoking status and prior statin use with adjustment for BMI.

4.5.5 Exploring the residuals to assess the fit of the model

From a plot of the residuals of the efferocytosis data against the fitted values from the model it was possible to detect a pattern with largest variance at large fitted value (figure 4.14). The above analysis was therefore re-run with log transformed efferocytosis values (figure 4.15). The residual plot from the log transformed

data showed largest variance at small fitted values. Several data points were identified as outliers and were removed (figures 4.16 and 4.17). In each case these represented anomalous experimental replicates from a set of replicates of an individual subject's efferocytosis experiment. The modelling was re-run with these replicates removed and the resulting residual plot was satisfactory – i.e. was consistent with a model which fitted the data (figure 4.18).

4.5.6 Final model characteristics

The final model that best explained this non-transformed data with outliers removed data was an interaction between smoking status and statin use adjusted for BMI. The model accounted for 72.0% of the variation in the data of which 83.1% was explained by inter-subject variation and 17.0% by intra-subject (experimental replicates) variation. The log likelihood of the model was -119.4. The parameter estimates for the interactions in this final model can be seen in table 4.5 and a graphical representation of the interaction of the 3 covariates can be seen in figure 4.19.

Table 4.3 Univariate analysis of covariates versus efferocytosis

Explanatory Variables	Regression coefficient	95% confidence interval	Adjusted R ²	<i>p</i>
Statin use	34.98	19.33 to 50.62	0.497	0.0001
Smoker	10.13	2.7 to 17.6	0.62	0.01
Ex-smoker	39.93	24.6 to 55.2		<0.0001
Never smoker	26.17	12.1 to 40.3		0.0009
Gender	19.7	2.3 to 37.12	0.18	0.03
BMI	1.77	0.116 to 3.43	0.167	0.037
Oral steroid use	-21.56	-47.26 to 4.14	0.09	0.096
Macrolide use	-21.03	-46.8 to 4.77	0.08	0.105
Admission CAP-sym score	-0.426	-1.1 to 0.25	0.03	0.203
COPD	-12.67	-33.10 to 7.76	0.03	0.21
Presenting aPTT waveform	-0.52	-1.39 to 0.35	0.03	0.227
Late apoptosis	-0.9	-2.49 to 0.69	0.019	0.25
Early apoptosis	-0.16	-0.55 to 0.23	-0.012	0.394
Combined apoptosis	-0.102	-0.44 to 0.24	-0.029	0.538
Pneumococcal blood culture positive	-0.42	-41 to 12.6	-0.03	0.57
Presenting pro-calcitonin	0.44	-1.17 to 2.05	-0.03	0.574
Inhaled steroid use	-4.85	-29.3 to 19.6	-0.041	0.683
Presenting CRP	-0.01	-0.08 to 0.06	-0.04	0.689
CURB65	1.15	-8.58 to 10.89	-0.047	0.81
Age	-0.03	-0.64 to 0.58	-0.05	0.9
Influenza infection	-1.15	-24.2 to 21.9	-0.05	0.93
Middle lobe consolidation	0.81	-26.8 to 28.4	-0.05	0.95

Mean % efferocytosis was calculated from replicate wells for each subject. Using linear modelling we performed a univariate analysis to determine which clinical covariates were associated with efferocytosis. Variables in the lower, shaded part of the table showed no statistically significant association with efferocytosis. Macrolide use refers to macrolides used in the treatment of the acute pneumonia.

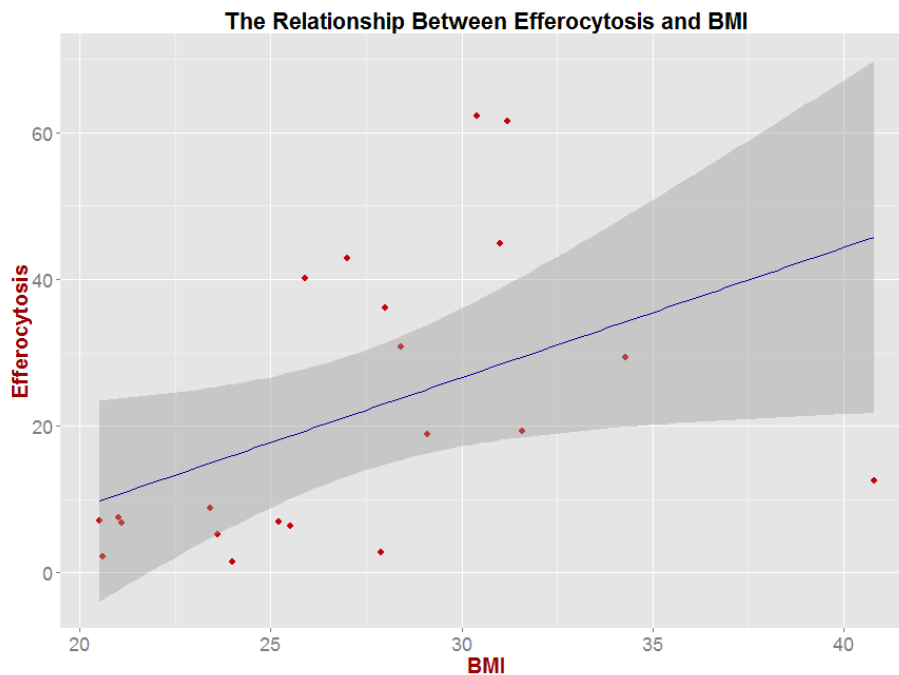


Figure 4.9 Relationship between BMI and efferocytosis



Figure 4.10 Relationship between age and efferocytosis

This page and next - representative plots of the univariate analysis of observed variables with efferocytosis. Fig.4.9 A positive association between BMI and efferocytosis ($p=0.036$). Blue line represents the fitted linear model with the shaded area showing the 95% confidence intervals. Fig. 4.10 No significant association was found between age and efferocytosis ($p=0.9$). Fig.4.11 Men had higher efferocytosis than women ($p=0.03$). Fig. 4.12 Smoking status was associated with efferocytosis ($p<0.01$). Fig.4.13 Statin use was associated with efferocytosis ($p=0.0001$).

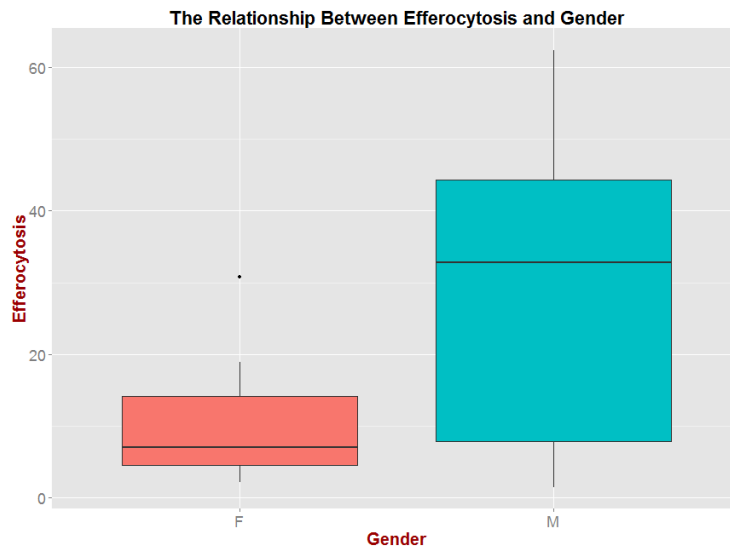


Figure 4.11 Relationship between gender and efferocytosis

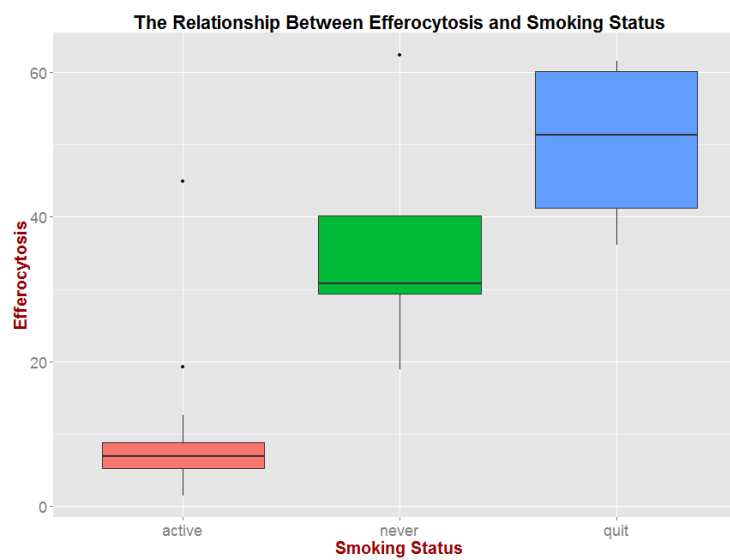


Figure 4.12 Relationship between smoking and efferocytosis

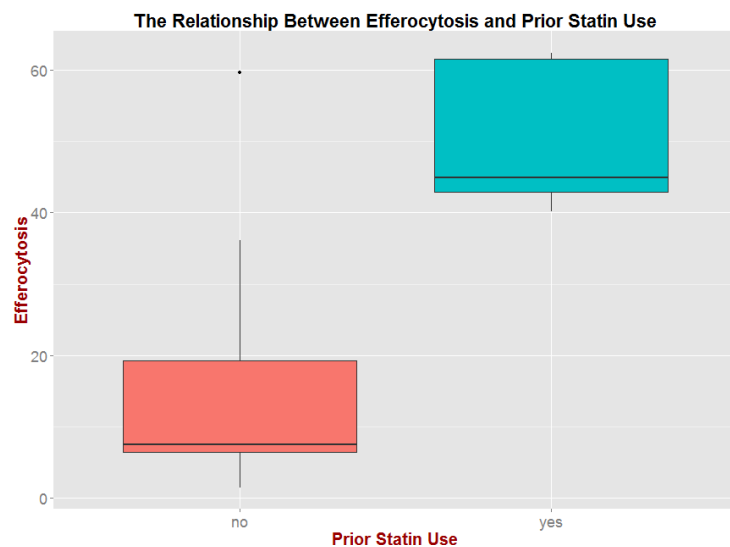


Figure 4.13 Relationship between statin use and efferocytosis

Table 4.4 Deriving the final model by backwards stepwise linear regression

Model	Parameters	Log Likelihood of Model	Comparison of Models	
1	Statin + Smoking + Gender + BMI + macrolide + flu	-120.9		
2	Statin + Smoking + Gender + BMI + flu	-121	Anova 1 v 2	P=0.75 (no difference – lose macrolide)
3	Statin + Smoking + BMI + flu	-122	Anova 2 v 3	P=0.16 (no difference – lose gender)
4	Statin + Smoking + BMI	-122.1	Anova 3 v 4	P=0.77 (no difference – lose flu)
5	Statin + BMI	-131.1	Anova 4 v 5	P=<0.0001 (favours 4 keep smoking)
6	Statin + Smoking	-124.2	Anova 4 v 6	P=0.04 (favours 4 keep BMI)
7	Smoking + BMI	-134.5	Anova 4 v 7	P=<0.0001 (favours 4 keep statin)
8	Statin * Smoking + BMI	-119.5	Anova 4 v 8	P=0.05 (favours 8 – keep interaction)

This regression was based on data from 20 of the 22 subjects. The two exclusions had missing values for some of the covariates of interest.

Model 1 is the maximal model including all the statistically significant covariates from the univariate analysis.

Model 2 has the term “macrolide” removed as it was least significant in model 1. When models 1 and 2 were compared using analysis of variance (anova), there was no statistically significant difference (i.e. $P > 0.05$) indicating “macrolide” makes little contribution.

The above process was repeated until the three variables smoking, statin and BMI remained; all three were shown to significantly increase the log likelihood of the model when included.

Model 8 includes an interaction (symbol *) between smoking and statin and this produced a significant increase in the log likelihood by comparison with model 4 (which had the same variables but no interactions).

Table 4.5 The parameter estimates from the final efferocytosis model

Final Model (model 8)	Interactions	Estimate	Standard error	Log likelihood of model
Statin * Smoking + BMI	Smoking active no statin	-7.9	7.4	-119.5
	Smoking active statin	26.4	10.5	
	Smoking never no statin	8.5	9.4	
	Smoking never statin	35.0	8.9	
	Ex-smoker no statin	21.8	9.9	
	Ex-smoker statin	35.0	9.21	
	BMI	0.6	0.3	

A linear mixed effects model took each of the six combinations of smoking status and statin use and summarised the relationship by one of six parallel lines. Each line had slope 0.6 and intercepts, as given in the “Estimate” column of the table, representing the estimated mean efferocytosis value for subjects in each interaction group. The precision of these estimates is indicated by the standard errors.

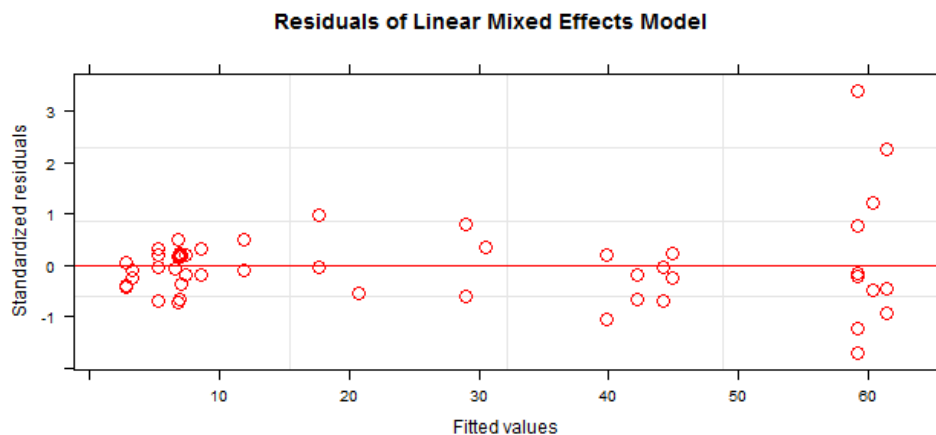


Figure 4.14 Residuals versus values fitted by the efferocytosis model

The residuals of the efferocytosis data plotted against the fitted values from the final LME model. The “horn-shaped” distribution (with the horn bell to the right) suggests this model is not a good fit for the data. This may be caused by the non-normal distribution of the efferocytosis data or by a number of outliers that are influencing the fit of the model.

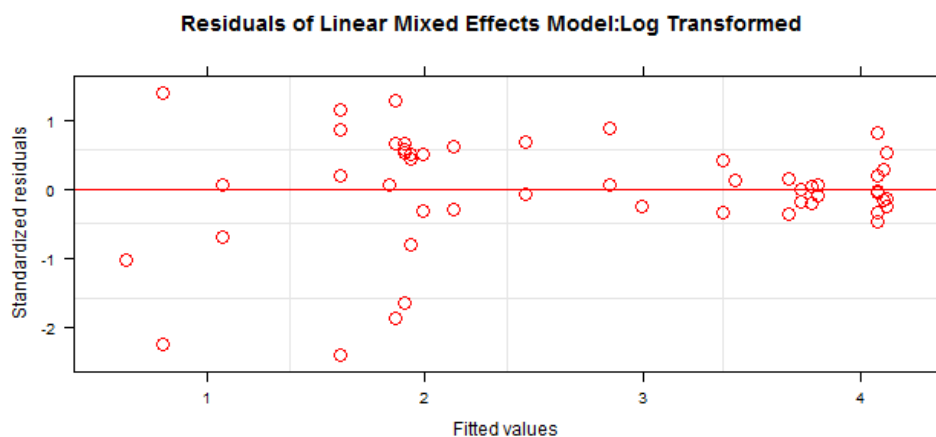


Figure 4.15 Residuals versus fitted values from modelling log transformed efferocytosis data

This is generated from the same model using log transformed efferocytosis values and residuals and shows a reversed pattern with increasing variance – this time at lower fitted values.

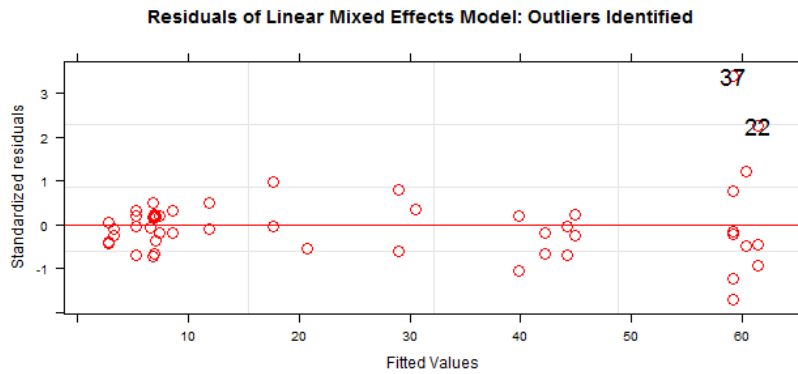


Figure 4.16 Two experimental replicates are identified as outliers

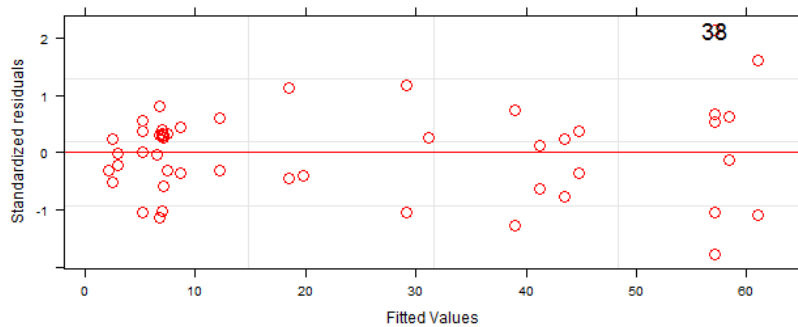


Figure 4.17 A further outlying experimental replicate

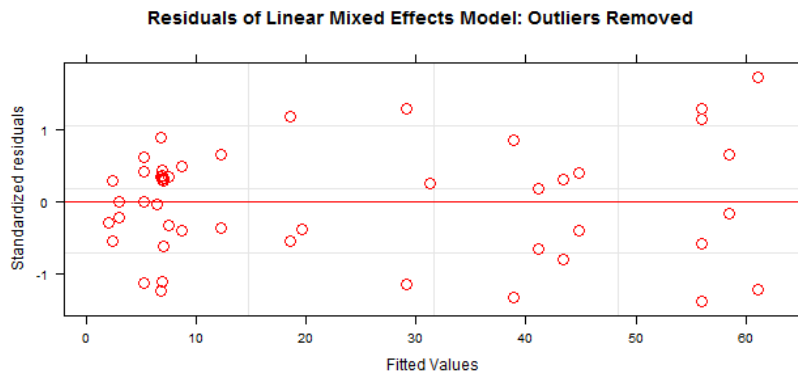


Figure 4.18 An acceptable residual plot following removal of outliers

Figure 4.16 is identical to plot 4.14 but this time two data-points have been identified as having a residual value outside of the 95% CI for all residuals. These points have been labelled with their row-names in the data-frame.

Figure 4.17 point 37 and 22 have been removed and repeated analysis reveals point 38 is an outlier in this new distribution.

Figure 4.18 with point 38 removed the analysis is re-run and no outlying residual values are found. This distribution of residuals now looks more random, indicative of a better model fit.

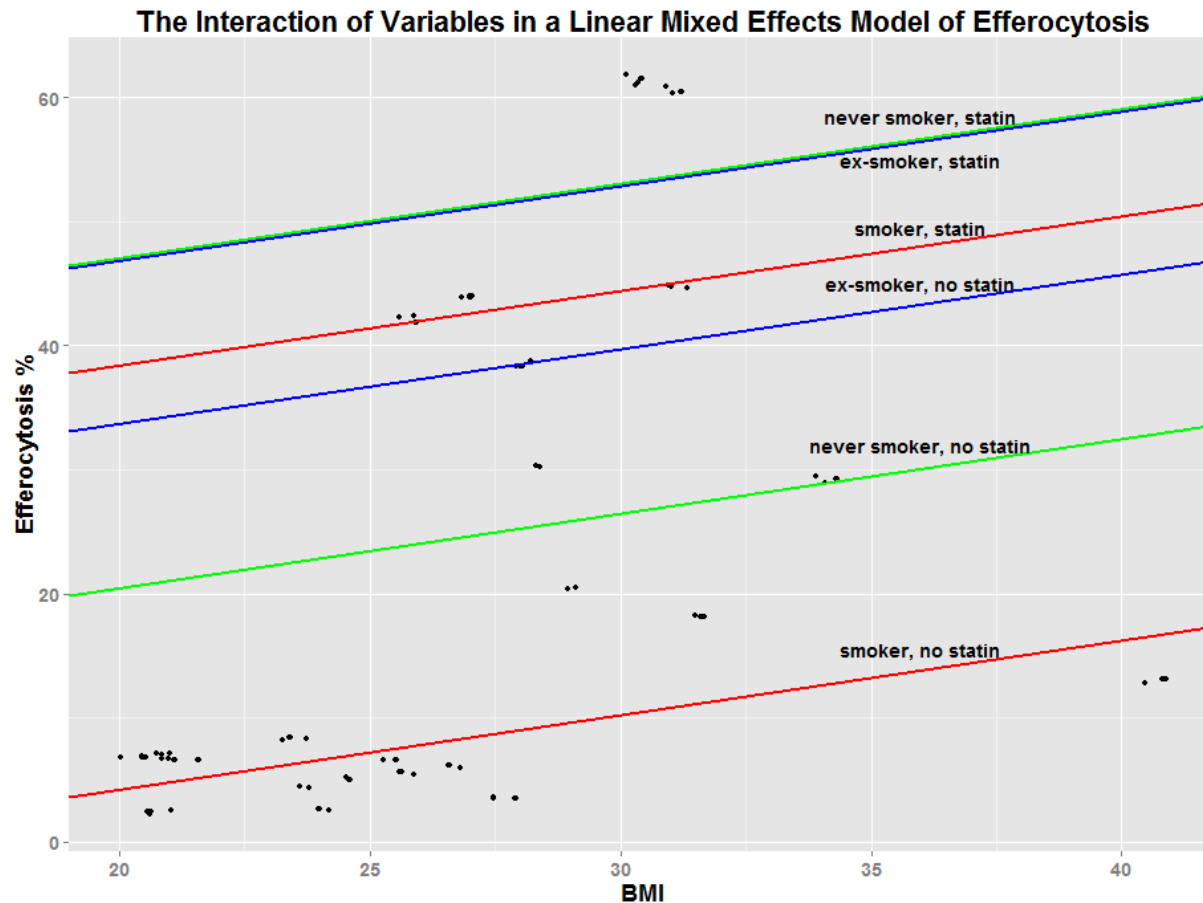


Figure 4.19 Graphical representation of the final linear mixed effects model of efferocytosis

The result of the linear regression was a model that included the interaction of smoking and statin with adjustment for BMI.

The efferocytosis values fitted by the model are on the Y axis with BMI on the x axis. Points are “jittered” to avoid overlap

The interactions of each level of smoking and statin are represented by 6 straight lines. These lines are displayed in colour pairs based on smoking status.

The y intercepts of each line are derived from the model as is the BMI dependent slope of 0.6.

From these lines it can be seen that without statins the differences between smoking status are large but with statins the differences are smaller. The magnitude of the statin effect is greatest in smokers.

4.6 Discussion

4.6.1 Summary of results

The results show that in this cohort of patients recovering from CAP, variation in efferocytosis was best explained by an interaction between smoking status and prior statin use with the model being adjusted for BMI which had a positive association with efferocytosis. Efferocytosis was negatively associated with smoking and positively associated with prior statin use and the effect of statin was maximal in those who smoked although this finding was not statistically significant.

4.6.2 What were the strengths of this work?

4.6.2.1 *Study design and cohort characteristics*

As described in chapter 3, the subjects enrolled in this study were carefully characterised. It is therefore possible to extrapolate these results to other study cohorts with similar characteristics and, with certain caveats (see below), to the generality of adult patients with CAP.

4.6.2.2 *Neutrophils*

The use of neutrophils as the apoptotic target in the efferocytosis assay is more pathophysiologically appropriate, in the context of CAP, than the immortalised T cell lines that are commonly used. Moreover, because we used autologous neutrophils we were able to control for the possibility that unknown 'self' receptors may play a role in the recognition of apoptotic cells by macrophages. There are various ways to derive apoptotic neutrophils and the most commonly used methods are induction by exposure to UV light or reagents such as corticosteroids.[149] The advantage of these techniques is that a consistently high proportion of a population of exposed neutrophils can be rendered apoptotic in a short period of time. However, both of these methods are artificial

and don't necessarily replicate the state of neutrophils in a lung recovering from an acute inflammatory insult. In vivo, due to their terminally differentiated nature, all neutrophils will naturally progress towards apoptosis but the rate at which they apoptose depends on many factors including how recently they have arrived in the lung, the local cytokine profile and whether or not they have encountered a pathogen.[99] This natural progression to apoptosis can be approximated ex-vivo by prolonged culture in media containing human serum, as was done here. Whilst this method of apoptotic induction is likely to be closer to the in vivo situation, patient factors determine that a varied proportion of apoptotic cells are produced. In order to control for the proportion and degree of apoptosis in each subject's neutrophils we quantified these apoptotic parameters in the cells offered for efferocytosis. Despite there being a wide range of apoptosis between subjects' neutrophils, in the univariate analysis there was no association between the proportion of apoptotic neutrophils and rates of efferocytosis. This finding may be a function of the 5:1 ratio of neutrophils to macrophages – even if a smaller proportion of neutrophils were apoptotic, each macrophage was never far from a potential target.

4.6.2.3 Replicates

From the BAL to the cytometer the alveolar macrophages were pipetted and washed 10 times and transferred between 5 pieces of plastic ware with each of these steps introducing the potential for laboratory variation between assays. However, in 18/22 subjects we had enough cells to run at least one replicate well of the efferocytosis experiment. This enabled an accurate assessment of the contribution of experimental variation to the final efferocytosis result by specifying 'subject id' (a surrogate here for

replicate) as a random effect in the mixed effect model. This has two benefits: - first we can confidently conclude that the effect of laboratory error on the variability of the efferocytosis data was minor (17.0% of data variation) in comparison to the inter-subject variation (83.1% of variation). Secondly, a detailed knowledge of the extent of assay variation gives us the ability to impute more accurate estimates of effect size and standard deviation for sample size calculations in future studies involving measures of efferocytosis.

4.6.2.4 Simplicity of the cytometry

Our flow-cytometric method of estimating efferocytosis was based on gating macrophages by their light scatter characteristics from a plot of all cells harvested from the efferocytosis well. This method has the advantage of requiring fewer preparatory steps than gating strategies based on staining surface markers and enabled us to study fresh, unfixed, unstained macrophages. As well as adding steps to an already involved SOP, immunologically labelling cell surface markers can activate cell processes that may affect efferocytosis and by avoiding this we can remove a source of misinterpretation.

4.6.3 What were the limitations of this work?

4.6.3.1 Study design and cohort characteristics

The design of the Pneumonia Aetiology and Severity Study (PASS) imposed certain limitations on the degree to which the results of these experiments can be extrapolated. PASS screened thousands of acute admissions across two hospitals to recruit 169 patients with pneumonia (see chapter 2 more a more in depth discussion of PASS). Primarily because of ineligibility due to co-

morbidity, only 22 patients contributed cells to the efferocytosis study. Of these only 5 were never smokers and of the smokers all but one was not taking a statin. Therefore analysis of sub-groups had no statistical power. It would be interesting to see which factors - age, BMI, statin and macrolide use for example - influence efferocytosis in a larger group of non-smokers and whether the trends seen here with statin use are replicated in larger cohorts.

The majority of patients with CAP in the UK and similar developed nations are treated in primary care. Since PASS sampled a population admitted to hospital we do not know which factors most influence efferocytosis in CAP patients treated in the community. In our analysis smoking had a dominant effect on efferocytosis, even when adjusted for factors such as and COPD, steroid use and initial CAP severity, factors which could be expected to be more influential in a hospital population than in primary care. Given this, it may be that smoking has a more dominant effect in a community population with fewer and less severe comorbidities and milder CAP. PASS also excluded the most severe CAP cases that were unable to complete the CAP-sym questionnaire due to being moribund or ventilated on critical care. It may be that effects of the intubation, ventilation and severe sepsis dominate in that group and that smoking and statins are relatively less influential.

All subjects who contributed to the efferocytosis data were caucasian apart from one – a distribution that reflects the population served by our hospitals. It is therefore not possible to extrapolate these results to the rest of the world or indeed many areas of the UK where racial factors may be influential.

4.6.3.2 Timing and longitudinal effects

We chose one month following admission from hospital for the timing of our efferocytosis experiment since this was felt to best balance, on the one hand, the desire to capture the recovery phase of CAP, and on the other the risks associated with bronchoscopy in unstable patients. It was clear to the author that earlier, awake, research bronchoscopy and BAL would be unsafe and poorly tolerated in many patients recovering from CAP. Restrictive spirometry and cough were a very common finding at discharge and would have led to poor cell yields even in those who could safely complete the procedure.

Three of the subjects were recovering from CAP which had been radiologically reported as involving the right middle lobe. Since we consistently lavaged the middle lobe it is possible that the recent local inflammation of that lobe may have affected rates of efferocytosis in those three patients when compared to those whose inflammation was radiologically elsewhere. Any difference associated with middle lobe consolidation was small as no statistically significant difference was detected in the univariate analysis. Two possible interpretations of this are that despite the radiological appearances of lobar disease the CAP was in fact widespread leading to relative similarity between middle lobe and non-middle lobe pneumonias with respect to efferocytosis. An alternative explanation is that any differences relating to lobe of CAP had resolved and we were in fact measuring efferocytosis in stable state. Further studies of a different design would be required to tease these possibilities apart.

To confidently conclude that we were assessing efferocytosis under the influence of recovery – rather than in stable state, it

would be necessary to perform repeat bronchoscopies on each subject. This was an ambition for this study but only 2 subjects successfully contributed paired efferocytosis data and an analysis of these was not attempted. What we have learnt is that a much larger cohort would be needed to capture enough subjects willing and able to accept repeat bronchoscopy.

4.6.4 Comparison with other published work

4.6.4.1 *Mechanistic studies of cigarette smoke on efferocytosis*

A number of studies have investigated the mechanisms by which cigarette smoke leads to impairment in efferocytosis. Hodge et al. demonstrated that smoking has a detrimental effect on the efferocytosis of apoptotic alveolar epithelial cells (immortalised cell line) by alveolar macrophages. [150] The effect was greatest in smokers with established COPD, but was significant even in smokers who were otherwise healthy. The effect of smoking was at least partly reversible. Efferocytosis improved when those with COPD stopped smoking reaching higher levels than 'healthy' active smokers but did not return to the levels of never smoking controls. Kirkham et al showed that monocyte derived macrophages had a greater propensity to adhere to surfaces coated in extracellular matrix proteins that had been exposed to cigarette smoke extract (CSE). [151] Reactive carbonyl groups in CSE led to modification of these proteins and macrophages that were adherent to them showed reduced efferocytosis of apoptotic autologous neutrophils. More recently Minematsu et al. have shown that exposure to CSE leads to impairment of RAC1 and consequently impairment of the cytoskeletal rearrangements required for phagocytosis – including efferocytosis. [152] Noda et

al studied the efferocytosis of apoptotic human neutrophils by mouse alveolar macrophages. They found that pre-treatment of macrophages with cigarette smoke extract (CSE) impaired the function of the enzyme histone deacetylase (HDAC) a consequence of which was inhibition of RAC and impairment of efferocytosis.[153] More recently Petrusca et al have shown that cigarette smoke inhibits the enzyme ACDase which catalyses the conversion of ceramide to sphingosine. This results in an increase in sphingosine which in turn has an inhibitory effect on HDAC6 and reduced efferocytosis. [154] In summary, cigarette smoke affects several molecular pathways that lead to the activation and membrane localisation of a key enzyme RAC – the function of which is to facilitate the cytoskeletal rearrangements needed for efferocytosis. RAC is a member of the family Rho GTPases and the finding that cigarette smoke has an effect on these is intriguing in the light of the results of this study since, as will be seen below, one of the ‘off-line’ effects of statins is their effect on the balance of Rho-GTPases.[155]

4.6.4.2 Statin effects on efferocytosis

Statins are reversible inhibitors of the enzyme HMG-CoA reductase which is involved in cholesterol biosynthesis in the liver. They are extensively prescribed to reduce LDL-cholesterol (LDL) with the aim of cardiovascular disease risk modification. However, statins have many “off-line” effects, i.e. those beyond their desired primary pharmacologically intended effect.[156] Some of these effects are thought to be responsible for highly beneficial effects beyond their lipid lowering potential. The JUPITER study demonstrated that, in 17802 subjects with normal LDL but raised CRP, rosuvastatin led to a significant

reduction in cardiovascular events, suggesting an anti-inflammatory role.[157]

One statin effect is the inhibition of isoprenylation which is a key mechanism in the post translational modification of many proteins including the Rho GTPase family. Exposure to statins inhibits the isoprenylation of RAC and RhoA leading their inactive forms to sequester in the cytosol.[156] RAC facilitates efferocytosis and RhoA is inhibitory. [158] This has led several investigators to study the effects of statins on efferocytosis. Morimoto et al found that lovastatin inhibited the membrane localisation of RhoA and RAC1. The effect was greater on RhoA and the balance of these two antagonistic enzymes was such that efferocytosis of apoptotic neutrophils was potentiated. [102]

Taken together the studies above suggest that smoking reduces efferocytosis by the impairment of RAC1 whereas statins alter the membrane balance of RhoA and RAC1 in such a way as to increase efferocytosis. This provides a possible mechanistic explanation for the findings of our study – that smoking impairs efferocytosis but statins can moderate the effect.

4.6.4.3 BMI and lung disease and efferocytosis

Several studies have demonstrated strong associations between BMI and risk of CAP. Phung et al reviewed the relationship between BMI and risk of developing CAP and found that those who were underweight had an 80% increase in their risk of developing CAP whereas those who were overweight had an 11% reduction in risk. The risk increased again, though not statistically significantly, in those categorised as obese.[159]

Our results suggested a statistically significant association between BMI and efferocytosis such that as BMI increased so did efferocytosis and this association has not been shown previously. If increased efferocytosis is regarded as beneficial then one would expect increased BMI to be associated with better outcome in CAP. The literature supports this with several studies consistently demonstrating reduced mortality in those with high BMIs. Kalon et al compared mortality between those who were under weight, normal weight, overweight or obese. They demonstrated a statistically significant reduction in mortality, in the obese patients (BMI>30) compared to those with normal weight and this was not replicated in those who were overweight or underweight.[160]

It may be that differential rates of efferocytosis are in part responsible for the association between BMI, risk and outcome in CAP. Studies have suggested a role for the adipokine adiponectin in modulating inflammatory responses in acute and chronic inflammatory conditions including those of the lung.[161] Some of these studies demonstrate direct effects of adiponectin on innate immune cells and a possible mechanistic link between adiponectin levels and efferocytosis may be worth exploring. However, in other inflammatory lung diseases the association between efferocytosis and BMI is negative and it is clear the interactions are complex.[162]

4.6.5 Implications

4.6.5.1 Future assay development

It is now clear that alveolar macrophages have distinct immunological properties that distinguish them from

macrophages resident at other anatomical sites, and that their phenotype is plastic. We did not attempt a phenotypic examination of our cells but it would be fascinating to explore the initial phenotype recovered from the subjects and to relate this to the potential for efferocytosis and to patient related factors – in particular the effect of prior statins and steroid use on phenotype.

In the literature and by personal communication with experts we were unable to find a single reagent or simple panel of reagents that could reliably separate human alveolar macrophages from autologous apoptotic neutrophils. CD16b is said to be a specific marker of neutrophil granulocytes but is lost during apoptosis. A combination of CD14 and 16 have been used to distinguish the two cell types but some cells in both populations express both. It was beyond the scope of this study to enter into a systematic validation of new surface marker combinations and our solution was to use the distinct size and granularity differences between the populations to identify a gate that excluded the vast majority of neutrophils while capturing macrophages. Light scatter has been used in this way in previous studies [148], however this strategy excludes a proportion of macrophages whose light scatter overlaps with that of the neutrophils and we were therefore not able to assess efferocytosis in this population. A future project would be to identify a panel of reagents that would enable the two cell types to be sorted enabling the efferocytotic potential of the whole macrophage population to be assessed.

4.6.5.2 Clinical implications

On the basis of this data, subjects with low BMI have lower rates of efferocytosis and could therefore be expected to recover more slowly and may benefit from enhanced follow-up and, in the

future, from pro-recovery therapy in the form of dietetic advice and supplementation, pro-resolution medication and or physical rehabilitation. This association has not been proven by this study but the data would suggest it should be studied in future observational studies

The detrimental effects of smoking are well known and epidemiological studies have demonstrated that up to 50% of pneumococcal pneumonia cases in smokers can be attributed to smoking [22]. Clearly prevention by promoting smoking cessation is the best strategy but in order to develop interventions to improve recovery from CAP the mechanisms involved must be elucidated. This is reinforced by evidence which suggest that although some BAL parameters improve within the first month following smoking cessation, the alveolar macrophage cytometric abnormalities described in figure 4.6 remain for at least 15 months.[163] This implies that even if patients were to give up smoking at the time of developing pneumonia and remain abstinent throughout their recovery their alveolar macrophage function may remain abnormal. The demonstration of a link between smoking, statins and efferocytosis provides a potential target for trials of treatment. In chapter 5 the effect of efferocytosis on outcomes following CAP will be explored.

The effect of statins in the context of patients with CAP is controversial. The proposed benefits can be split into reduced risk of developing pneumonia [164,165] and improved outcome subsequent to the onset of pneumonia [166,167]. From a therapeutic perspective it is not clear if any potential benefit is seen acutely [168] or whether the effects are mediated during the convalescent phase [169]. Moreover, there is significant debate in

the literature as to what extent all these potential benefits are due to confounding as a result of the healthy user effect [170,171]. What is clear is that statins do have a range of measurable immune-modulatory effects. Murine pneumonia models have shown that, when fed to mice, statins have a wide range of effects such as reduced consolidation due to reduced neutrophil migration – but the overall impact on mortality is small [172]. All authors are consistent in suggesting that the only way to solve the question of whether statin effects are beneficial in pneumonia is to conduct randomised control trials. It will be vital that these trials are designed to take into account the available mechanistic data. Subjects will vary in their response to statins and trials should be designed to test for effects in those most likely to derive benefit and from the evidence of this study that would be smokers.

5 WHICH PATIENT FACTORS ARE ASSOCIATED WITH SPUTUM MICROBIOTA IN CAP?

5.1 Introduction

The concept of a bacterial microbiome and contemporary work on describing this in the lower airway was discussed in chapter 1. An analysis of the bacterial microbiota of sputum from patients with community acquired pneumonia (CAP) has only been published once previously in a cohort of 45 patients recruited in China.[173] That work focussed on defining bacterial community differences between health, CAP and Hospital Acquired Pneumonia (HAP). Those distinctions are important but in addition it would be useful to determine if the range and relative abundance of various bacteria differs in association with other clinical features. Understanding these differences may give an insight into the biology of pneumonia but may also have clinical implications for the future. The ability to refine future empirical antibiotic therapy based on how clinical factors influence the bacterial patterns in CAP may lead to improved outcomes. We therefore conducted an analysis of the bacterial microbiota of sputum collected from patient in the Pneumonia Aetiology and Severity Study (PASS) and related the microbiota to clinical variables.

5.2 Hypotheses

- a) The bacterial community composition of sputum from patients with CAP is influenced by pre-existing patient characteristics.
- b) The abundance of individual bacterial taxa in sputum from patients with CAP is associated with pre-existing clinical characteristics.

5.3 Methods

5.3.1 Subjects and samples

The study team collected sputum samples from subjects who were able to self-expectorate at the time of enrolment into the Pneumonia Aetiology and Severity Study (PASS). Sputum was collected into security sealed, gamma irradiated pots and within one hour of collection these were frozen at -80°C without the addition of any additives or processing. These samples were independent of those taken at the discretion of the clinical teams for clinical microbiological investigation.

5.3.2 DNA extraction

Sputum samples were defrosted in batches of 24 or 48 and kept on wet water ice. DNA extraction was performed using the proprietary 'FastDNA™ SPIN Kit for Soil' from MPbio. The kits were used in accordance with the manufacturer's instructions and using a standard operating procedure optimised by the National Heart Lung Institute (NHLI). In brief: - 300 μL of mucopurulent sputum was added to lysing matrix tubes which were pre-filled with buffers to protect and solubilise DNA following cell lysis. Cell lysis was achieved using an MPbio FastPrep®-24 bench-top homogeniser (figure 5.1). The resulting lysate underwent prolonged high-speed centrifugation to pellet the lysing beads and cell debris. The supernatant was mixed with protein precipitation solution and pelleted. The DNA containing supernatant was removed from the protein pellet, added to a suspension of DNA binding matrix, and left to adhere. The binding matrix was placed in a tube containing a fine filter and centrifuged. The liquid component passed through the filter leaving the DNA laden matrix above the filter. Finally the DNA was eluted from the

binding matrix by re-suspending the matrix in 100 μ L DNAase free water and centrifuging. The matrix remained above the filter and the 100 μ L of concentrated DNA solution was removed from the bottom of the spin-tube and stored at -80°C.

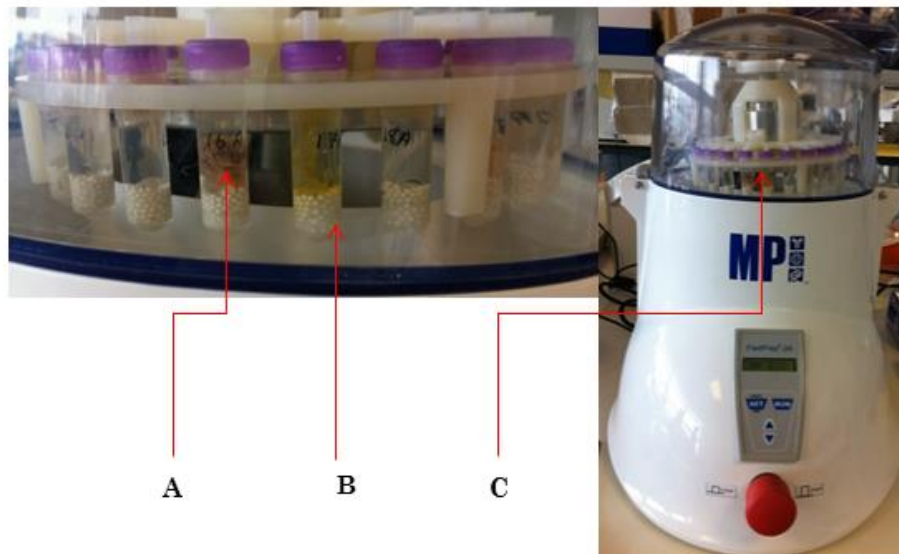


Figure 5.1 Lysing sputum to obtain DNA

A) An aliquot of each subject's sputum is placed in a pre-labelled lysing matrix tube along with stabilisation buffers.

B) Optimised mix of ceramic and silica beads of various sizes mechanically degrade cell walls when sample is agitated.

C) 24 samples are clamped in place in the carousel of a 'bead beater'. Samples are homogenised by rapid, multi-directional movements which lead to bead impaction and cell lysis.

5.3.3 16S rRNA PCR

To identify which bacterial taxa were present in the sputum samples we amplified the V3-V5 regions of the 16S rRNA gene (see section 1.6.3 for background). PCR preparations were made in a dedicated PCR hood in a dedicated room in which no DNA template was stored. We used a 25µl PCR based on a standard operating procedure (SOP) that had been optimised by the department of Genomic Medicine at the NHLI. The SOP included a proprietary DNA polymerase master-mix from New England BioLabs Inc. The following reagents were mixed to create a stock solution and then 23µl was added, to each well of a 96 well PCR reaction plate:-

- Q5[®] master High Fidelity Master Mix containing:
 - DNA polymerase,
 - a source of Mg⁺⁺,
 - a proprietary buffer
 - deoxynucleotides
- High purity molecular grade water
- 16S rRNA V3-5 forward primer
5'CCGTCAATTYMTTTRAGT3'

Next 1µl of “bar-coded” reverse primer 5'CCTACGGGAGGCAGCAG3' was added to each well – the same primer with a different bar code for each well.[174] The use of a bar-coded primers meant the 16S rRNA amplicons generated by the PCR reaction would include in their sequence a short nucleotide string (analogous to a bar code) which was unique to that sample. This bar-code would eventually allow amplicons derived from individual samples to be identified (by their bar

code) from a mixture of many samples. Lastly, 1µl of template DNA (DNA derived from each sputum sample to act as a template for replication during the PCR reaction) was added to 94 of the 96 wells. The 95th well had no template and acted as a negative control and the 96th well had 1µl of template DNA derived from a pure culture of *Vibrio natriegens*, a halophile (salt loving) bacteria first isolated from salt marshes and not known or suspected of being present in the lower airway. The PCR plate was then sealed and briefly centrifuged before being placed on the thermal cycler. Reaction steps for the 16S rRNA PCRs were:-

95°C for 2 minutes then 35 cycles of :-

95°C for 20 seconds

50°C for 30 seconds

72°C for 5 minutes

For each sputum derived DNA sample, the above was repeated 4 times i.e. for each sample we conducted four separate but identical PCR reactions in four separate plates.

5.3.4 Quality control of PCRs

When each PCR plate had completed its thermal cycling we took aliquots from a sub-set of the samples (one row) and performed an agarose gel electrophoresis (figure 5.2). This quality control step checked for the presence of a DNA band of an approximate weight equivalent to that of the 16S rRNA gene in the positive control i.e. the PCR had been successful. It checked for an absence of a band in the negative control – i.e. the PCR reagents were not grossly contaminated with bacterial DNA. And finally it checked that at least some of the samples had positive bands – i.e. the quantity of template DNA had generated an adequate amplicon level to take forward to sequencing.

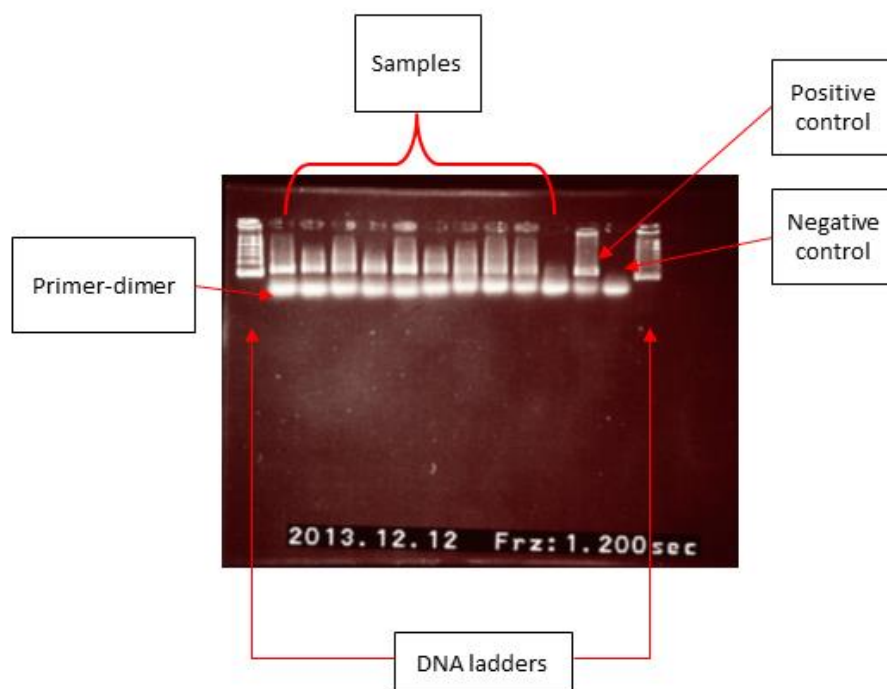


Figure 5.2 Agarose Gel Electrophoresis of a 16Sr RNA PCR

DNA is stained with a fluorescent intercalating nucleic acid stain called 'Gel Red' the fluorescent intensity of which increases when bound to DNA. Images are captured using a UV illuminator.

5.3.5 Pooling PCR products

Once the four, replicate PCR plates were completed and had passed the ‘gel’ quality control steps, the contents of all four plates were carefully transferred to the corresponding well of a fresh 96 well plate. This process of pooling four separate but identical PCRs has several advantages. It increases the amount of template DNA that is sampled, and by doing so increases the likelihood that bacterial species present at low concentration in the original sputum sample will be detected. Pooling has also been thought to be beneficial as each PCR, though prepared identically, will in fact yield subtle, but important *qualitative* differences in the spectrum of amplicons produced and by repeating the PCR four times, the full spectrum of possible products is realised. Recent evidence suggests that of the two arguments for pooling, increasing the absolute amount of template is probably the most important.[175]

5.3.6 Quantification of PCR products

The absolute quantity of double stranded DNA (dsDNA) amplicons in each well containing the products of four pooled PCRs was measured using a kit called Quant-iT™ (Invitrogen) according to the manufacturer’s protocol. This assay uses a proprietary, fluorescent DNA stain called PicoGreen®. This reagent has a strong selectivity for dsDNA and the degree of fluorescence in a sample is therefore directly proportional to the concentration of dsDNA.

5.3.7 Pooling all samples for multiplex-sequencing

Next we created a final “pool” containing amplicons derived from all the original sputum samples. Each sample needed to contribute equimolar concentrations of amplicon. The

concentration of dsDNA amplicons in each well of the plate containing pooled PCR products was known from the Quant-iT™ assay; therefore a calculation determined the aliquot volume of each well to be transferred to a single DNA free tube.

5.3.8 Purification prior to sequencing

Each of the completed PCR reaction wells above will have contained unused deoxyribonucleotides (dNTPs), primers, and primer-dimers (see figure 5.2). These unwanted residual nucleic acids will have been transferred through to the final pool of samples. To remove these from our final pool we used a kit called Agencourt® AMPure® XP. This size-selection purification assay involves creating a suspension of magnetic beads within the pooled PCR solution. The beads selectively bind dsDNA above 100 base pairs in length, leaving the rest in solution. By placing the reaction tube next to a strong magnet the beads are drawn into a pellet on the side of the tube allowing the contaminated solution to be carefully pipetted away. The tube is removed from the magnet and the DNA loaded beads washed with 70% ethanol. The tube is replaced near the magnet and the beads re-pelleted allowing the ethanol and dissolved contaminants to be removed. Finally the DNA is eluted off the beads, the beads re-pelleted and the purified dsDNA amplicon containing solution removed.

5.3.9 Quantification of the final amplicon pool

The final step in preparing a multiplexed pool of amplicons to take forward to sequencing is to quantify the concentration of dsDNA in the pool. This is done using the Quant-iT™ kit as in section 5.3.6 above.

5.3.10 FLX-454 sequencing of 16S-rRNA gene

An aliquot was taken of the sample pool. Adapter sequences were attached to each DNA fragment and the double stranded fragments split into single strands. These single stranded DNA library fragments were mixed with oil, capture beads and a water-based solution of PCR reagents. Mixing and shaking took place and each hydrophilic bead became surrounded by a droplet of the PCR mixture. Each of these droplets itself was suspended in an envelope of oil. Each oil droplet therefore became an oil-isolated 'reaction vessel' capable of carrying out a PCR of the bead-bound DNA library contained within. Thermal cycling took place and at the end of this process the bead within each droplet of oil was loaded with millions of 'clonal' copies of its original DNA sequence.

These beads were then sunk, one bead per well, into the wells of a PicoTitrePlate (PTP) by centrifuging the plate. In the wells with the DNA library beads were smaller polymerase coated beads capable of facilitating the generation of complementary strands of DNA against the single stranded DNA templates.

Nucleotides were washed across the plate in sequence multiple times. As each nucleotide-mix fell into a PTP reaction well it was added to the template DNA at the complementary position(s). The insertion of a complementary nucleotide triggered a luciferase mediated reaction generating light. Light was detected on a silicon sensor with the quantity of light being proportional to the number of nucleotides added; i.e. the addition of two adjacent TTs created more light than one T.

The read out of the sequencer was thus a list of sequential light intensity measurements occurring in relation to the flow of

particular nucleotides across the plate and corresponding to a yes/no for the addition of that nucleotide - and if 'yes' how many iterations for that nucleotide were added. From this 'flow-gram' the nucleotide sequence of the DNA attached to the bead within an individual well was computationally inferred. Quality information relating to each nucleotide in the sequence was incorporated in the output so that sequences based on lower quality detection could be identified and discarded.

5.3.11 Curating the raw sequence Data

Pre-processing of the raw sequencing data was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline for analysis.[176] Standard flowgram format (.sff) files derived from the Roche-FLX sequencer were provided from the DNA Sequencing Facility of the University of Cambridge. sff files were then used as the input for the QIIME analysis pipeline. Within QIIME, the programme AmpliconNoise was used to de-noise and de-multiplex the samples and Perseus was used to remove chimeras.[177] Operational taxonomic units (OTUs) were assigned by clustering sequences at a 97% identity threshold in the UCLUST program and the most abundant sequence within an OTU cluster was chosen as the representative sequence for that OTU. The Silva SSU Ref ND database (version 111) was used for taxonomical assignment of each OTU by using an 80% bootstrap confidence around a representative sequence from each OTU.[178] PyNAST was used for alignment of representative sequences by accessing the Silva reference set.[179] FastTree was used for phylogenetic tree reconstruction from a representative sequence of each OTU.[180]

5.3.12 Contaminants

It is now clear that many commercial DNA extraction kits have low levels of contamination by bacterial DNA.[181] It is possible to draw the wrong conclusion regarding microbiota diversity if these contaminants are not identified and removed.[182] Certain bacterial OTUs are consistently found in PCRs of kit reagents and in low biomass experimental samples. We removed these from the list of OTUs in our samples prior to further processing. The OTUs removed were from the following taxonomic groups:-

All bacteria of the phylum:-

Cyanobacteria

All bacteria from the following bacterial orders:-

Caulobacterales

Rhodobacterales

Rhizobiales

Rhodospirillales

Rhodocyclales

Deinococcales

Sphingomonadales

Sphingobacteriales

Methylophilales

Myxococcales

All bacteria of the families:-

Oxalobacteraceae

Comamonadaceae

5.3.13 Data analysis

5.3.13.1 *Alpha diversity*

To address hypothesis one, we considered how diverse each sputum sample was in terms of distinct OTUs. This per sample

diversity is referred to as alpha diversity, which is an ecological term. Alpha diversity was originally used to refer to the ecology of an individual 'site' (e.g. rock-pool) within a larger region (e.g. beach containing many rock-pools).[183] Using this analogy, in this sample set each sputum sample was a site which had certain pre-existing characteristics e.g. subject age, smoking status and comorbidities. The alpha diversity of each sputum sample was expressed in a number of ways. The total number of distinct bacterial OTUs in each sputum sample was referred to as species "richness" such that a sample with 50 OTUs had greater richness than a sample with 10 OTUs. The relative proportions of these OTUs in each sample were referred to as "evenness". A sample with 50 OTUs, where one of the OTUs accounted for 90% of all reads (16S rRNA sequences), was less "even" than a sample with 10 OTUs, where each OTU accounted for 10% of the total number of reads. Finally, Shannon's Diversity Index was calculated for each sample. This index takes into account both the richness and evenness of the sample when describing diversity. Shannon's index is a numerical quantification of the uncertainty in predicting the next component (originally letters, here OTU) to be identified from a composition (originally alphabetically coded message, here sputum sample), such that a higher number indicates more uncertainty, suggesting a more diverse community of OTUs.

The alpha diversity measures are formally defined below:

Formulas,

$$H = -\text{SUM} [(p_i) * \ln(p_i)]$$

$$E = \text{Evenness} = H/H_{\text{max}}$$

Where,

H = **Shannon's diversity**

SUM = Summation

p_i = proportional abundance of OTU = number of reads of OTU
i/total number of reads for all OTUs in that sample

* = symbol for multiply

$\ln(p_i)$ = natural log of p_i

S = number of species = **Richness**

H_{max} = maximum diversity possible = $\ln(S)$

Values for each of the three measures of alpha diversity (“richness”, “evenness” and “Shannon”) were calculated for all sputum samples and mean alpha diversity values for samples, grouped by patient characteristic (e.g. smoking status), were compared.

5.3.13.2 Rarefaction

Since sequencing more reads from a sample inevitably increases the number of distinct OTUs that are identified, the richness (number of distinct OTUs) attributed to a sample is highly influenced by technical variation. To get around this, the alpha

diversity measures described above were calculated on a rarefied data-set. This means that, for each sputum sample, the software randomly sampled from the complete set of reads available for that sample and chose a specified sub-set of reads to represent that sample. The rarefaction level, that is the number of reads to be randomly chosen from each sample, was high enough to be representative but not so high that many samples fell below the rarefaction level and were excluded from the analysis. In this way, the total number of reads for each sputum sample was adjusted to an even sequencing depth.

5.3.13.3 Compositional analysis

To address the second hypothesis we regarded the complete, un-rarefied set of reads from all samples as a composition. Data is compositional when the relative proportions of the components are of interest but the absolute value of each component (for example reads) is non-informative.[184,185] This is the case with 16S rRNA data where the absolute number of reads per OTU, per sample and for the pool as a whole is governed primarily by the sequencing platform (as discussed in 5.5.3.3). When considering each component of the composition as a proportion of the whole it is helpful to consider the components as summing to 1 then, if the count for one gene/bacteria goes up, then it must force the count of another one down - proportionally. Data of a compositional nature require an initial transformation – since the absolute difference between values is not of interest. The transformation should be one which renders the data scale-invariant, a property which would allow comparisons to be made across different sequencing platforms that generate total read numbers which are orders of magnitude different.[186]

Aitchinson proposed a number of different transformations, including the population centred log-ratio (clr). To determine the centred log ratio (clr) the count for each component is divided by the geometric mean of the counts for all components and then the logarithm of result is taken. Equivalently, if we observed counts n_1, n_2, \dots, n_m , for features 1, 2, ..., m , with a total read count of $N = n_1 + n_2 + \dots + n_m$, then we can scale to proportional abundances by dividing each individual read count by N . That is we calculate $p_i = n_i / N$ for $i = 1, 2, \dots, m$, such that $p_1 + p_2 + \dots + p_m = 1$. With this, the CLR transformed data are given by

$$c_i = \log[p_i / g(p_1, p_2, \dots, p_m)],$$

where $g(\cdot)$ represents the geometric mean: $(p_1 p_2 \dots p_m)^{1/m}$.

Once transformed by the clr the data have another useful property in that they can be subset without disturbing the relative difference between components. For example, if we wanted to explore OTUs at the taxonomic level phylum and restricted our analysis to just those OTUs belonging to the phylum firmicutes, we would want the relative proportions of OTUs within that subset analysis to be the same as they would be if we determined their proportions in the complete dataset; the clr facilitates these comparisons.

A characteristic of 16S rRNA sequence data is that it contains many zeros. For example, in many of our sputum samples OTUs that have been detected in at least one sample have read counts of zero in most other samples. Zeros are a problem when using the centred log ratio owing to the fact that the logarithm of zero is undefined. We adopt a two-stage procedure for handling zero counts as proposed by Fernandes et al.[186] The first step is

remove any components from the data if the counts are zero for all samples, based on the reasoning that they are uninformative for the purposes of the analysis. The second step is to assume that the observed read counts for a given sample are subject to sampling variation. We do so by assuming that the counts n_1, n_2, \dots, n_m are realisations from a multinomial distribution—a generalisation of the ubiquitous binomial distribution for dichotomous data—with event probabilities p_1, p_2, \dots, p_m . Also note that the classical maximum likelihood estimates of $p_i = n_i / N$ (as defined above earlier) are inaccurate in the case of very small (or zero) read counts.[187] To overcome this, Fernandes et al. (2013, 2014) consider the event probabilities as unknown random variables and model them in a Bayesian framework. A non-informative conjugate Dirichlet($\frac{1}{2}, \frac{1}{2}, \dots, \frac{1}{2}$) prior distribution is used. The posterior distribution for p_1, p_2, \dots, p_m conditional on the data is then Dirichlet($n_1 + \frac{1}{2}, n_2 + \frac{1}{2}, \dots, n_m + \frac{1}{2}$). From the posterior distribution, several (128 in our case) Monte Carlo samples of the vector (p_1, p_2, \dots, p_m) are drawn, which are interpreted as proportional abundances for the components. Monte Carlo sampling is a statistical technique for random sampling from probability distributions.[188] For each of the Monte Carlo draws, the clr transformation is applied (as described above), leading to so-called ‘relative abundance values’. With the transformed data, standard statistical tests can be applied, such as Welch’s *t*-test or the Wilcoxon-Mann-Whitney *U*-test, to derive *P*-values. The *P*-values are then averaged over the Monte Carlo draws, which we report as the test *P*-value. In addition to *P*-values *q*-values are also returned which represent adjustments for multiple testing and used the Benjamini-Hochberg method. The analysis was performed using the R package ALDeX2.[189]

5.4 Results

5.4.1 General

The 169 PASS subjects provided 86 acute sputum samples of which 9 were deemed inadequate (had no obvious mucopurulence) and 77 went through to sequencing. Characteristics of the subgroup with sequenced sputum can be compared to the remainder of the PASS cohort in table 5.2. Age, gender and comorbidities were similar between the two groups. However, nearly twice as many (50%) of those with sequenced sputum were active smokers when compared to those without sequenced sputum (26.9%). Sputum samples yielded between 130 and 4065 copies (reads) of the v3-v5 region of the 16S rRNA gene. A rarefaction level of 549 reads was chosen and this meant excluding one sample (with 130 reads) from the analysis of alpha diversity. Following rarefaction to 549 reads and removal of singletons (reads that appeared only once in only one sample), 774 OTUs were identified across all sputum samples. The median per-sample was 43 (IQR 23-62).

5.4.2 The most abundant OTUs

Exploratory analysis revealed that the dominant bacterial OTU in this study of community acquired pneumonia was *Haemophilus_617*. This was determined by analysing the read counts for all OTUs in all 76 samples together – analogous to pooling all 76 sputum samples into one pot, then mixing and sequencing the ‘pneumonia super-sample’. The second most abundant OTU was *Veilonella_1328*. Bacteria of the genus *Veilonella* are Gram negative anaerobic cocci. They exist as commensals of the gut and oral mucosa. The most abundant streptococcal OTU was less than half as abundant as

Haemophilus_617. The top 20 OTUs in are displayed in Figure 5.3 and counts for each of the top 20 most abundant OTUs can be seen for each subjects sputum sample in appendix 3.

5.4.3 Important species with low abundance

We specifically explored the abundance of certain OTUs due to their prominence in the pneumonia literature. *Mycoplasma pneumoniae* occurs in epidemic cycles during which it is responsible for large numbers of relatively mild cases of CAP. Pneumonia due to *Legionella pneumophila* is associated with contact with infected water and can cause a severe pneumonia. *Staphylococcus aureus* is associated with prior influenza infection, underlying lung disease and pneumonia among patients on Intensive Care units. *Mycoplasma pneumoniae* and *Staphylococcus aureus* were present at low levels of abundance and *Legionella pneumophila* was not detected in any samples. *Legionella pneumophila* was detected in one patient by urine antigen testing (see chapter 3) but this patient did not provide sputum for sequencing. Table 5.1 (next page) compares the abundance of these genera to the genera *Streptococcus* and *Haemophilus*.

Table 5.1 Genera *Mycoplasma*, *Legionella* and *Staphylococcus*

Genus	Number of OTUs within this genus	Most abundant OTU in this Genus	Total number of sequencing reads across all samples for most abundant OTU	Mean read count per sample for most abundant OTU
<i>Haemophilus</i>	41	<i>Haemophilus_617</i>	8429	218
<i>Streptococcus</i>	73	<i>Streptococcus_4318</i>	3644	94
<i>Mycoplasma</i>	5	<i>Mycoplasma_3100</i>	174	4.5
<i>Staphylococcus</i>	5	<i>Staphylococcus_2814</i>	143	3.1
<i>Legionella</i>	0	NA	NA	NA

5.4.4 The relative abundance of individual OTUs per-sample

Next we explored the relative abundance of OTUs in individual samples. To do this, read counts for each individual OTU in a sample were converted to a percentage of the total read count for that sample. To reduce the dimensionality of the data to a level that could be explored graphically, for each sample, we grouped together, into a bin called 'other' all OTUs that made up 12% or less of the total for that sample. The relative abundances within and across samples could then be displayed in a stacked bar-chart (figure 5.4). This bar-chart shows some samples were more diverse than others i.e. they were composed of many OTUs but each individual OTU accounted for a small proportion of the sample. Other samples were less diverse and were dominated by a single highly abundant OTU.

5.4.5 The relative abundance of selected OTUs in distinct clinical groups

Next we explored how the abundance of several distinct OTUs varied between different clinical groups. The choice of OTUs to display was based on the data displayed in figure 5.4 and prior assumptions about the importance of certain bacteria; for example the species of the genera *Streptococcus*, *Haemophilus* and *Pseudomonas* have previously been described as key pathogens in lower respiratory tract infections and CAP. The three most abundant streptococcal OTUs were explored further as all previous studies of CAP have highlighted that *Streptococcus pneumoniae* is a key aetiological agent in CAP. The most abundant OTU from the genera *Moraxella*, *Haemophilus*, *Pseudomonas* and *Klebsiella* were all chosen for further exploration as each was seen (in figure 5.4) to be dominant in at least one sputum sample and previous studies have identified

species from these genera as significant respiratory pathogens. Differences in relative abundance of these OTUs were most apparent when subjects were divided into those who had or did not have prior pulmonary disease. Results are displayed in figures 5.5 to 5.10.

Table 5.2 Clinical characteristics of PASS subjects with and without sequenced sputum

Characteristic		Sequenced Sputum n=76	No Sputum n=93	P
Median Age, (IQR)		68.5 (49 – 76)	71 (55-79)	0.1 *
Male, n (%)		42 (55.3)	46 (49.5)	0.7 #
Smoking, n (%)	Active	38 (50)	25 (26.9)	0.7 #
	Quit	28 (36.8)	40 (43.0)	
	Never	10 (13.2)	22 (23.7)	
Modified Charlson Comorbidity Index n (%)	0	23 (30.3)	32 (34.4)	0.8 #
	1	32 (42.1)	38 (40.9)	
	2	8 (10.5)	10 (10.8)	
	3	10 (13.2)	7 (7.5)	
	4	2 (2.6)	4 (4.3)	
	5	1 (1.3)	1 (1.1)	
	6	0	1 (1.1)	
Prior statin use, n (%)		21 (27.6)	35 (37.6)	0.7 #
Prior pulmonary disease, n (%)		46 (60.5)	44 (47.3)	0.7 #
CURB65, n (%)	0	22 (28.9)	18 (19.4)	0.8 #
	1	13 (17.1)	26 (28.0)	
	2	22 (28.9)	28 (30.1)	
	3	18 (23.7)	18 (19.4)	
	4	1 (5.3)	3 (3.2)	
	5	0	0	
Presenting CRP median (IQR)		150 (81 – 235.5)	144 (43-249.5)	0.3 ≠
BMI median (IQR)		25.5 (22.2-29.7)	25.9 (23.0-30.9)	0.5 ≠
Flu, n (%)		9 (17)	9 (16.7)	0.7 #
Pneumococcal bacteraemia n (%)		5 (6.8)	3 (3.7)	0.7 #

*Wilcoxon rank-sum test

#Chi Squared test

≠Welch's t test

Data was incomplete for the highlighted clinical variables, n for those groups is indicated below:-

Smoking status, no bronchoscopy group, n=139

CRP, sputum group n=75 no sputum group, n=91

BMI, sputum group, n=62 no sputum group n=60

Flu, sputum group, n=53; no sputum group, n=54

Pneumococcal bacteraemia, sputum group, n=74 no sputum group n=82

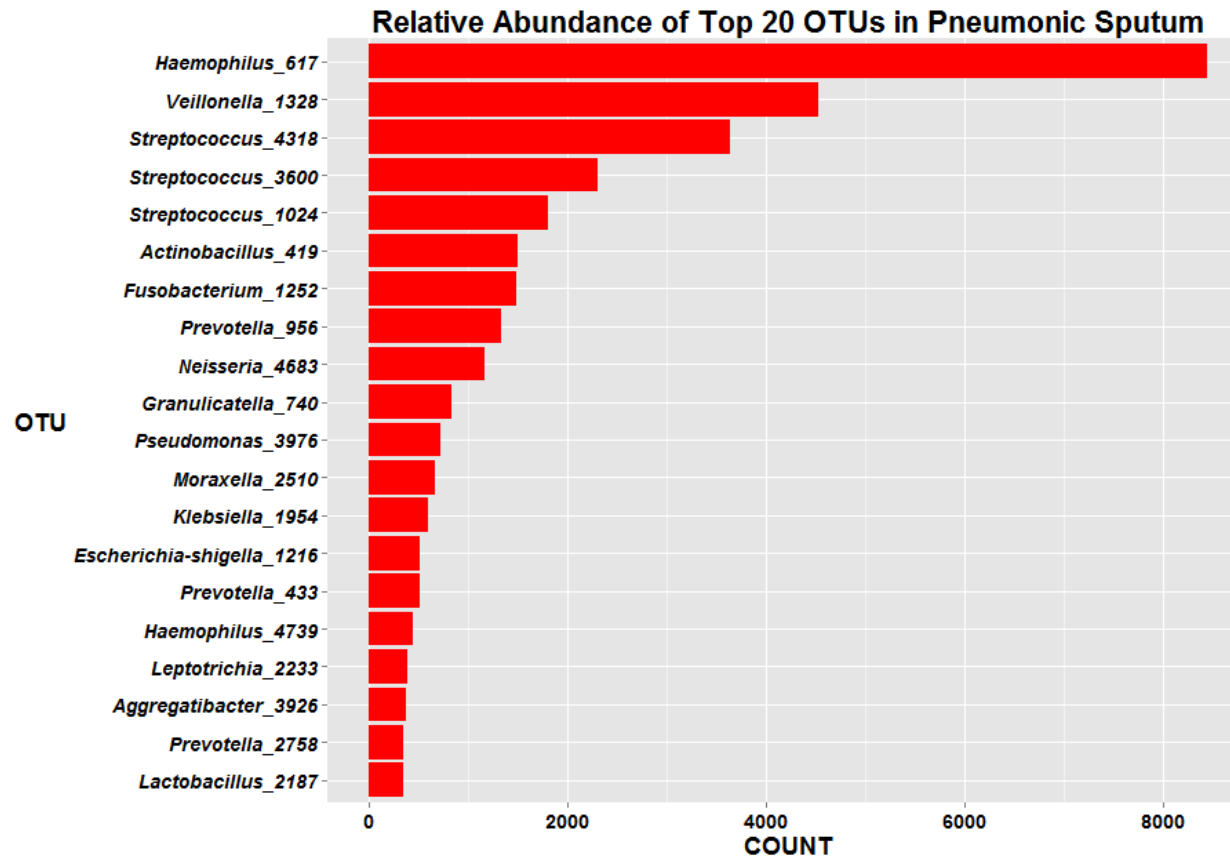


Figure 5.3 Top 20 OTUs

In this figure, all reads from all 76 sputum samples are pooled.

The bar lengths represent the number of reads attributed to each OTU (bacterial species) in the pool.

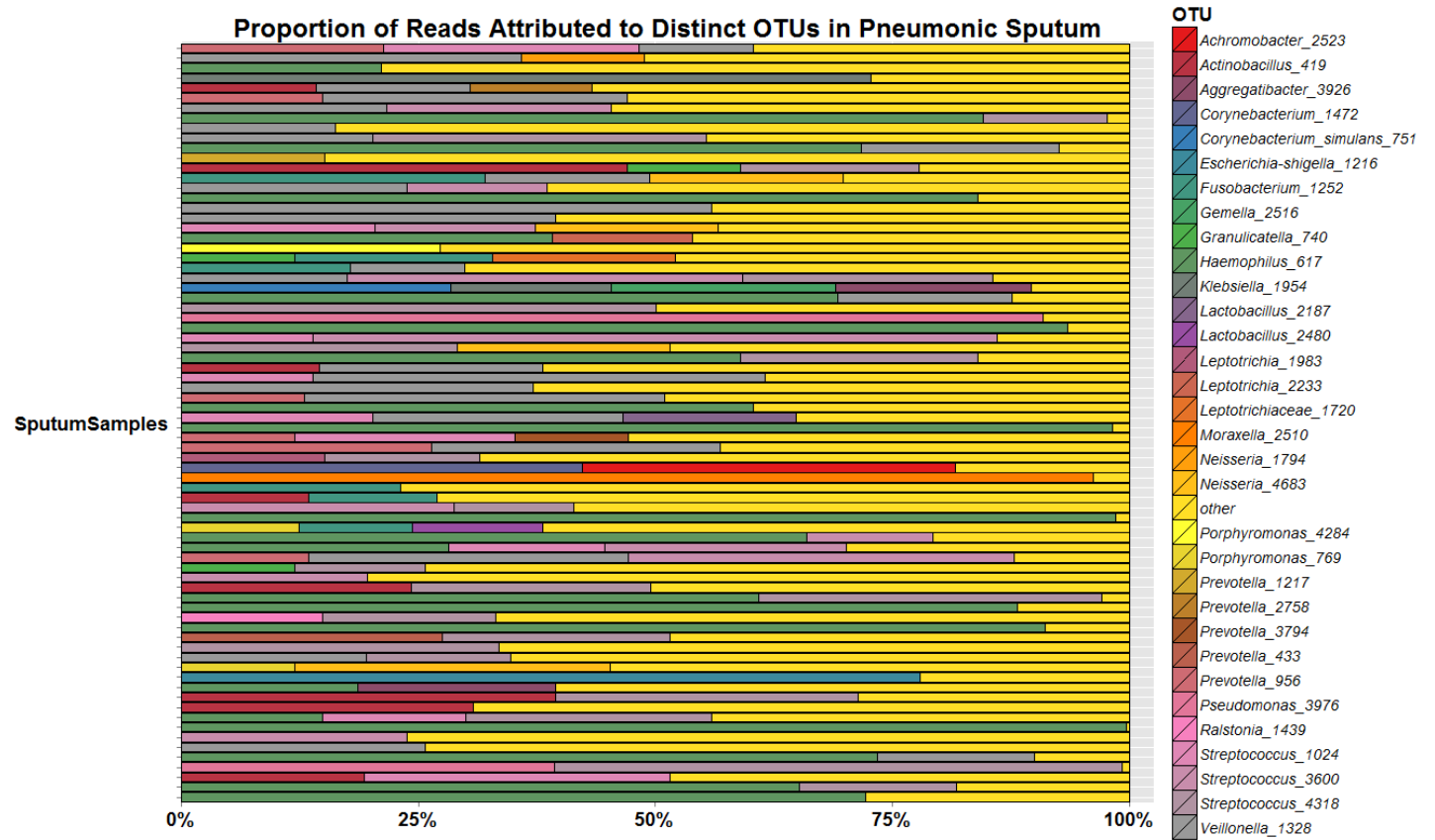
This demonstrates that, in this cohort of patients with CAP, *Haemophilus_617* was dominant and was more than twice as abundant as any Streptococcal species (OTU).

Figure 5.4 Proportional abundance of OTUs in sputum samples

Each stacked bar represents one of 76 sputum samples.

The coloured bar segments represent distinct bacterial OTUs and their proportional abundance in that sample. OTUs that represent less than 12% of a sample were binned into a group called “other”.

Some samples are seen to be diverse with a large proportion of OTUs assigned to “other” whereas other samples are almost entirely dominated by a single OTU.



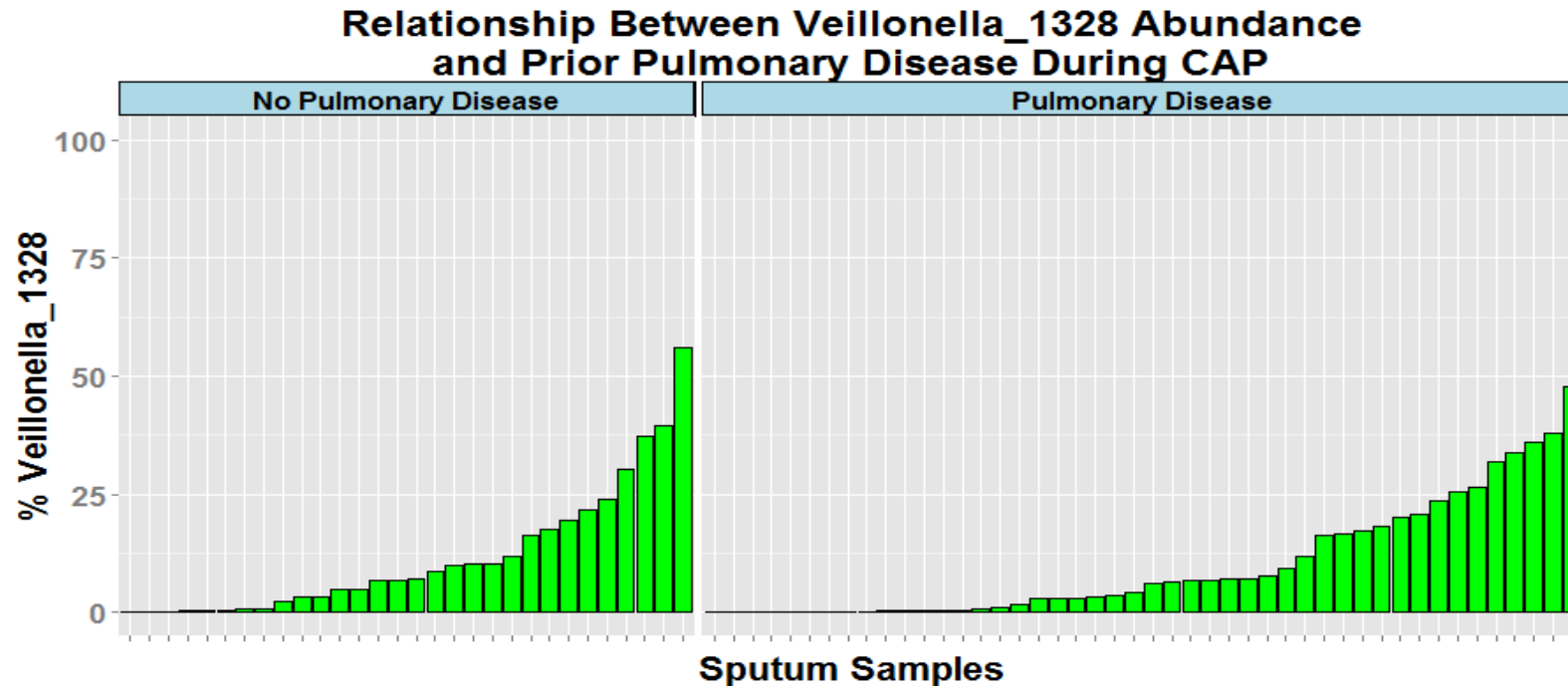


Figure 5.5 Abundance of *Veillonella*_1328 in sputum of subjects with and without pulmonary disease

Each bar represents a sputum sample. Bar heights represent the % of reads in a sample attributed to *Veillonella*_1328. *Veillonella*_1328 was one of the most commonly detected OTUs but was rarely a dominant OTU and only once did it represent more than 50% of the reads from a sample. The pattern of abundance between those with (n=46) or without (n=30) prior pulmonary disease was similar.

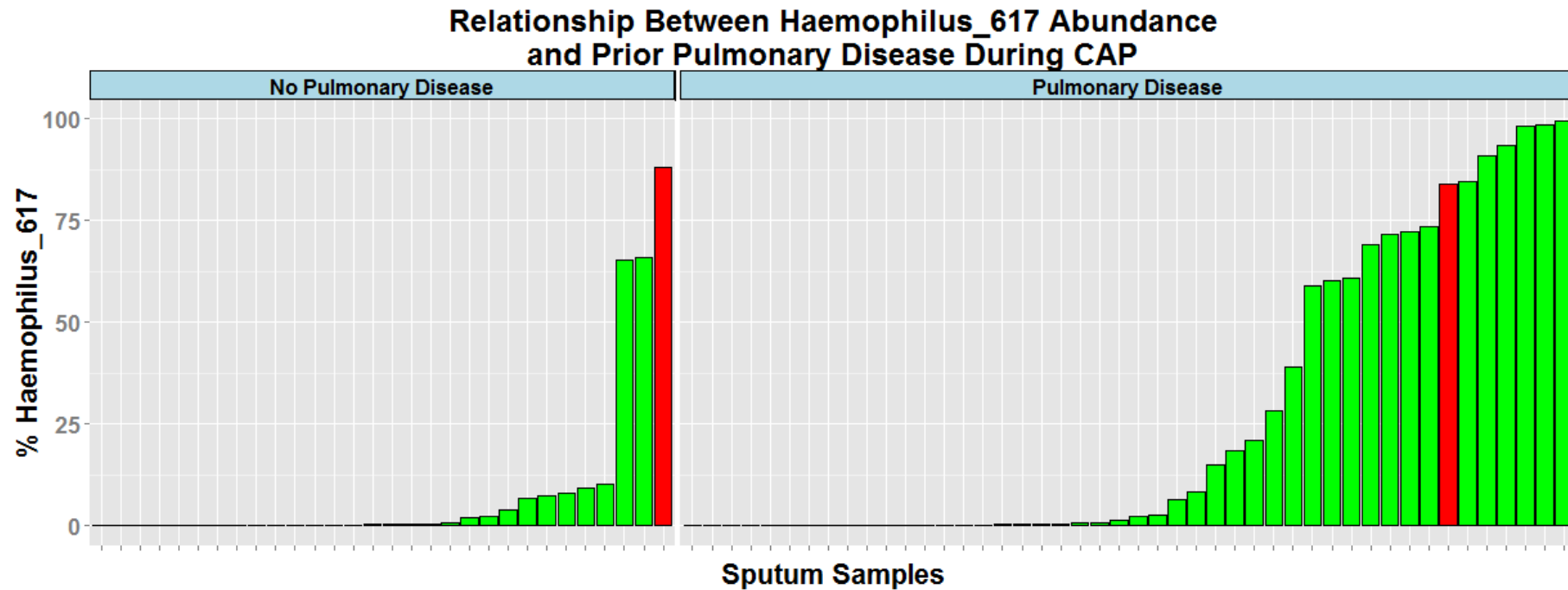


Figure 5.6 Abundance of *Haemophilus_617* in sputum of subjects with and without pulmonary disease.

The bars represent the proportion (%) of reads in that sample that were attributed to *Haemophilus_617*. Bars coloured red are from subjects whose sputum grew *Haemophilus influenzae*. In 14/46 (30%) of those with prior pulmonary disease *Haemophilus_617* was the dominant OTU with >50% of reads compared with 3/30 (10%) of those who did not have pulmonary disease before they developed CAP.

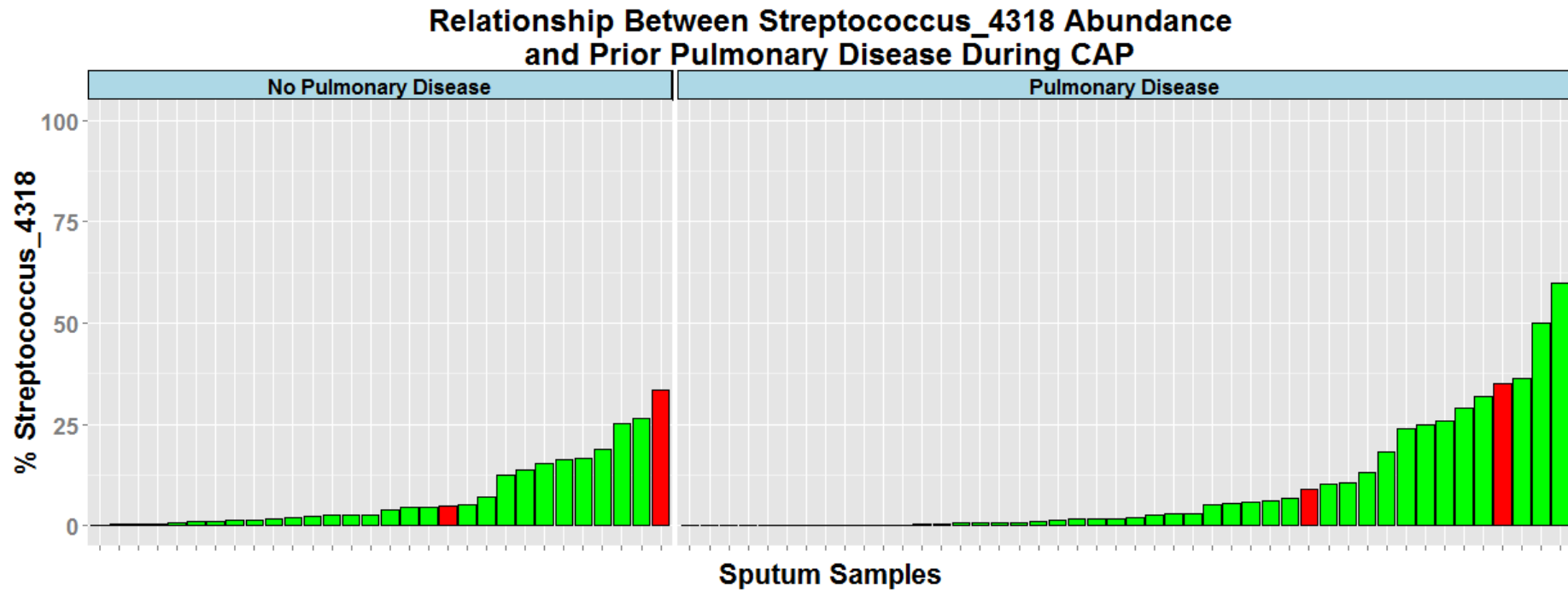


Figure 5.7 Abundance of *Streptococcus_4318* in sputum of subjects with and without pulmonary disease

The bars represent the proportion (%) of reads in that subjects sample that were attributed to *Streptococcus_4318*. Bars coloured red are from subjects who grew *Streptococcus pneumoniae* in their blood cultures (one subject did not have a blood culture). *Streptococcus_4318* was rarely dominant with an abundance >50%, in only one sample.

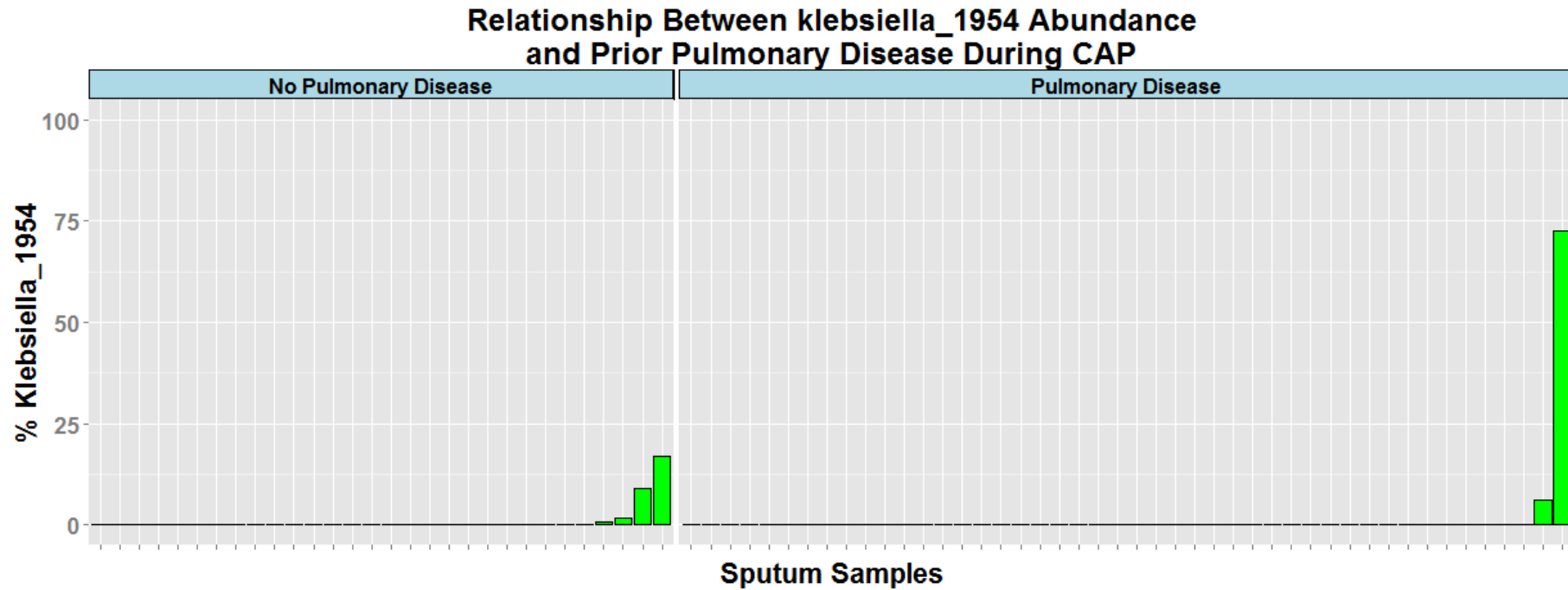


Figure 5.8 Abundance of *Klebsiella_1954* in sputum of subjects with and without pulmonary disease

The bars represent the proportion (%) of reads in that sample that were attributable to the OTU *Klebsiella_1954*. This OTU was identified infrequently but on one occasion was highly dominant with nearly 75% of all sequences being from the OTU *Klebsiella_1954*.

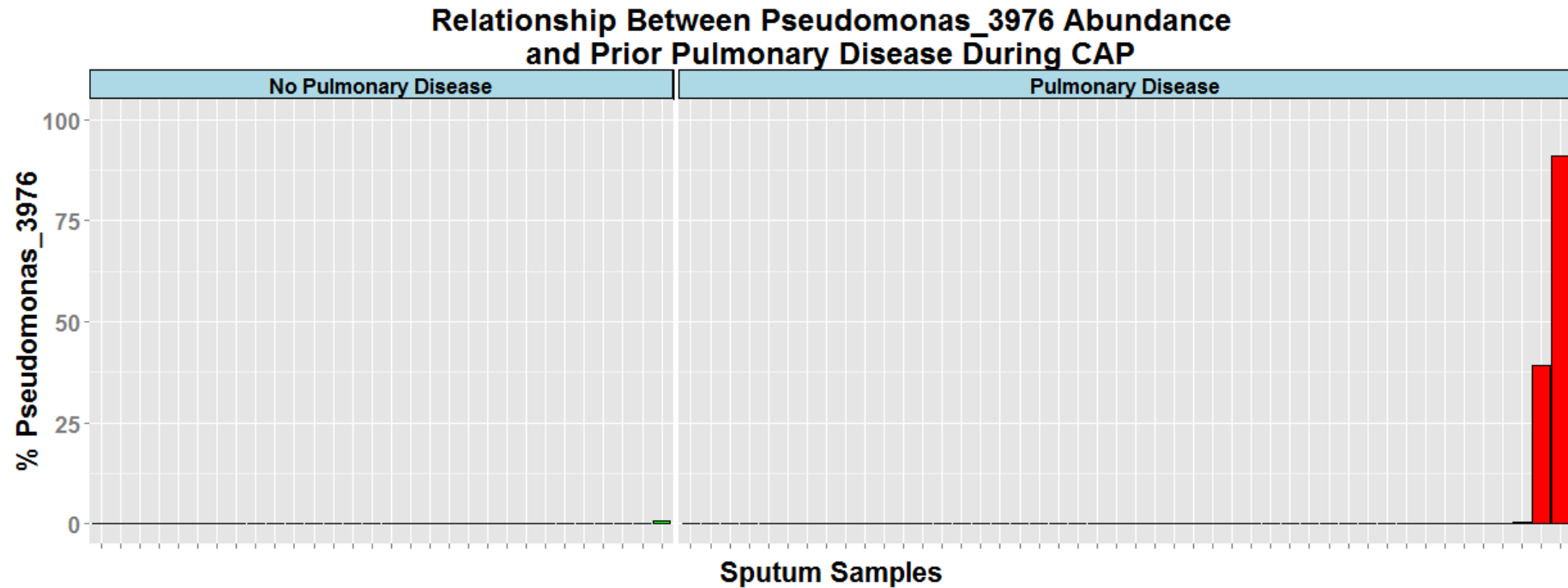


Figure 5.9 Abundance of *Pseudomonas_3976* in sputum of subjects with and without pulmonary disease

The bars represent the proportion (%) of reads in that sample that were attributable to the OTU *Pseudomonas_3976*. This OTU was identified rarely from subjects without prior pulmonary disease. On one occasion *Pseudomonas_3976* was highly dominant with nearly 90% of all sequences being from this OTU. Red bars represent samples from subjects whose sputum grew *Pseudomonas aeruginosa* in the NHS microbiology lab.

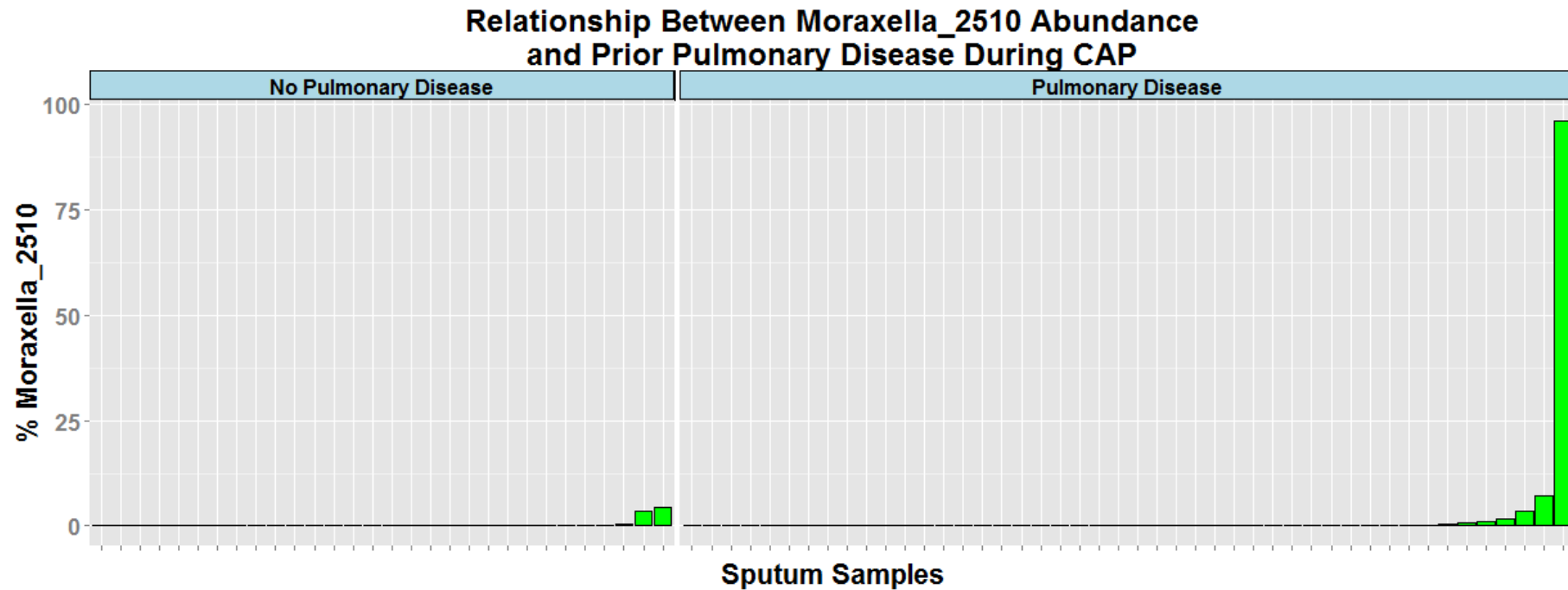


Figure 5.10 Abundance of *Moraxella_2510* in sputum of subjects with and without pulmonary disease

The bars represent the proportion (%) of reads in that sample that were attributable to the OTU *Moraxella_2510*. *Moraxella* was rarely identified in this set of samples suggesting it is not a commensal organism in either the upper or lower respiratory tract. In one sample it displayed 'pathogenic' behaviour by being completely dominant with >90% of all sequences in that sample being attributed to that single OTU.

5.4.6 Alpha diversity

To explore the diversity of bacterial OTUs in each sample we calculated the species 'Richness' (number of different OTUs), 'Evenness' (how evenly distributed the OTUs were) and 'Shannon's' diversity index. Several clinical characteristics were independently associated with each measure of alpha-diversity (see tables 5.3 – 5.5). Backwards stepwise multiple linear regression (table 5.6) demonstrated that the richness of species in the sputum of subjects with CAP was associated with age and prior statin use (figure 5.11 and 5.12). Multiple-linear regression revealed that underlying pulmonary disease was independently associated with the evenness ($p=0.001$) and Shannon's diversity index ($p=0.002$) of bacterial species in a subject's sputum after adjustment for other clinical factors (Figures 5.13 and 5.14 and tables 5.7 and 5.8).

5.4.7 Distribution of individual bacterial OTUs

The exploratory analysis of figures 5.4-5.10 revealed several individual bacterial operational taxonomic units (OTUs) that appeared to be associated with prior pulmonary disease. However, when the un-rarefied dataset was subjected to compositional analysis using the ALDeX2 packages, none of the OTUs were significantly associated with any of the clinical parameters tested after correction for multiple testing (Benjamini-Hochberg method) Table 5.9.

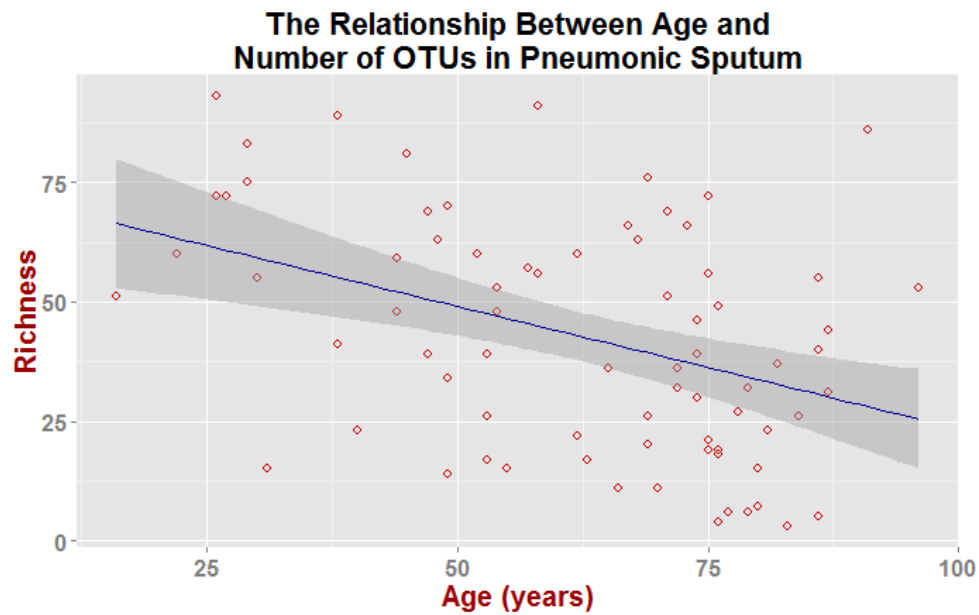


Figure 5.11 The relationship between age and species richness

Each circle represents a sputum sample. The y axis indicates the number of bacterial OTUs in that sample. The line demonstrates a linear model of the relationship between richness and subject age ($p=0.0003$) and the shaded area shows the 95% confidence interval around the position of that line.

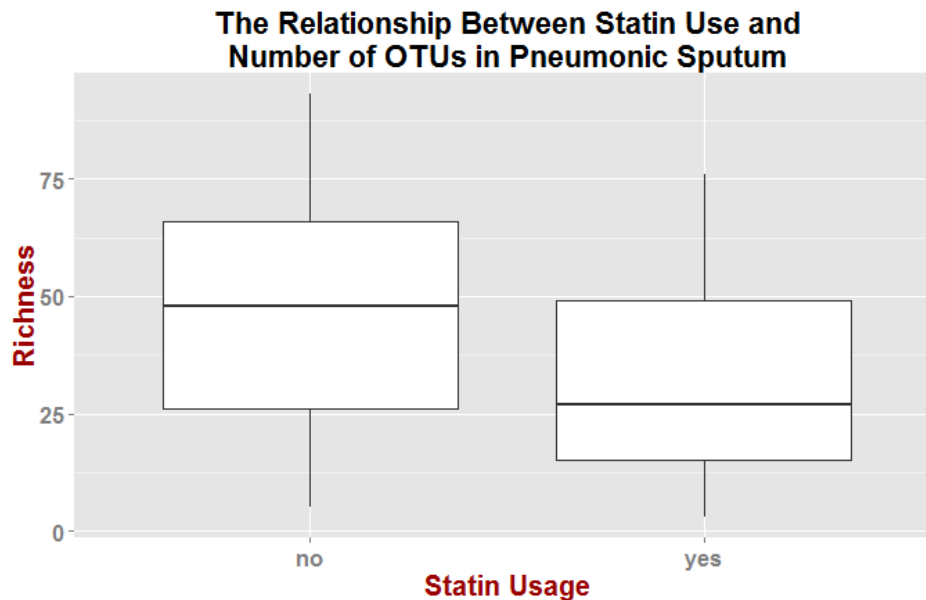


Figure 5.12 Relationship between richness and prior statin use

The relationship between the 'Richness' (number of OTUs) in the samples and statin usage ($p=0.01$). The box limits are defined by the inter-quartile range of values for that group and the horizontal line is the median value. Whiskers extend to 1.5x the inter-quartile range.

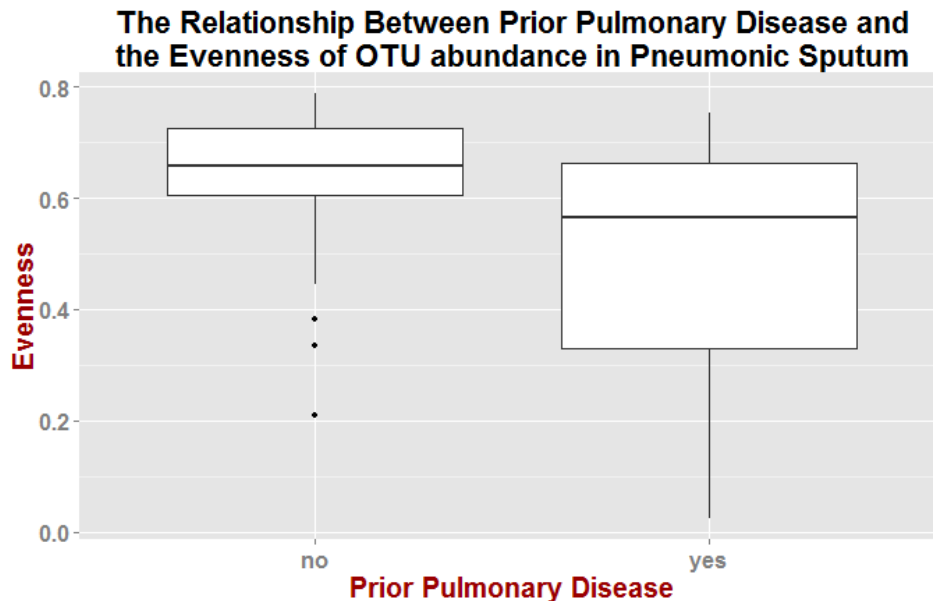


Figure 5.13 Relationship between evenness and prior pulmonary disease

Those with no pulmonary disease had more even distribution of species abundance ($p=0.001$). Whiskers extend to 1.5x the inter-quartile range and points outside of the whiskers indicate sputum samples whose evenness value is outside of 1.5 x the inter-quartile range.

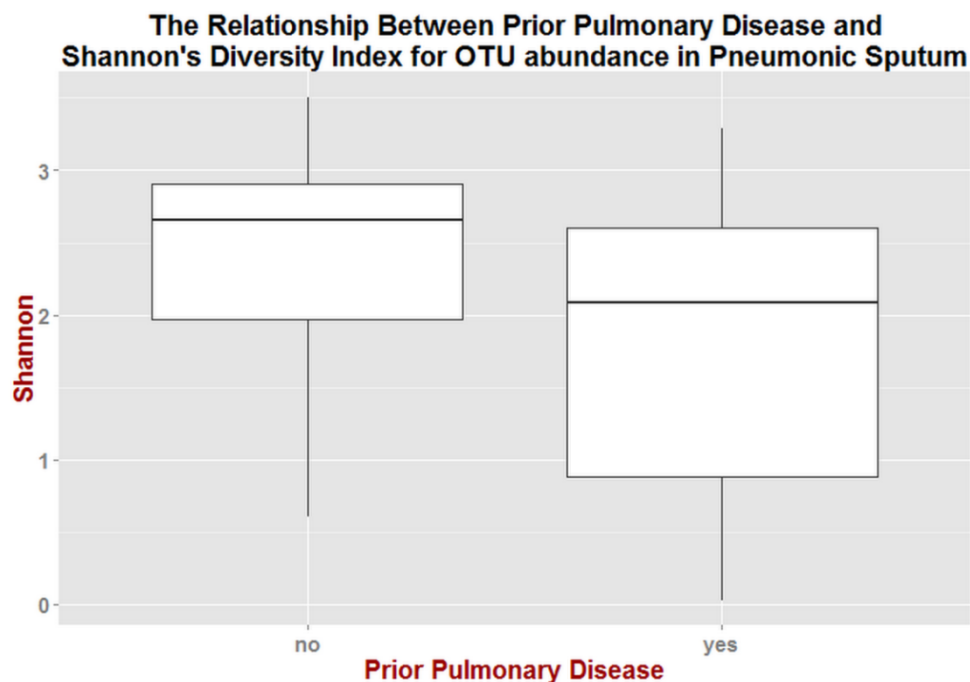


Figure 5.14 Relationship between prior pulmonary disease and bacterial species diversity in pneumonic sputum.

Those with no prior pulmonary disease a higher species diversity ($p=0.002$).

Table 5.3 Univariate analysis of richness and clinical variables

Explanatory variable		Estimate	Standard error	<i>P</i>
No influenza		40.7	3.4	0.04
Influenza		17.9	8.3	
BMI		(40.8)	(15.4)	0.9
		0.07	0.6	
No statin		46.9	3.2	0.001
Statin		-15.4	6.0	
Age		(74.6)	(8.8)	0.0003
		-0.5	0.1	
Charlson comorbidity index	0	51.3	5.0	
	1	-7.7	6.5	0.2
	2	-17.3	9.8	0.08
	3	-20.1	9.0	0.03
	4	-22.3	17.5	0.2
	5	-24.3	24.3	0.3
Never smoker		52.9	7.4	
Ex-smoker		-18.7	8.7	0.03
Active smoker		-6.6	8.4	0.4
No prior pulmonary disease		50.7	4.3	0.02
Prior pulmonary disease		-13.2	5.5	
Female		37.6	4.1	0.09
Male		9.4	5.5	
No antibiotics pre-admission		43.6	2.9	0.3
Antibiotics pre-admission		-11.4	10.3	

Table 5.4 Univariate analysis of evenness and clinical variables

Explanatory variable		Estimate	Standard error	<i>P</i>
BMI		(0.5) 0.002	(0.1) 0.005	0.7
No influenza Influenza yes		0.6 0.05	0.03 0.07	0.5
No statin Statin yes		0.5 -0.008	0.03 0.05	0.9
Never smoker Ex-smoker Active smoker		0.6 -0.05 -0.03	0.06 0.08 0.07	0.5 0.7
No antibiotics pre-admission Antibiotics pre-admission		0.5 -0.1	0.02 0.09	0.3
Female Male		0.5 0.1	0.03 0.04	0.02
Age		-0.003	0.001	0.02
Charlson comorbidity index	0	0.6	0.04	
	1	-0.1	0.05	0.05
	2	-0.1	0.08	0.2
	3	-0.1	0.07	0.09
	4	-0.3	0.1	0.06
	5	-0.1	0.2	0.5
No prior pulmonary disease Prior pulmonary disease		0.6 -0.1	0.03 0.04	0.001

Table 5.5 Univariate analysis of Shannon's diversity and clinical variables

Explanatory variable		Estimate	Standard error	<i>P</i>
No influenza		2.1	0.1	0.2
Influenza		0.5	0.3	
BMI		(1.8) 0.008	(0.6) 0.02	0.7
No pulmonary disease		2.5	0.2	0.002
Prior pulmonary disease		-0.7	0.2	
Age		(3.1) -0.02	(0.4) 0.006	0.004
Charlson comorbidity index	0	2.4	0.2	0.08
	1	-0.5	0.3	
	2	-0.6	0.4	
	3	-0.7	0.4	
	4	-1.1	0.7	
	5	-0.8	1.0	
Female		1.8	0.2	0.03
Male		0.5	0.2	
Statin		2.1	0.1	0.3
Statin yes		-0.3	0.2	
Never smoker		2.3	0.3	0.2
Ex-smoker		-0.4	0.4	
Active smoker		-0.2	0.3	
No antibiotics pre-admission		2.1	0.1	0.3
Antibiotics pre-admission yes		-0.5	0.4	

Table 5.6 Multiple linear regression of bacterial species richness in pneumonic sputum and clinical variables

Model	Parameters	Log Likelihood of Model	Comparison of Models by Likelihood Ratio	
1	Statin + flu + age + prior pulmonary disease	-226.0		
2	Statin+flu+age	-226.9	ANOVA 1 v 2	$p=0.2$ no significant difference so drop prior pulmonary disease
3	Statin+age	-228.6	ANOVA 2 v 3	$p=0.08$ no significant difference so drop flu
4	Age	-231.2	ANOVA 3 v 4	$p=0.03$ significant difference so keep statin
5	Statin*age	-228.5	ANOVA 3 v 5	$p=0.7$ no significant interaction

Model 1 is the maximal model including all variables that were significantly associated with richness in the univariate analysis. Model 2 has the least significant variable (prior pulmonary disease) from model 1 removed. An analysis of variance (anova) of model 1 and 2 demonstrates that there is no statistically significant difference between the two models indicating prior pulmonary disease contributes little. Repeating the process anova demonstrates that statin but not age should be included as a main effect in the model. Model 5 includes an interaction term (*) and ANOVA demonstrates there is no statistically significant interaction between statin and age. In summary age and statins are the most important factors in determining species Richness in these samples.

Table 5.7 Multiple linear regression of bacterial species evenness in pneumonic sputum and clinical variables

Model	Parameters	Log Likelihood of Model	Comparison of Models	
1	Age + gender + prior pulmonary disease	16.7		
2	Gender + prior pulmonary disease	15.9	Anova 1 v 2	$p=0.2$ no significant difference lose age
3	Prior pulmonary disease	14.9	Anova 2 v 3	$p=0.2$ no significant difference so lose gender

Model 1 is the maximal model including all variables that were significantly associated with evenness in the univariate analysis. Model 2 has the least significant variable (age) from model 1 removed. An analysis of variance (anova) of model 1 and 2 demonstrates that there is no statistically significant difference between the two models indicating age contributes little. Repeating the process anova demonstrates that gender contributes little and that only prior pulmonary disease has a statistically significant association with evenness after adjustment for the other variables.

Table 5.8 Multiple linear regression of Shannon's diversity of pneumonic sputum and clinical variables

Model	Parameters	Log Likelihood of Model	Comparison of Models	
1	Age + gender + prior pulmonary disease	-63.5		
2	Age + prior pulmonary disease	-64.9	Anova 1 v 2	$p=0.1$ not significant lose gender
3	Prior pulmonary disease	-66.8	Anova 2 v 3	$p=0.06$ not significant lose age

Model 1 is the maximal model including all variables that were significantly associated with evenness in the univariate analysis. Model 2 has the least significant variable (gender) from model 1 removed. An analysis of variance (anova) of model 1 and 2 demonstrates that there is no statistically significant difference between the two models indicating gender contributes little. Repeating the process anova demonstrates that gender contributes little and that only prior pulmonary disease has a statistically significant association with Shannon's diversity after adjustment for the other variables.

Table 5.9 The relationship between individual bacterial OTUs or genera and clinical variables

Variable	Test	OTU identified $p < 0.05$	p	q	Genus identified @ $p < 0.05$	p	q	Interpretation
Smoking status	glm	None identified	NA	NA	Neisseria_4683	KW 0.02	BH 0.5	Not significant following correction
			NA	NA		glm 0.03	BH 0.5	
Age by centile	glm	Fusobacterium_1252	KW 0.02	BH 0.9	Parvimonas_175	KW 0.02	BH 0.5	Not significant following correction
			glm 0.05	BH 0.4		glm 0.03	BH 0.3	
Modified Charlson co-morbidity index	glm	None identified	NA	NA	None Identified	NA	NA	NA
Flu	ttest	None identified	NA	NA	None identified	NA	NA	NA
Anti-biotics pre-admission	ttest	None identified	NA	NA	None Identified	NA	NA	NA
Gender	ttest	None identified	NA	NA	None Identified	NA	NA	NA
Pulmonary co-morbidity	ttest	None identified	NA	NA	None identified	NA	NA	NA
Statin	ttest	None Identified	NA	NA	None Identified	NA	NA	NA

Relationship between either individual bacterial OTUs (species) or bacterial genus and clinical parameters. glm = generalised linear model. KW = Kruskal Wallis. BH = Benjamini-Hochberg method for correcting for multiple comparisons.

5.5 Discussion

5.5.1 Summary of results

This study of the bacteria present in the sputum of patients with CAP found statistically significant associations between patient characteristics and measures of ecological diversity. After adjustment for other clinical factors, the number of bacterial species in a sample (richness) was inversely related to a patient's age and was decreased if they were taking statins. The proportional representation of OTUs was less 'even' in patients with prior pulmonary disease. Combining the concepts of richness and evenness, and after adjustment for other clinical associations, the overall ecological diversity of bacterial species, as measured by Shannon's diversity index, was decreased in the presence of prior pulmonary disease. In this set of samples, the OTU *Haemophilus_617* had the highest number sequences detected (reads) and was also the OTU which was most frequently dominant in individual samples. Statistically significant associations with patient characteristics were not seen with individual bacterial species or at the higher taxonomic level of bacterial genera.

5.5.2 What were the strengths of this work?

Sputum remains the primary sample of interest when determining the microbiological aetiology of community acquired pneumonia (CAP) and this is the largest study to date of the sputum bacterial microbiota of CAP. The subjects were well characterised and were representative of CAP treated in our region and in similar regions throughout the world (see section 3.2). Sputum samples were collected within 24 hours of admission to minimise the impact of antibiotic on the bacterial

DNA in sputum. The size of the sample set and the extent of the clinical data available enabled us to adjust our models for confounding variables and to identify statistically significant factors that determine bacterial diversity in pneumonic sputum. The use of the new ALDEx2 package in the analysis of this data enabled us to apply a statistically robust approach to the compositional analysis of species and genus level associations and enabled us to account for technical variation introduced by the sequencing platform.

Both of the a-priori hypotheses were adequately addressed.

- i. The range of bacteria in a sputum sample from a patient with CAP is influenced by subject specific characteristics. The most influential factor is the presence of prior pulmonary disease.
- ii. Several bacterial OTUs were identified more frequently and at higher abundance in subjects with particular characteristics. However, we were not able to predict from a subject's baseline characteristics which individual OTU was likely to dominate their sputum sample.

5.5.3 What were the limitations of this work?

5.5.3.1 *Incomplete data*

169 subjects were recruited to PASS but only 76 sputum samples were sequenced, representing 45% of the cohort. It is well recognised that only a minority of patients with CAP, as we currently define it, produce sputum and that sputum production is less likely in the elderly.[190] Ewig et al. conducted a study to directly assess the utility of sputum as an aetiological diagnostic specimen for CAP.[191] They found that only 22% of patients were able to self-expectorate on admission, rising to 34% within

24 hours of admission. The eligibility criteria for the Ewig et al study were very similar to those for PASS.

In PASS, the subjects with sequenced sputum appeared different to those without in some key respects, although none of these observed differences achieved statistical significance. A higher proportion (50% v 26.9%) of the sequenced group were active smokers. The sequenced group were more likely to have pre-existing pulmonary disease (60.5% v 47.3%) and were more likely to have a pneumococcal bacteraemia (6.8% v 3.7%). The implications of these differences, given the major findings of this work, are that we now know more about the microbiota of CAP in those with chronic lung disease but still have a limited understanding of the lung bacterial microbiota in patients with CAP who are non-smokers without prior lung disease.

5.5.3.2 Lack of species level identification of key pathogens

The bacterial genera *Streptococcus* and *Haemophilus* cannot be further sub-divided by 16S rRNA sequencing. Thus *Streptococcus mitis* and *Streptococcus pneumoniae* cannot be distinguished nor can *Haemophilus influenzae* be separated from *Haemophilus haemolyticus*. As a consequence two key pathogens in the context of lower respiratory tract infection and CAP are not clearly characterised in this study. Extended sequence analysis and comparison with a species-specific quantitative PCR of the sample would enable us to make the distinctions above but were beyond the scope of this study. However, some inferences can be made as to the likely species of some OTUs in this data by comparing with NHS clinical laboratory cultures of the samples. For example, several of the samples in which *Haemophilus_617* was dominant were also reported as growing *Haemophilus influenzae* in culture.

Moreover, the distribution pattern of *Haemophilus_617* – being more abundant in those with prior pulmonary disease and being the dominant OTU overall in a cohort with very high rates of smoking, is in line with previous associations between *Haemophilus influenzae*, smoking and pulmonary disease.[192] It is therefore likely that the operational taxonomic unit *Haemophilus_617* is *Haemophilus influenzae*.

Inferences about which OTU represents *Streptococcus pneumoniae* are more difficult. *Streptococcus_4318* had the greatest number of reads of all the streptococcal OTUs and had the highest levels of abundance per-sample of all *Streptococci*. However, in contrast to other recognised respiratory pathogens it was rarely dominant and only once represented more than 50% of the total sequences in a sample. The distribution pattern of *Streptococci* were similar between subjects grouped by clinical characteristics and when the pneumococcus was grown from the blood there was no association with high streptococcal abundance in sputum. *Streptococcus pneumoniae* was not grown from any of the sputum samples sent to the hospital lab.

These findings should be validated with larger studies as they may be an artefact of our study design. For example subjects in this study had received variable amounts of antibiotic prior to submitting their sputum – some had received nearly 24 hours of intravenous antibiotic – and it may be that this suppressed the pneumococcal signal to a greater degree than for other OTUs. Against this is the finding that 5 subjects grew pneumococcus in their blood but had negative sputum cultures. If antibiotic were the cause of negative sputum cultures it would be expected that the blood cultures would also be culture negative. An alternative explanation is that this is a true finding, in which case it gives a

clue as to the pathophysiology of pneumococcal disease. The assumption that the dominant species in a sample from a sick patient is the causative pathogen may not always be true; it is possible that pneumococci do not need to achieve airway dominance in order to cause disease.

5.5.3.3 Sequencing platform

In 2011, when this study was funded, the state of the art 16S rRNA gene sequencing platform for microbiota studies was Roche's FLX version of their 454 technology. Subsequently this platform has largely been superseded by the latest developments in Illumina sequencing.[193] The differences between the two platforms are essentially that 454 is able to sequence longer sections of the 16S rRNA gene (read-lengths now up to 1000 base pairs vs. Illumina which now has a maximum of 300) whereas the Illumina platforms generate many times more reads per run (several billion reads per Illumina run vs maximum 700,000 with FLX-454). More reads are equivalent to greater magnification for a microscope – it enables us to detect more of what is present in a sample, including rare bacteria whose signal would have been lost within a complex sample from which we had fewer reads. Also, Illumina is now significantly cheaper than 454 sequencing. However, the longer read lengths of 454 technology still confer some advantages in terms of accurate identification of individual species within certain genera, and it is unlikely that the major conclusions of this chapter would be different if the samples had been Illumina sequenced.[194] Moreover the bulk of the published work on sputum microbiota and respiratory microbiota in general has used 454 sequencing making comparisons with the work presented here valid.

5.5.4 Comparison with other published work

5.5.4.1 *Pneumonic sputum*

There is only one previous study of the bacterial microbiota of sputum from unventilated patients with CAP. Chen et al. collected sputum from 45 immuno-competent patients who had been hospitalised for radiologically confirmed CAP.[173] None of the patients had received antibiotic in the community although it is not clear if they had received any hospital antibiotic prior to the submission of their study sample. In contrast to our study, this group performed a prolonged sputum lysis step (18 hours incubation with sodium hydroxide) prior to chemical (as opposed to our mechanical) cell lysis and DNA extraction. They used a different 16S rRNA PCR protocol which involved an initial PCR using 50ng of template then then a second PCR using the PCR products from the first as substrate. Their sequencing used the same Roche 454-FLX platform as ours.

Chen et al. found that the bacterial genus *Streptococcus* had the highest average abundance per sample at 20.6% with the genus *Haemophilus* being ranked 11th with an average abundance of 1.65% per sample. In our data the genus *Streptococcus* was ranked second with an average abundance of 23.3% and the genus *Haemophilus* was top with an average of 23.7% per sample. However, the clinical implication of abundance at the taxonomic level of genus in the context of CAP is very limited since the Genus *Streptococcus* is composed of a large number of distinct species (68 OTUs in our data) and many of those detected in the sputum will not be causally linked to patient's pneumonia.

Our work went beyond the genus level and showed that, per sample, there were wide variations in abundance at the species

(OTU) level and distinct patterns began to emerge. The most abundant Streptococcal OTU was very frequently isolated but rarely achieved high levels of dominance. The most abundant *Haemophilus* OTU was less frequently isolated, but in some samples accounted for nearly all the bacteria isolated and this was most commonly the case in samples from patients with chronic lung disease. Rates of lung disease are not specified in the Chen paper but 10% of subjects whose sputum was sequenced were active smokers as compared to 50% of ours. This is further evidence that smoking and the consequent development of pulmonary disease may well account for the high abundance of *Haemophilus* in our study.

5.5.4.2 Sputum from subjects with chronic lung disease

Pneumonia is inflammation caused by infection in the lung and a criticism of sputum as a specimen for the description of lower respiratory tract infection is that, given the identification of a large number of bacterial species in a sputum sample how would we distinguish between those that originated in the ‘diseased’ lung from those that were from the ‘healthy’ mouth? Recent work comparing bacterial microbiota in a range of samples from patients with cystic fibrosis in stable state has shown that using 16S rRNA sequencing, oral wash and sputum samples were virtually identical in some individuals.[195] In other patients the sputum microbiota diverged from the oral washes – suggesting a unique lung microbiota. Where the oral wash and sputum differed there was an association with poor prognostic features such as increased markers of airway inflammation. The conclusion was that, despite coming from the lung, ‘healthy’ sputum had similar bacteria to the mouth, and where differences existed between mouth and lung microbiota these could be easily

detected i.e. the mouth signal did not 'swamp' the lung signal. The implication of that study for our work is that in the case of CAP, although some of the sequences detected in sputum will have come from the mouth, the lung signal will not have been lost. Indeed many of our subjects had sputum which contained a very dominant OTU and in all cases these OTUs were well known respiratory pathogens. Venkataraman and colleagues have recently applied ecological theory to model the lung microbiota from healthy and disease lungs and their results suggest that, as proposed above, in health the low-biomass lung microbiota is highly influenced by dispersal from the mouth microbiota.[196] In disease states they found the microbiota could not be explained by a 'neutral model' and their data suggested that selective pressures were acting on the microbiota enabling certain species to thrive at the expense of others. This fits with our findings that those with pulmonary disease had lower species diversity.

Previous studies have demonstrated that conventional culture techniques for the identification of *Haemophilus influenzae* have high false negative rates. Wood-Baker et al. found that culture identified *Haemophilus influenzae* in 5/36 (14%) sputum samples from patients with stable COPD where as a targeted PCR found 14/36 (39%) were positive.[197] Garcha et al. showed that during exacerbations of COPD bacterial biomass of sputum, as measured by quantitative PCR (qPCR) increases by as much as 20 fold.[198] Using species specific qPCR they showed that the most common isolate at exacerbation and in stable state was *H. influenzae*. This is relevant to our work since many of the subjects in our study had COPD and there is significant overlap between the clinical syndromes of 'infective exacerbation of COPD' and 'community acquired pneumonia in a patient with COPD'.

5.5.4.3 *Bronchoalveolar lavage of HIV infected patients with CAP*

One study has investigated the bacterial microbiota of bronchoalveolar lavage (BAL) samples taken from HIV+ve patients being treated for CAP.[199] 60 BAL samples from Ugandan patients initially thought to have CAP were compared with 15 BAL samples from patients in San Francisco who were thought to have CAP. None of these samples were taken during the acute phase of CAP and all patients were well into their antimicrobial treatment regime. Moreover, when final diagnoses were assigned to each patient – 2 months after discharge – only 7/60 Ugandan patients were felt to have “probable bacterial pneumonia” with 35/60 having been diagnosed with TB, 2/60 with PCP, 4/60 with pulmonary Karposi’s sarcoma and 20/60 in whom there was not enough data to evaluate the probability of CAP. Of the San Francisco patients 7/15 had “probable bacterial pneumonia”, 7/15 had PCP, 1/15 had fungal pneumonia, 1/15 had CMV and 1/15 had pulmonary Karposi’s. The total list of diagnoses for the San Francisco cohort came to 17/15 implying that several subjects had multiple primary diagnoses – although which had more than one is not specified. Bacterial microbiota from all 60 Ugandan subjects was compared with all 15 subjects from San Francisco. Due to the heterogeneity of diagnoses (with only the minority having CAP) and the duration of pre-sampling antibiotic it is very difficult to draw meaningful conclusions as to the bacteria that were present during acute CAP.

5.5.5 Clinical implications of this work

The clinical questions this study tackles are, what are the aetiological agents of CAP – and are they the same for everyone? If we could comprehensively answer these questions then we

could stratify our empirical prescribing guidelines by incorporating patient characteristics thus making them more personalised. Microbiota studies that directly tackle such clinical questions have the potential to significantly improve clinical practice but care must be taken not to over-reach when drawing conclusions and in particular to avoid assuming causality where none has been established.[200] We found a much larger range of bacteria in sputum from patients with CAP than has been described in culture based studies. The ecological analysis of this data found that those with pre-existing lung disease had lower species diversity in their sputum. Low species diversity can occur when a sample contains a single dominant species. When we explored the relationship between individual species and prior pulmonary disease we showed that a single species from Gram negative genera such as *Moraxella*, *Klebsiella*, *Pseudomonas* and particularly *Haemophilus* was more likely to dominate.

Our current empirical antibiotic regime is based on the assumption that *Streptococcus pneumoniae* is the most common cause of pneumonia in all groups. The backbone of treatment is therefore a β -lactam with sicker patients also receiving a macrolide to broaden the spectrum of cover. However, resistance to β -lactams is increasingly common among *Haemophilus influenzae* which also possesses an efflux pump which makes it inherently resistant to macrolides.[201] Our work supports recent calls for more intensive study of *Haemophilus influenzae* as an under recognised pathogen.[202] If future studies confirm that in patients with prior lung disease *Hemophilus influenzae* is a common cause of CAP then a randomised control trial of doxycycline vs. amoxicillin as first line treatment for CAP in the context of prior lung disease would inform changes to guidelines.

6 LONGITUDINAL ANALYSIS OF RECOVERY USING THE CAP-SYM QUESTIONNAIRE

6.1 Introduction

The CAP-sym (community acquired pneumonia-symptom) questionnaire provides a quantitative measure of symptoms and was devised as a patient based tool for measuring outcome following community acquired pneumonia (see section 1.4.3).[58] By recording the CAP-sym questionnaire at a number of time-points following an index case of pneumonia, recovery can be compared between individuals.[60]

Statistical modelling uses mathematics to produce a function (model) that can explain how a series of observed responses have been generated.[203,204] A successful statistical model generates an approximation to the behaviour of data derived from a study using as few input variables as possible. The input variables which produce the best fitting model give clues as to the mechanisms underlying the phenomenon being studied. An example of this is a model describing the pattern of *forced expiratory volume in 1 second* (FEV1) measurements obtained from Danish children with cystic fibrosis (CF).[205] In that study 70448 FEV1 measurements on 448 CF patients were modelled with clinical data. The derived model described the degree to which an initial FEV1 measurement predicted future variability in FEV1 and demonstrated that decline in FEV1 was related to pancreatic status, *Pseudomonas aeruginosa* infection and year of birth.

Using a similar approach, we recorded the CAP-SYM score at multiple time points for each subject recruited to the Pneumonia Aetiology and Severity Study (PASS) and created a model to test the following hypothesis:-

6.2 Hypothesis

Using a statistical model of CAP-sym scores, recovery following community acquired pneumonia can be predicted from baseline clinical characteristics.

6.3 Methods

6.3.1 Recording and calculating CAP-sym scores

CAP-sym is an 18 question questionnaire (see figure 6.1). During its validation, CAP-sym was completed by trained interviewers who asked the questions and recorded the answers. Each question is phrased in a similar way, “in the last 24 hours how much have you been bothered by (e.g.) shortness of breath?” The subject can choose one of six possible answers and each answer carries a numerical score:

I do not have the symptom (scores 0),

not bothered at all (scores 1)

a little bothered (scores 2)

moderately bothered (scores 3)

bothered quite a bit (scores 4)

extremely bothered (scores 5).

If the scores for each of the 18 component answers are summed they come to a maximum score of 90 which represents the worst a patient could possibly feel with respect to these symptoms. The total score for the questionnaire therefore represents the burden of pneumonia-related symptoms felt by that patient in the 24 hours preceding the completion of the questionnaire.

In the Pneumonia Aetiology and Severity Study (PASS) the CAP-sym questionnaire was conducted by a trained study team member. For a detailed description of PASS protocol see chapter

2. Briefly, medical admissions were screened for possible community-acquired pneumonia (CAP). Patients were eligible if they volunteered consent to join the study within 24 hours of their first dose of in-hospital antibiotic and did not meet any exclusion criteria. Subjects were followed for up to one year. At the time of enrolment subjects conducted the CAP-sym questionnaire twice. The first iteration of the questionnaire represented their symptoms in the previous 24 hours i.e. the day of admission to hospital with CAP. The second was completed *thinking back 30 days* prior to admission and represented how they felt before the pneumonia began. CAP-sym was repeated at the next in-hospital study visit, two days following recruitment, then again on the day of discharge. Due to the practical limitations of the study if the patient was discharged prior to day 2 or if day 2 or discharge fell at the weekend then a study visit was not completed and the CAP-sym was not recorded. CAP-sym was conducted at each of the study out-patient follow-up visits which were at one month, six months and one year following recruitment.

6.3.2 Statistical Analysis

The score for each of the 18 questions in the CAP-sym questionnaire was summed and the total (the CAP-sym score) recorded against the time-point for that subject. We compared our mean values of CAP-sym to those of the multi-centre study used to validate the CAP-sym score to confirm that our values were of similar magnitude and that the trend was comparable. Next, an exploratory analysis involved graphical representation of all subjects' CAP-sym trajectories followed by representations of individual trajectories to draw out trends. Transformations such as plotting ranked median CAP-sym residuals were also

explored.[204] To begin to explore the relationship between clinical variables and median recovery for the group we initially defined recovery as the % difference in CAP-sym score between presentation and at the one month follow-up visit. However, this is a naive measure of recovery and does not take into account the patients pre-morbid baseline symptom level nor does it correct for repeated measures on the same individual over time.

Therefore we next attempted to use linear modelling to describe the data. A linear model is one where each term in the prediction equation is either a constant or the product of a parameter and a predictor variable, hence

$$Y = \alpha + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_k X_k + Z$$

where α is a constant, β_1, \dots, β_k are parameters, X_1, \dots, X_k are the predictor variables and Z is an independent error term. The nature of our data was found to be such that, after initial explorations, linear modelling was deemed inadequate to describe the patterns of recovery. As a consequence, non-linear modelling was applied. Non-linear models take many forms but are defined as non-linear by not conforming to the linear form described above. An initial non-linear model was created without the pre-morbid (t-30) CAP-sym scores. This model was used to better understand the symptom trajectory from admission to recovery. The fit of this basic non-linear model was assessed.

Once an adequate model framework was achieved the model was re-parameterised to include the pre-admission (t-30) CAP-sym score. With this more elaborate model we were able to assess how covariates affected the degree of recovery from peak symptoms to

the mean baseline (pre-morbid) symptom level for the group. The covariates tested were those that had been shown to be significant in chapters 4 and 5 i.e. age, smoking status, statin use and prior pulmonary disease.

➤ **Introduction to the CAP-Symptom Questionnaire to be read to the patient.**

Patients with pneumonia sometimes experience symptoms or problems which we are evaluating as part of the study in which you are currently participating. We would therefore like to ask you a few questions about your own current experience in that respect. I am going to read you a list of symptoms or problems. For each of them, I will ask you the extent to which the symptom/problem has bothered you in the past 24 hours: not at all, a little, moderately, quite a bit or extremely. If you have not had the symptom/problem in the past 24 hours, please let me know.

Overall, the interview will only take a few minutes and the questions are simple to answer. Please remember that you should answer in reference to what happened in the past 24 hours. Thank you very much in advance for your participation.

Please read each item to patient and circle the number that corresponds to how much the patient has been bothered by the symptom/problem IN THE PAST 24 HOURS.

In the past 24 hours, how much have you been bothered by:						
	Patient did not have the symptom/problem	Patient had the symptom/problem and it bothered him/her...				
		Not at all	A little	Moderately	Quite a bit	Extremely
*1. Coughing?	0	1	2	3	4	5
*2. Chest pains?	0	1	2	3	4	5
*3. Shortness of breath?	0	1	2	3	4	5
4. Coughing up phlegm/sputum (secretion from the chest)?	0	1	2	3	4	5
5. Coughing up blood?	0	1	2	3	4	5
*6. Sweating?	0	1	2	3	4	5
*7. Chills?	0	1	2	3	4	5
*8. Headache?	0	1	2	3	4	5
*9. Nausea?	0	1	2	3	4	5
10. Vomiting?	0	1	2	3	4	5
11. Diarrhea?	0	1	2	3	4	5
12. Stomach pain?	0	1	2	3	4	5
*13. Muscle pain?	0	1	2	3	4	5
*14. Lack of appetite?	0	1	2	3	4	5
*15. Trouble concentrating?	0	1	2	3	4	5
16. Trouble thinking?	0	1	2	3	4	5
*17. Trouble sleeping?	0	1	2	3	4	5
*18. Fatigue?	0	1	2	3	4	5

* Indicates items that are included in the CAP-Sym 12.

Figure 6.1 The CAP-sym questionnaire

6.4 Results

6.4.1 Initial exploratory analysis

6.4.1.1 *Trajectory of recovery for the PASS cohort*

Mean CAP-sym scores for the PASS cohort at each time-point are shown in table 6.1. The scores at presentation were similar to those obtained in the multicentre study in which the CAP-sym questionnaire was validated.[206] Compared to the validation study recovery scores were slightly higher at comparative time-points. The distribution of scores is revealed in figure 6.2 as is the median recovery trajectory for the group.

6.4.1.2 *Associations between smoking status and PASS CAP-sym scores over time*

We next plotted the summary CAP-sym scores for the PASS cohort over time but divided the subjects up by key variables to determine if, going forward, there were likely to be associations that could be explored by statistical models. Due to the prominence of smoking status in the cohort and in the analyses of chapters 4 and 5, CAP-sym scores for PASS subjects were grouped by smoking status and we observed how they varied over time (see figure 6.3). The pattern that emerged was of higher symptoms scores for smokers than ex-smokers who in turn had higher scores than never smokers. This pattern was repeated at every timepoint.

6.4.1.3 *The relationship between efferocytosis and Haemophilus_617 abundance on symptom recovery at one month*

We next asked how the two key experimental variables explored in chapters 4 and 5 influenced recovery. There were limited data

available for both efferocytosis (22 subjects) and *Haemophilus* dominance (76 sequenced sputum samples) and we therefore began with the simple measure of % recovery of presenting symptoms at one month. Figure 6.4 illustrates the non-statistically significant trend towards increased recovery with increasing efferocytosis. Figure 6.5 demonstrates that subjects with a sputum dominated by the OTU *Haemophilus_617* had a statistically significantly worse recovery at one month than those in whom *Haemophilus_617* was present, but not dominant.

6.4.1.4 Exploration of individual recovery traces

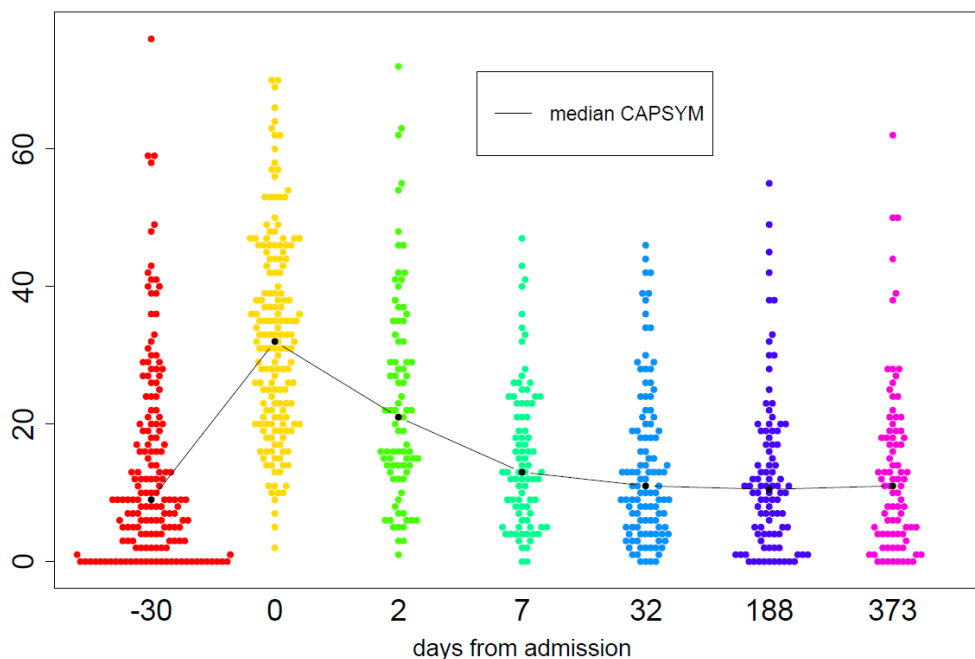
The trajectory of each PASS subject's symptoms is displayed in figure 6.6. Most subjects had a sharp rise in symptoms from 30 days prior to admission to presentation with CAP. In most cases, symptom scores then fell rapidly over the next few days and were close to, but above base-line by discharge. By one month most subjects had resolved most of their symptoms and if that was the case the symptom score remained generally around baseline throughout follow-up to one year. However, some patients did not follow this trend. Figure 6.7 plots the residuals of the CAP-sym scores over time and picks out 10 randomly chosen subject traces. The residual was calculated by subtracting the mean CAP-sym value for all subjects at a given time point from an individual's CAP-sym score at that time-point and dividing the answer by the standard deviation of all scores at that time point. This transformation standardises the scores and enables the degree to which patients' trajectories differ from one another at each time-point to be made more obvious. Some individuals actually described feeling better on admission than 30 days prior to admission, suggesting that the admission was part of a longer

running illness. Others felt worse on day 2 than on day 0 suggesting they had presented early in the illness and that symptoms were still evolving. Some patients went home with a higher symptom burden than others and some actually felt worse at follow-up than on discharge.

Table 6.1 PASS CAP-sym compared to the validation cohort

Clinical stage	Time, days	PASS CAP-sym score (SD)	CAP-sym validation study groups	
			Standard treatment score (SD)	Moxifloxacin score (SD)
Pre-morbid	-30	13.6(14.5)	NA	NA
Enrolment	0	32.8(14.6)	33.9 (13.6)	34.3 (13.2)
Mid-treatment	2	23.8(15.1)	20.6 (11.0)	20.9 (11.8)
Discharge	Variable	15.3(10.6)	12.0 (10.3)	13.5 (11.5)
Early follow-up	28-35	13.6(11.8)	9.6 (10.8)	10.1 (10.9)
Medium term follow-up	160-200	12.6(11.8)	NA	NA
Late follow-up	345-385	13.3(12.7)	NA	NA

Symptomatic Recovery by CAPSYM Score

**Figure 6.2 Median CAP-sym scores for the PASS cohort**

A skewed distribution of CAP-sym scores was seen at all time-points. Median values were joined to describe a summary recovery trajectory for PASS. The mean time in days of each time-point was indicated on the x axis however the scale was compressed for ease of plotting. All CAP-sym values (y axis) were jittered around the time-point to prevent over-plotting of points.

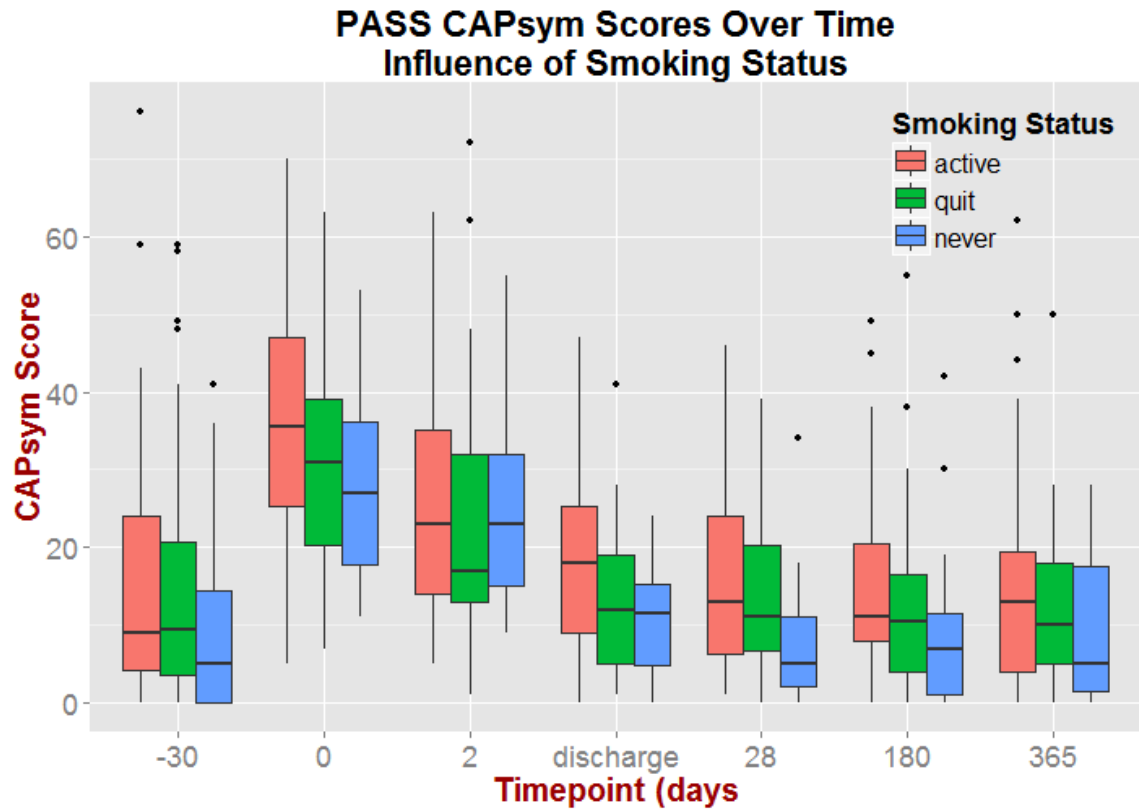


Figure 6.3 The influence of smoking status on the trajectory of symptom recovery for PASS cohort

At every timepoint active smokers have a higher median level of symptoms than ex-smokers who have a higher burden of symptoms than never smokers. Boxes describe the interquartile range (IQR) with a black line for the median value. Whiskers extend to 1.5x the IQR.

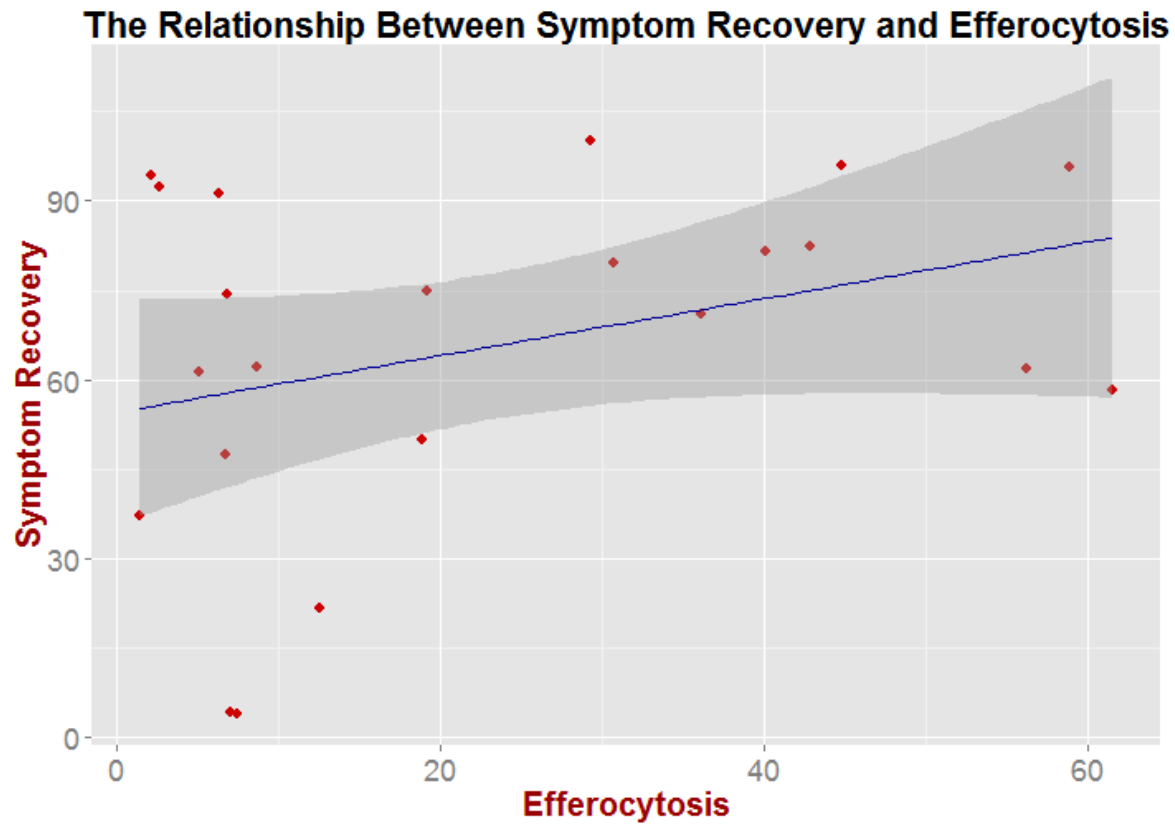


Figure 6.4 Exploration of the relationship between symptom recovery and efferocytosis

Recovery was calculated as the percentage improvement in CAP-sym score at one month compared to the CAP-sym score at presentation. This recovery measure is then plotted against the efferocytosis result for 22 subjects (see chapter 4). The result is a non-statistically significant trend towards increasing recovery with increasing efferocytosis as displayed by the blue line (shaded area = 95% CI around the position of the line).

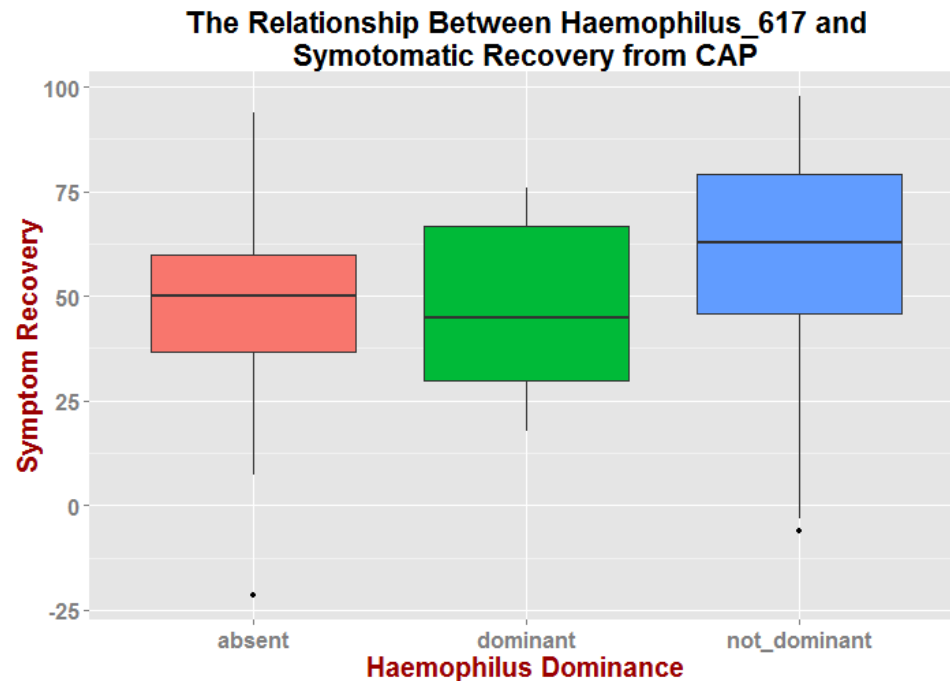


Figure 6.5 Exploration of the relationship between the OTU *Haemophilus_617* and symptomatic recovery from CAP.

Recovery was calculated as the percentage improvement in CAP-sym score at one month compared to the CAP-sym score at presentation. PASS subjects are divided into those in whom *Haemophilus_617* represented >50% of all reads in their sputum (dominant), <50% of all reads in their sputum (non-dominant) or was not detected in their sputum by 16S rRNA sequencing (absent). Those without *Haemophilus_617* in their sputum and those in whom *Haemophilus_617* was non-dominant both had significantly ($p=0.005$) greater symptom recovery than those in whom *Haemophilus_617* was dominant. There was no difference in recovery between the non-dominance group and the absent group.

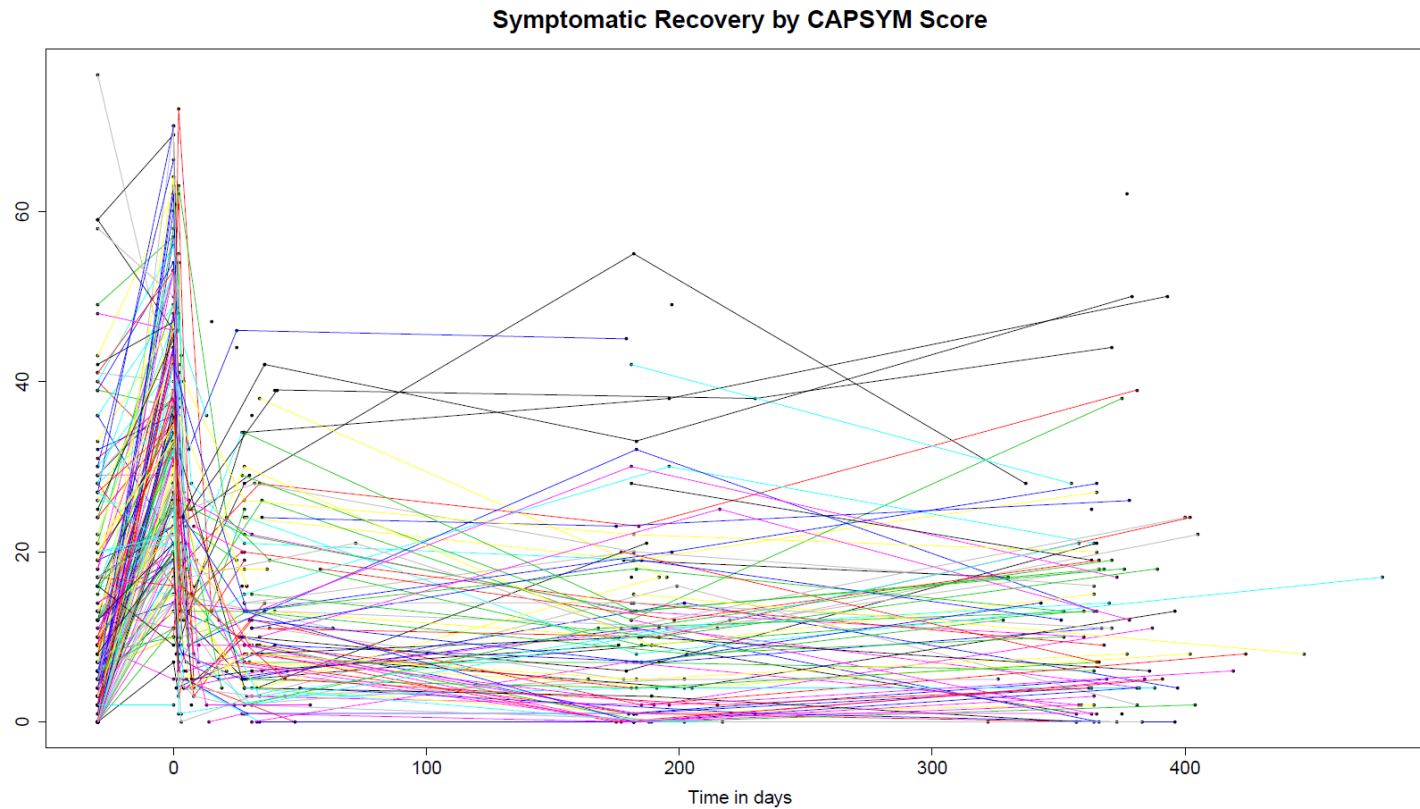


Figure 6.6 Individual subject's CAP-sym traces for the PASS cohort

Most subjects' symptoms followed a pattern of rapid initial recovery following admission then sustained low level symptoms. However a significant minority had an alternative pattern with some being highly symptomatic out to one year.

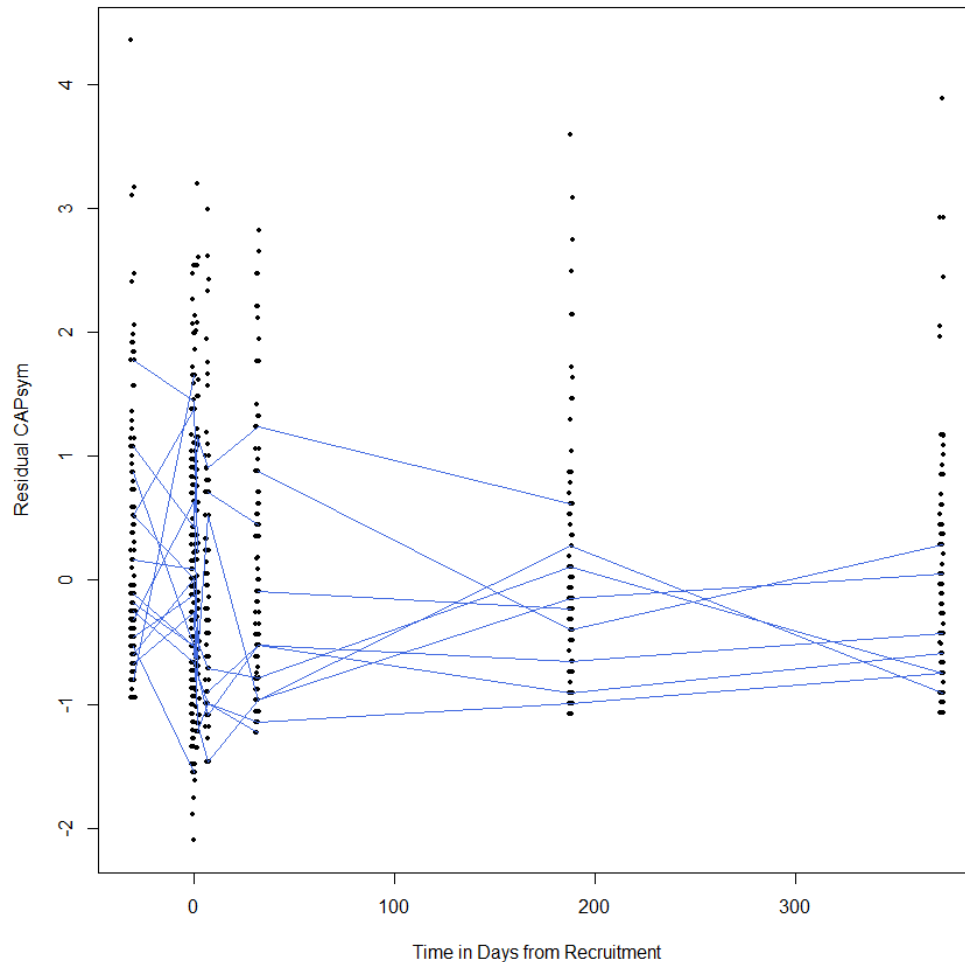


Figure 6.7 Variation in recovery revealed by residuals of CAP-sym scores

In figure 6.3 CAP-sym scores were transformed to residuals by the following formula:-

$$y^{*}_{ij} = (y_{ij} - \bar{y}_j) / s_j$$

where

y was a CAP-sym score

i was a particular subject

j referred to a given time-point

\bar{y}_j was the mean of all subjects' CAP-sym scores at time j

s_j was the standard deviation of all CAP-sym scores at time j

The means of each subject's residuals were then calculated and ranked. The traces above represent a selection of traces from the lowest mean residual to the highest. Within these, substantial variation in recovery pattern can be seen.

6.4.2 Base Non-linear Model

The following are the algebraic arguments for the initial non-linear model we used to describe the CAP-sym recovery data from the Pneumonia Aetiology and Severity Study (PASS). Let Y_{ij} denote the j th CAP-sym value for subject i and t_{ij} the corresponding time in days since admission. Model was:-

$$Y_{ij} = \alpha + \beta \exp(-t_{ij}/\gamma) + U_i + Z_{ij} \quad (1)$$

where α is the mean CAP-sym score at maximum recovery and $\alpha+\beta$ is the mean CAP-sym score at maximum symptoms. The parameter γ controls the average rate of recovery: the smaller the value of γ the more rapid the recovery. The exponential function captures the general shape of the recovery curve. $U_i \sim N(0, \nu^2)$ are independent subject-specific random intercepts and $Z_{ij} \sim N(0, \tau^2)$ are independent residuals. Regarding variance (Var), this model implied that $\text{Var}(Y_{ij}) = \nu^2 + \tau^2$ and that the correlation between pairs of CAP-sym measurements on the same subject was equal to ρ where

$$\rho = \nu^2 / (\nu^2 + \tau^2).$$

The above model was applied to the PASS CAP-sym values. The estimated values of the model parameters are shown in table 6.2

Table 6.2 Parameter Estimates for the Base Non-Linear Model of CAP-sym Scores

Parameter	Maximum Likelihood Estimates	Standard Error
α	13.7	0.9
β	19.0	0.9
γ	2.2	0.3
τ^2	87.3	5.8
ν^2	87.0	13.0

A plot of the above model is shown in figure 6.8 and a diagnostic plot of the residuals versus fitted values is seen in figure 6.9..

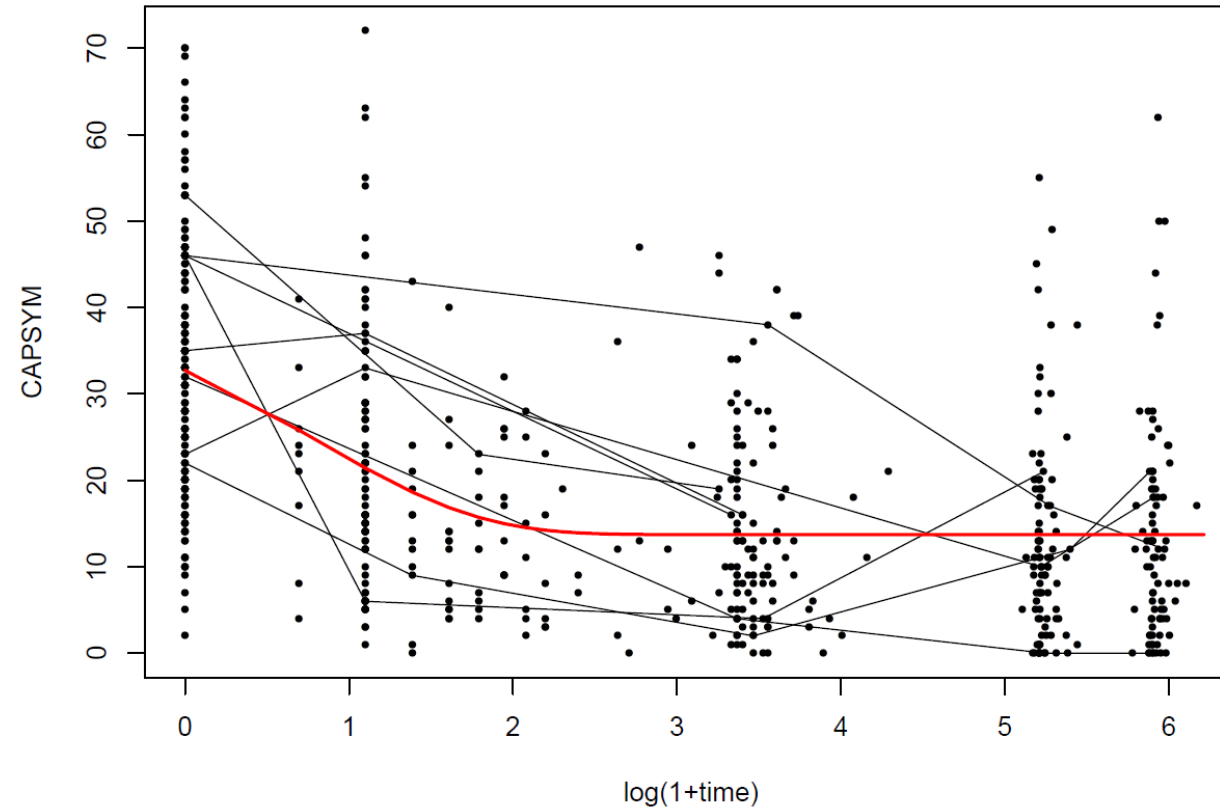


Figure 6.8 Non-linear Model of CAP-sym Scores from Admission to Recovery.

Dots represent the raw CAP-sym scores and black lines pick out the traces of 8 randomly selected subjects. The red line shows the trajectory of the mean CAP-sym score from the non-linear model (see 6.1.3) using the calculated parameters shown in table 6.2.

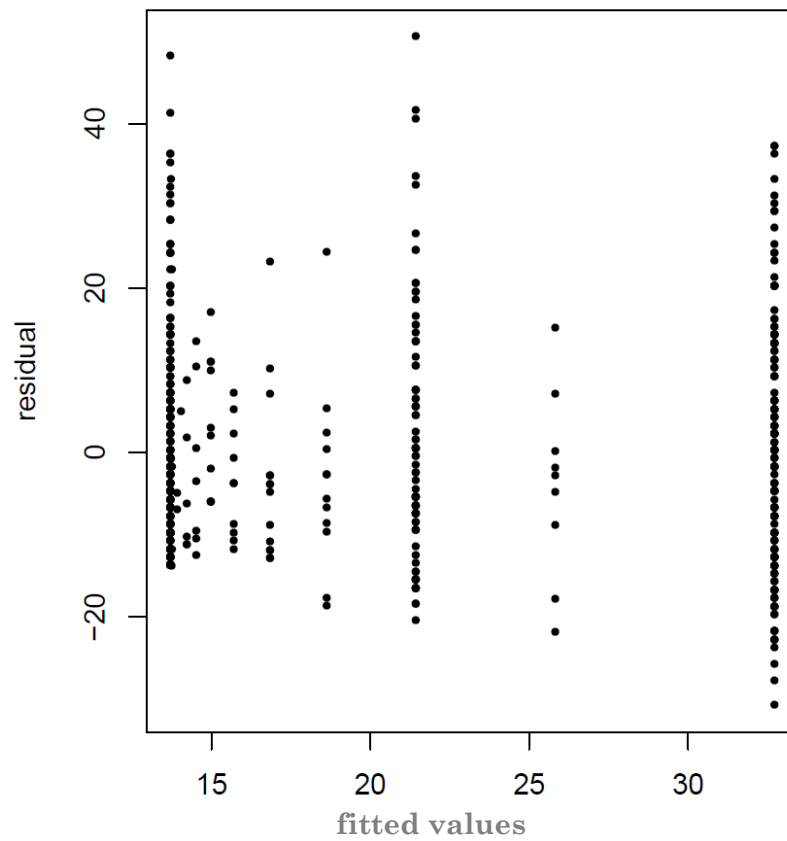


Figure 6.9 Diagnostic Plot for Initial Model: Residuals vs. Fitted

The Residual is calculated for all CAP-sym values and these are plotted against the values derived from the non-linear model. No systematic change in the distribution of residuals was seen across the fitted values suggesting the model was an acceptable fit.

6.4.3 Incorporating pre-morbid symptom level into the model

The model described in 6.4.2 did not take into account the recorded values for the CAP-sym score which subjects provided to represent how they felt 30 days prior to admission. The model of recovery was therefore refined by including these values so that recovery became the degree of symptom resolution with respect to a pre-pneumonia baseline level of symptoms. Thus the new model was:-

$$Y_{ij} = \begin{cases} \delta + U_{ij} + Z_{ij} : t_{ij} < 0 \\ \alpha + \beta \exp\left(-\frac{t_{ij}}{\gamma}\right) + U_{ij} + Z_{ij} : t \geq 0 \end{cases}$$

Where now δ is the average pre-pneumonia (t-30) CAP-sym score and the other parameters are as in equation (1) (see 6.1.3). From this it can be seen that, if α is bigger than δ , recovery is, on average, only partial, whereas if α is smaller than δ , on average patients' long-term state of health is better than their pre-admission state. This model is represented schematically in figure 6.10

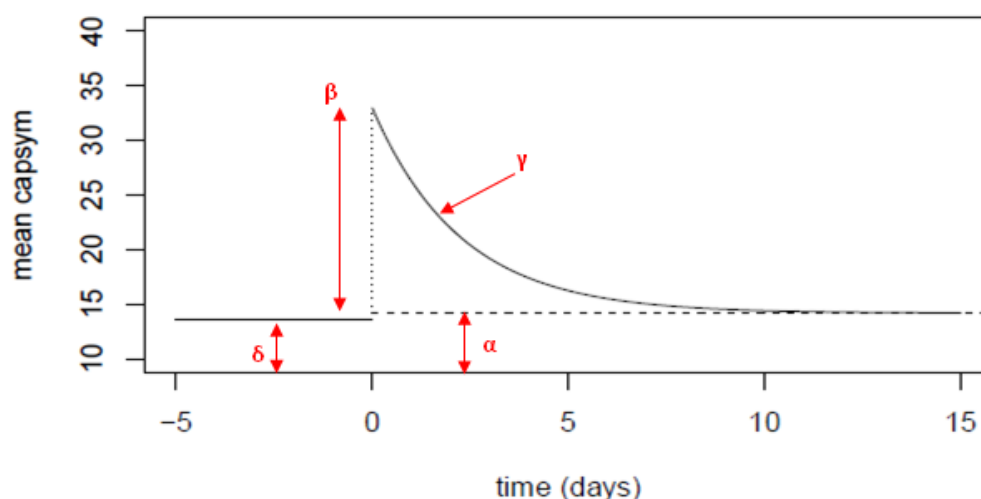


Figure 6.10 Schematic Representation of Non-linear model of CAP-sym Scores

6.4.4 Adding covariates to the model

Next we explored how clinical covariates affected the model. We chose to limit this analysis to an exploration of the degree to which the covariates affected the average CAP-sym score after recovery i.e. α in figure 6.6.

In the model formula in 6.4.4 α was replaced with α_i :-

$$\alpha_i = \alpha \exp(x_{1i}\theta_1 + \dots + x_{ki}\theta_k) \quad (2)$$

where

i denotes a subject

$\theta_1, \dots, \theta_k$ are parameters

x_{1i}, x_{ki} are the values of the k covariates for subject i

The covariates fitted to the model were prior statin use, prior pulmonary disease, smoking status (coded as 0,1,2 for never, quit and active) and age (which was centred at 65 years).

Table 6.3 Univariate Effects of Covariates on the Non-linear Model

Covariate	Maximum Likelihood Estimate	p
Age	-0.01	<0.001
Prior Pulmonary disease	-0.06	0.520
Prior Statin Use	-0.21	0.045
Smoking	0.17	0.013

From the data in table 6.3 it can be seen that the effect of age was to *reduce* the magnitude of α i.e. as people get older their maximum recovery to baseline was greater. The effect of smoking was in the opposite and led to an *increase* in α from never smokers

to smokers such that smokers had a larger residual level of symptoms than non-smokers.

Finally we performed a multiple regression to adjust each covariate for one another. We used a likelihood ratio test to judge the significance of each covariate in the presence of others. Results are displayed in table 6.4.

Table 6.4 Results of Multivariate Analysis of Covariates and CAP-sym Recovery

Covariates	Log-likelihood	p
None	2913.4	
Age	2902.7	<0.001
Age+Smoking	2901.1	0.069
Age+Smoking+statin	2900.6	0.351
Age+Smoking+Statin+Pulmonary Disease	2900.6	0.739

On the basis of this analysis we chose a model including the effects of age and smoking. The parameter estimates for this model are shown in table 6.5.

Table 6.5 Parameter Estimates for the Final Non-Linear Model of Recovery from CAP

Parameter	Estimate
α	11.649
θ (age)	-0.011
θ (smoking)	0.011
β	19.043
γ	2.229
δ	13.614
τ^2	89.910
ν^2	85.313

6.5 Discussion

6.5.1 Summary

Using the CAP-sym questionnaire we described the symptom kinetics of recovery from community-acquired pneumonia (CAP). Considering the group as a whole, subjects presented with a high burden of symptoms. These symptoms diminished rapidly over 7 days, resolved to near baseline by one month and remained at this level up to one year. On a subject by subject basis, patterns of recovery varied considerably. Non-linear modelling and multiple regression revealed that age and possibly smoking status influenced symptomatic recovery, but with opposite effects. Older people tended to recover more completely and smokers less so. Note, however, that the age effect is unequivocally significant ($p < 0.001$) whereas the smoking effect, after adjusting for age, does not reach the conventional 5% level of significance ($p = 0.069 > 0.05$)

6.5.2 What are the strengths of this work?

This is the first study to model symptomatic recovery from CAP. Previous studies have described mean recovery among groups of patients at a range of time-points.[53,55,59] However, most doctors are aware that some patients take longer to recover than others. And whilst these previous studies enable generalisations to be made about large groups of patients, they do not tell us about the causal factors involved in differential rates of recovery; nor do they help us inform individual patients as to their likely recovery trajectory. This study moves us closer to personalised medicine by modelling individual recovery trajectories through the random effect term U_i in equation (1) and the covariate adjustments in equation (2). The cohort has been shown to be

representative (chapter 3) and this enables us to generalise our results to similar cohorts and populations.

6.5.3 What are the limitations of this work?

6.5.3.1 *Exclusions*

The CAP-sym questionnaire requires patients to be able to answer questions and as a consequence some of the most sick – i.e. those who were expected to die soon after admission or those who were intubated – were excluded from the study. As a consequence, this is a study of moderate-to-severe CAP and cannot be extrapolated to the most severe cases. However, intubation itself is known to influence recovery and may have confounded our attempts to determine CAP-specific effects if these patients had been included.[207] Similarly those who were excluded because of cancer would have had their recovery confounded by the effects of the cancer. Larger studies powered to investigate differential effects in these sub-groups could confirm these effects.

6.5.3.2 *Model parameterisation*

The statistical techniques used in the work were advanced. As a consequence we limited the analysis in several ways. The final model allows covariates to influence alpha – the long-term level of symptom recovery – but not the other recovery parameters e.g. rate of recovery (gamma) or peak symptoms (alpha+beta). Moreover, in the final model the baseline symptom score, delta, with which alpha can be compared is not allowed to depend on covariates and so is effectively an average for the group as a whole. This is clearly an un-representative way to parameterise the model. As such the final model is best considered as a proof of

principle for the technique and could be refined with further work, including investigation of how covariates affect each of the parameters in the model. However, a cautionary point is that over-elaborate models fitted to sparse data tend to have poor predictive performance, hence extension of the model may need data from a larger cohort of patients.

6.5.4 Clinical implications

The finding, albeit not conventionally significant, that smoking is likely to lead to worse symptomatic recovery from CAP enables us to make patient-centred decisions and give personalised advice. It delineates a group of patients who may gain greater benefit from clinical follow-up and active rehabilitation. It may also enable future research to be focussed on the mechanistic links between smoking and symptoms, which may in turn lead to therapeutic trials.

The finding that as you get older you are more likely to fully recover from CAP is at first perplexing since 'traditional' outcomes, such as mortality, are unquestionably worse in the elderly. There are several possible explanations for this result. The first is that it may be an artefact of our cohort and study design. It is possible that older people who volunteered for PASS were generally fitter than average and therefore predestined to do well. Certainly the in-patient mortality in PASS was lower than in some previously reported studies (table 3.5). Against this is the fact that, once cancer and those with terminal disease at presentation were accounted for, PASS was similar with respect to other outcomes (length of stay mortality and re-admissions) to other cohorts (table 3.5 and section 3.1.16). The age range of

PASS was representative of CAP in general and the level of co-morbidity was high rather than low. An alternative explanation is that this is a true result and reflects something particular about how patients report symptoms. It is possible that the elderly are more stoical and under-report symptoms when compared with their younger counter-parts. Several studies have reported this phenomenon previously.[208] This is potentially very important as our population is aging and rates of CAP in the elderly are high and increasing.[14] It will be important in future studies to carefully link symptoms with physiology and biomarkers of inflammation since low levels of reported symptoms may mask significant underlying disease. It may be that in the future, as some other authors have suggested, we should conceptualise pneumonia as a different disease in the elderly compared to the young.[209]

7 DISCUSSION

7.1 Key findings with reference to the stated aims

The aim of this thesis was stated in 1.9:-

‘to explore how efferocytosis and sputum microbiota vary depending on the clinical characteristics of patients with CAP and to relate these to symptomatic recovery’.

We found that in a cohort recovering from CAP, symptomatic recovery improved as patients got older but was worse in smokers than non-smokers. Smoking reduced the rate of efferocytosis but statins increased it and efferocytosis increased as BMI increased. The cohort had high rates of smoking and consequently high rates of underlying lung disease and *Haemophilus* was the dominant bacterial genus identified in the sputum. The sputum microbiota was less diverse when underlying pulmonary disease was present. These host-pathogen-symptom interactions are shown schematically in figure 7.1.

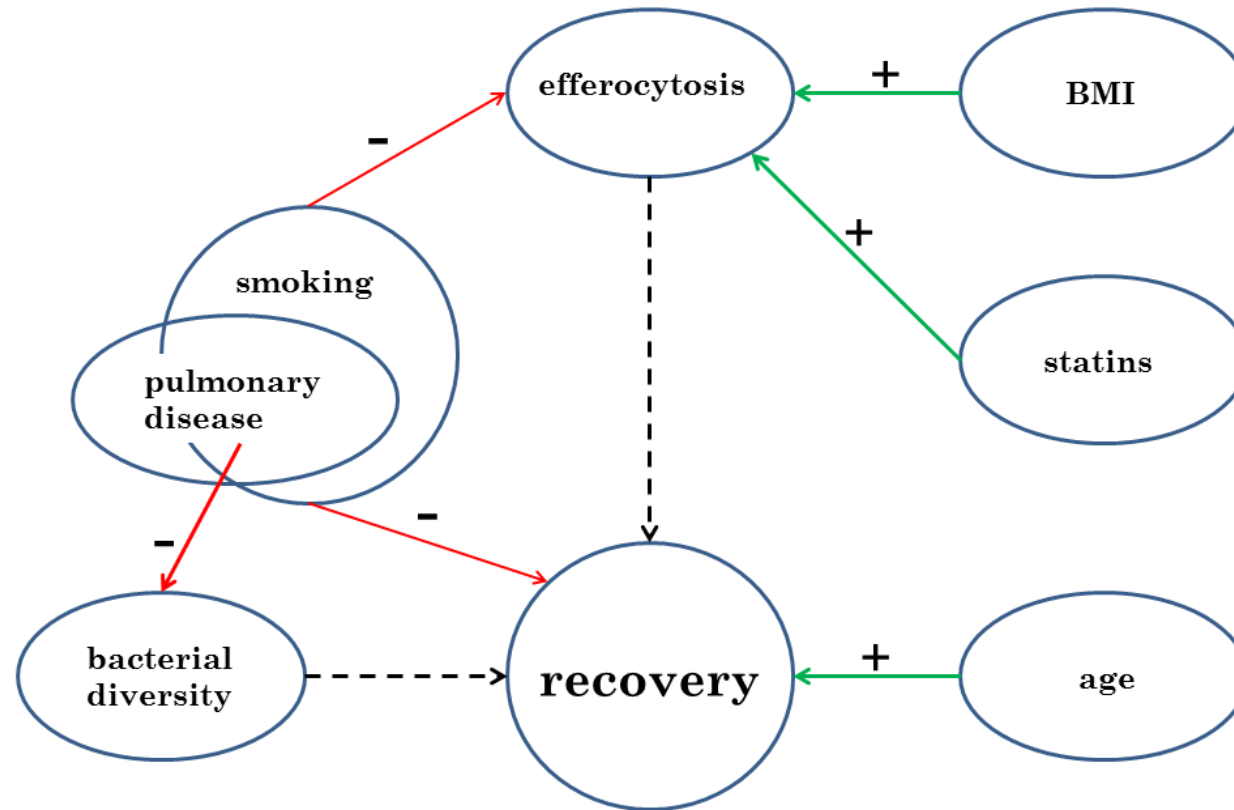


Figure 7.1 Schematic Representation of Host Pathogen Interactions in CAP Recovery
 Solid lines indicate a measured effect and dashed lines indicate a hypothetical interaction.

7.2 Strengths of this work

7.2.1 Efferocytosis

This is the first study to investigate ex-vivo efferocytosis during recovery from CAP. Patients were representative, alveolar macrophages were immediately cultured post-bronchoscopy, neutrophils were autologous and cytoplasmically stained rather than surface labelled. Multiple replicate wells enabled us to account for the experimental component of stochastic variation in the model and multiple regression accounted for confounding clinical variables. This comprehensive range of methodologies has not been combined previously in efferocytosis studies and the results are robust and novel.

7.2.2 Sputum microbiota

This was the largest CAP microbiota study and the first to link clinical variables to ecological measures of diversity in acute samples. Sputum samples were rapidly frozen and unadulterated by pre-processing prior to DNA extraction. The DNA extraction was mechanical and therefore un-biased and PCRs were quadrupled to enhance coverage of rare species. We removed contaminating OTUs and applied a cutting edge compositional analysis package. The size and rigour applied to this analysis yielded novel insights into the spectrum of bacteria in CAP sputum and will be highly relevant data on which to base future study designs.

7.2.3 Recovery by CAP-sym score

This is the first study to create a longitudinal model of symptomatic recovery from CAP. Previous studies of symptom recovery have compared mean group symptoms to generalise

about recovery at various time points. This study went further and by accounting for repeated measures on the same individual and created a model that could be used to explore the influence of clinical covariates. The model has raised important questions about the validity of comparing symptoms across the age spectrum and has provided useful framework for generating future hypotheses.

7.3 Limitations of this work

7.3.1 Potential for bias in this work

7.3.1.1 Low eligibility rates

PASS limited its recruitment to Monday to Friday 9am to 4pm due to resource constraints. As a consequence a large proportion of subjects treated for CAP by the hospitals during the time of PASS recruitment were ineligible. Some of the patients 'treated as CAP' would not have met our strict definition of CAP but the greatest proportion of ineligibility was a consequence of our strict adherence to the recruitment of patients early in their admission. This was scientifically beneficial but potentially limits the applicability of the results if those who could not be recruited represented a systematic bias. It is well known that patients who are admitted to hospital at night or at the weekend have worse outcomes and PASS in-patient mortality was low when compared to the national BTS CAP audits. However, in most other respects PASS subjects were remarkably similar to those audits and other published observational studies. Moreover, when malignancy, dementia and those who were moribund at presentation were accounted for PASS outcomes were very similar to other cohorts.

7.3.1.2 *Efferocytosis*

Since all the efferocytosis work was carried out by the author who also recruited, bronchoscoped and followed-up the study subjects it was predicted that I would have insight into the clinical background and recovery rate of the patient. There were three stages at which this knowledge could have introduced bias into the assay. The first was during the 'wet bench' section of the work. That involved isolating and culturing alveolar macrophages and then co-culturing the macrophages with neutrophils. It was during the co-culture that efferocytosis occurred but this process was not amenable to experimental influence and so was perceived to have little susceptibility to bias. Replicate wells of the efferocytosis experiment generated remarkably tight results indicating low levels of experimental variation. The second stage of the assay involved measuring the efferocytosis using flow cytometry. The measurement was performed mechanically along pre-set parameters and was therefore was not susceptible to researcher bias. The third stage of the assay was the analysis of the output data from the flow cytometry work. This involved a subjective, visual inspection of the pattern of the data and was potentially highly susceptible to researcher bias. For that reason the data was batch analysed at later date when there was less possibility of researcher recall of the recovery characteristics of individual, anonymously coded study subjects.

7.3.1.3 *Sputum microbiota*

Regarding the 16S rRNA PCRs and amplicon purification, these samples were all anonymised and multiplexed and there was little opportunity for the introduction of researcher bias at this stage.

Sequencing was performed by an external lab with no prior knowledge of the study design and subject characteristics. Likewise the assignment of OTU identities and the building of the phylogenetic tree that underpinned the data-set were performed by a researcher who was entirely removed from all aspects of the data other than the sample ID codes. Thus compartmentalisation of the microbiota work and the handling of samples in large batches will have effectively blinded the handlers to the data and reduced experimental variation. Two significant and unaccounted for areas of bias remain with the sputum microbiota work. One is that half the patients in PASS did not contribute to this data as a consequence of not producing sputum. This is not unusual for CAP but does mean that we have only described the microbiota in sputum producers and it is possible that non-sputum producers have a different spectrum of bacteria especially given the trends towards increased rates of pulmonary disease and smoking among the sputum producers. Future studies aimed at identifying the spectrum of potential pathogens in CAP will have to specifically address the issue of non-sputum production in their design. The second remaining bias in this study is the timing of samples with respect to antibiotic. All the subjects here had received at least one dose of antibiotic prior to providing a sputum sample. This is very likely to have altered the results. The effect of pre-hospital antibiotics was investigated in the linear regression and was not significant even as a univariate. Future studies could target patients in primary care presenting with CAP whilst antibiotic naïve; or in hospital, closely link to the initial triage teams so that patients with respiratory tract infections are sampled prior to antibiotic.

7.3.1.4 *CAP-sym bias*

Questionnaire data is prone to intra-subject bias and inter-observer bias. However the intra-subject variation was accounted for in the statistical modelling and during validation studies the CAP-sym questionnaire was shown to have low levels of variation between investigators.

7.3.2 **Limitations with respect to the study aims**

It was an ambition of this work to draw direct associations between efferocytosis, the bacterial ecology of sputum and patient symptoms. However the study was limited in its scope and findings by lower rates of recruitment than expected, the high level of comorbidities which rendered most subjects ineligible for bronchoscopy and by the fact only half of the subjects could produce sputum. As a consequence the CAP-sym model could not incorporate measures of efferocytosis or bacterial diversity as the numbers were too small to deliver meaningful answers. As a consequence, as indicated in figure 7.1, associations between symptomatic recovery, efferocytosis and bacterial diversity are hypotheses rather than direct estimates. In that diagram I hypothesise that the measured negative effect of smoking on symptoms may be mechanistically mediated by the measured negative effect on efferocytosis. In a similar fashion it may be that the reduced diversity seen in the sputum of those with pulmonary disease is mechanistically responsible for some of the negative effect of smoking on symptomatic recovery. What I have learnt from this study is that to generate enough data to directly test these associations would require a study of a different order of magnitude – based at multiple sites. However, the data

presented here are the ideal preliminary dataset to support applications to fund such ambitious studies.

7.4 How has this thesis contributed to field?

There is only a small literature regarding recovery from CAP. What studies there are have limited themselves to summary descriptions of whole cohorts. We went further and defined how clinical characteristics influence recovery. Moreover, no prior studies have married symptomatic recovery to host or pathogen mechanistic factors. The data presented here pave the way for larger future studies aimed at devising pro-recovery strategies tailored to the characteristics of individual patients.

7.4.1 What are the implications for policy and clinicians?

Current clinical guidelines offer very limited guidance regarding the management of recovery from CAP which reflects the limited amount of research in this area. The recently published NICE guidelines on pneumonia specifically excluded follow-up and post discharge management from their remit although they do specify recovery milestones. However the only specificity associated with these recovery targets is the suggestion that those with more severe illness may take longer to recover and this is based on studies mostly rated low-quality.[10] The British Thoracic Society Guidelines on Pneumonia suggest all patients should have a follow-up visit either in hospital or with a GP at 6 weeks.[6] The finding from our study that smoking has a negative impact on symptomatic recovery implies a change may be warranted to this current 'one size fits all' approach to managing the aftermath of CAP and that smokers should be treated differently to non-smokers. But if smokers deserve special attention what should

this be? Our study was not an intervention study and therefore does not provide the definitive answer but rather offers hypothesis generating preliminary results which may pave the way for further work (see 7.5).

The overwhelming weight of evidence from previous studies of the aetiological causes of CAP suggests that the most common bacterial pathogen is *Streptococcus pneumoniae* and this heavily influences antibiotic prescribing guidelines. However, the majority of this evidence was derived from studies where the detection of bacterial pathogens was limited to culture where some bacteria are more easily cultured than others and some cannot be cultured at all. There is therefore an inherent detection bias associated with our current understanding of the bacterial spectrum of CAP. Our study suggests that, in a cohort with high rates of smoking and lung disease, bacteria of the genus *Haemophilus* are much more abundant in the sputum of patients with CAP than was previously thought. It should be noted that this link does not imply causality and future studies would be needed to determine this.

7.5 Ongoing Research

A number of questions relating to the work described in this thesis are currently being pursued. Collaborative grants are being developed to investigate the microbiota of respiratory samples from patients with severe CAP that required ventilation in intensive care; to refine the CAP-sym model in large more diverse cohorts and to generate further insights into the effect of statins on macrophages and outcome from CAP. Another study will to explore patients' experiences of CAP managed by the NHS

with the aim of better understanding the processes and communication that frustrate or enhance the experience of being treated for CAP across the primary secondary care interface.

8 APPENDIX 1: PASS DOCUMENTATION

The following pages contain copies of regulatory documentation drawn from the 'Site Master File' for to the Pneumonia Aetiology Study (PASS).

National Institute of Health Research funding letter regarding the Doctoral Research Fellowship that funded PASS p218

Independent Scientific Review of DRF application p219 -220

NHS Research Ethics Committee approval for PASS p221 -222

Front section of contract between funder and sponsor of PASS p223

PASS patient information leaflet (PIL) p224-228

PASS patient information leaflet for bronchoscopy p229-232

PASS consultee information leaflet p233-238

PASS consent form p239

PASS consultee declaration form p240



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9th September 2010

Dr. Dan Wootton
Specialist Registrar Respiratory and General Medicine
Department of Respiratory Medicine
University Hospital Aintree

Dear Dr. Dan Wootton

Doctoral Fellowship - awarded August 2010

Our ref: NIHR-DRF-2010-03-154

Thank you for accepting your NIHR Fellowship award.

This is to formally confirm that the NIHR is currently preparing a contract between the Department of Health and University Hospital Aintree. This contract will support you to start your NIHR DRF on 1st Nov 2010 for a duration of 36 months.

In the meantime we are looking in detail at the finances of your award, the original submitted total of which was £223,617. NIHR TCC reserves the right to reject any costs that it considers unreasonable or not fully justified. We will be in touch if we have any queries. A payment schedule forms part of the contract and will set out the dates when your host institution will receive payment, quarterly in arrears. Once the contracts have been signed, the first payment will be released at the next quarterly payment date.

Individuals can, with the agreement of their employing organizations, establish start dates which fall before the formal contracting process is complete.

This award letter is a commitment from the NIHR to support you in a Fellowship.

If I can be of any further assistance, please do contact me.

Yours sincerely

Karen Fernando

Programme Manager

NIHR Trainees Co-ordinating Centre
 NIHR Fellowship Scheme Round 3 – Doctoral Research Fellowship
 SHORTLISTING
 Panel Assessment Form for Feedback to Applicants

Panel Member:**Applicant:** Dan Wootton**Application No:** NIHR/DRF/2010/03/154

Please read the application and:

1. Complete the check boxes (strong/average/uncompetitive) below.
2. For each section please provide a score on the following scale (1-5):
- 5: EXCELLENT 4: VERY GOOD 3: SATISFACTORY 2: UNCOMPETITIVE 1: POOR
3. At the end of the form please provide an overall recommendation of A; Definitely shortlist, B; Not sure or C; Reject.

Applicant	Strong	Average	Uncompetitive	N/A
Previous research experience or formal training (e.g. Masters)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Relevance of previous research experience	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Output from previous research experience	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Evidence of commitment to a research career	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Evidence of potential as a career researcher	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Score (1-5):	5			
Comments: An SR in Respiratory Medicine. No intercalated BSc but a strong candidate with some excellent research experience in challenging studies into infectious disease in Africa.				
Proposed Research	Strong	Average	Uncompetitive	N/A
Quality of proposed research project	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Scope of project suitable for PhD	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Relevance to the NHS of the proposed research	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Appropriateness of chosen research methods & design	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Appropriateness of statistical component(s)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Likely impact of research	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Where already registered, quality of doctoral research undertaken to date	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Score (1-5):	5			
Comments: A well thought out project addressing an important clinical question. The study will be nested within an existing, NIHR-funded study within the Liverpool BRC making recruitment feasible. Good collaborations have been established to support the molecular aspects. My only question is who will be carrying out the efferocytosis assays. Are they being run by the candidate, or is this what the service support costs of £78k are for?				

Site, Training and Supervision	Strong	Average	Uncompetitive	N/A	
Suitability of proposed training programme for proposed research	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Suitability and appropriate experience of proposed supervisor(s)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Suitability of the proposed academic host and institutional support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Score (1-5):	5				
Comments: The candidate has identified a series of training activities that suggest a good understanding of the requirements to carry out this study successfully. Environment and supervision are sound.					
Finance	Strong	Average	Uncompetitive	N/A	
Understanding of cost of proposed research (Section C, Part 7)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Justification for funding requested	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Overall value for money	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
Score (1-5):	4				
Comments: Two questions; (i) have the costs for the efferocytosis assays been included (assume so as part of the £11,160 consumables costs but this is not made clear. There is mention of support from UHA but this may need to be clarified at interview (ii) £13,500 seems a bit expensive for an incubator and -80 freezer.					
Overall recommendation – Please tick one of these boxes				Definitely shortlist (A)	<input checked="" type="checkbox"/>
				Not sure (B)	<input type="checkbox"/>
				Definitely reject (C)	<input type="checkbox"/>
Any other comments:					

NIHR Trainees Co-ordinating Centre
 NIHR Fellowship Scheme Round 3 – Doctoral Research Fellowship
 SHORTLISTING
 Panel Assessment Form for Feedback to Applicants

Panel Member:
Applicant: Wooton **Application No:** NIHR/DRF/2010/03/154

Please read the application and:

1. Complete the check boxes (strong/average/uncompetitive) below.
 2. For each section please provide a score on the following scale (1-5):
 - 5: EXCELLENT 4: VERY GOOD 3: SATISFACTORY 2: UNCOMPETITIVE 1: POOR
 3. At the end of the form please provide an overall recommendation of A, Definitely shortlist, B, Not sure or C, Reject.
 4. If you feel unable to complete this form for an applicant, please notify us (to lynne.penistone@nhrcc.org.uk) as soon as possible, but before **24th February 2010**. Please provide the name, institution and email address of 2-3 potential reviewers.
 5. Return completed forms to lynne.penistone@nhrcc.org.uk by **12th April 2010**.
- Your comments are necessary to inform the provision of anonymised feedback**

Applicant	Strong	Average	Uncompetitive	N/A
Previous research experience or formal training (e.g. Masters)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Relevance of previous research experience	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Output from previous research experience	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Evidence of commitment to a research career	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Evidence of potential as a career researcher	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Score (1-5):	5			

Comments:

Proposed Research	Strong	Average	Uncompetitive	N/A
Quality of proposed research project	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Scope of project suitable for PhD	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Relevance to the NHS of the proposed research	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Appropriateness of chosen research methods & design	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Appropriateness of statistical component(s)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Likely impact of research	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Where already registered, quality of doctoral research undertaken to date	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Score (1-5):	4			

Comments:

Site, Training and Supervision	Strong	Average	Uncompetitive	N/A
Suitability of proposed training programme for proposed research	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Suitability and appropriate experience of proposed supervisor(s)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Suitability of the proposed academic host and institutional support	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Score (1-5): 5

Comments:

Finance	Strong	Average	Uncompetitive	N/A
Understanding of cost of proposed research (Section C, Part 7)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Justification for funding requested	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Overall value for money	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Score (1-5): 3

Comments:

Overall recommendation – Please tick one of these boxes

Definitely shortlist (A)	<input checked="" type="checkbox"/>
Not sure (B)	<input type="checkbox"/>
Definitely reject (C)	<input type="checkbox"/>

Any other comments:



GIG
CYMRU
NHS
WALLES

Bwrdd Iechyd Prifysgol
Betsi Cadwaladr
University Health Board

North Wales Research Ethics Committee (Central and East)

G1/G2 Croesnewydd Hall
Croesnewydd Road
Wrexham Technology Park
Wrexham
LL13 7YP

Tel: 01978 726377

11 July 2011

Dr Stephen Gordon
Reader in Tropical Respiratory Medicine
Liverpool School of Tropical Medicine
Respiratory Infection Group
Liverpool School of Tropical Medicine
Pembroke Place
L35QA

Dear Dr Gordon

Study title: Pneumonia Aetiology and Severity Study (PASS): Study One. What are the causes of delayed recovery from community acquired pneumonia (CAP) in adults and could new technology for diagnosis and severity assessment play a role in improving recovery from CAP?
REC reference: 10/WNo03/40
Amendment number: AM02
Amendment date: 09 June 2011

The above amendment was reviewed at the meeting of the Sub-Committee held on 08 July 2011.

Ethical opinion

The Committee queried what mechanism was in place should any abnormalities arise in a healthy volunteer following bronchoscopy.

Following clarification of the strategy from Dr Wootton the committee requested a revised participant information sheet. The Committee approved the revised information sheet for healthy volunteers Version 1.1 08.06.11. Please ensure that the accompanying Consent Form refers to the correct version number.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Advertisement	1	08 June 2011
Participant Consent Form: Healthy volunteer for bronchoscopy	1	08 June 2011
Participant Information Sheet: Healthy volunteer for bronchoscopy	1.1	08 June 2011
Participant Information Sheet: Consultee Information Sheet	2	08 June 2011
Protocol	3	07 June 2011
Notice of Substantial Amendment (non-CTIMPs)		09 June 2011
Covering Letter		09 June 2011

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

10/WNo03/40:	Please quote this number on all correspondence
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Yours sincerely



PP

Professor Alex Carson
Chair

E-mail: Tracy.Hughes4@wales.nhs.uk

Enclosures: List of names and professions of members who took part in the review

Copy to: Mrs Michelle Mossa
Clinical Sciences Centre for
Research & Education
University Hospital Aintree
Lower Lane
L9 7AL

Dr Dan Wootton
Respiratory Research
3rd Floor Clinical Sciences Centre
University Hospital Aintree
Lower Lane
L9 7AL

North Wales REC (Central and East)

Attendance at Sub-Committee of the REC meeting on 08 July 2011

Name	Profession	Capacity
Professor Alex Carson	Associate Dean (Research)	Lay Plus
Mr Philip Richards	Associate Specialist - Surgery	Expert

Also in attendance:

Name	Position (or reason for attending)
Ms Tracy Hughes	Research Ethics Committee Co-ordinator

RESEARCH AGREEMENT

BETWEEN

SECRETARY OF STATE FOR HEALTH (1)

AND

**AINTREE UNIVERSITY HOSPITALS NHS
FOUNDATION TRUST (2)**

1

**SECTION 1
FORM OF AGREEMENT**

The Agreement is made between

THE SECRETARY OF STATE FOR HEALTH of Richmond House, 79 Whitehall, London, SW1A 2NS ("the Authority")

and

AINTREE UNIVERSITY HOSPITALS NHS FOUNDATION TRUST, of Longmoor Lane, Liverpool, L9 7AL ("The Contractor")

The Authority and the Contractor being together called "the Parties".

IT IS AGREED THAT:

1. The Contractor will host Dr Daniel Wootton (DRF-2010-03-154) to complete a National Institute for Health Research (NIHR) Doctoral Research Fellowship in accordance with the research and training programme specified in Section 3.
2. The Authority will pay the Contractor the Approved Cost as set out in Section 4 in respect of completing the Doctoral Research Fellowship. No payments will be made until the approvals sought pursuant to Condition 12 of Section 2 are obtained unconditionally.
3. This form of Agreement (Section 1) together with the attached Sections 2 to 6 inclusive are the documents which collectively form "the Agreement" (as defined in Section 2).
4. The contract effected by signing of this Form of Agreement constitutes the entire agreement between the Parties relating to the subject matter of the contract and supersedes all prior negotiations, representations or understandings.

2

Pneumonia Aetiology and Severity Study (PASS)

Participant Information Sheet

We would like to invite you to take part in our research study. Before you decide you should understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. One of our team will go through the information sheet with you and answer any questions you have.

What is the purpose of the study?

Pneumonia is an infection of the lungs which can be caused by a number of different bacteria and viruses. Traditionally patients have been told by their doctor that it takes 6 weeks to fully recover from pneumonia. It is now clear that some patients recover more quickly than this while others take much longer. In this study we want to investigate a group of patients who are admitted to hospital with pneumonia and follow their progress over 12 months to investigate reasons for different patterns of illness.

It is possible that particular bacteria or viruses or combinations of both lead to more severe pneumonia and a more prolonged recovery. In our study we will perform state of the art tests to try to find out if there are differences in the organisms present in the lungs of people with different patterns of pneumonia illness. We will investigate ways of predicting which patients become severely unwell and who takes longer to recover. For example it may be that some people's genetic make-up predisposes them to slow recovery from pneumonia if they have certain bacteria in their lungs. Investigating this will involve taking samples and recording clinical information at several points during admission and following discharge from hospital. We hope that the information generated by this study will lead to the development of new therapies to treat and improve recovery from pneumonia in the future.

Why have I been invited to take part?

We aim to recruit 400 patients with pneumonia for this study. Our research team works with doctors and a specialist nurse to identify suitable patients for the study. You have been invited to take part because the doctors looking after you think you have pneumonia.

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
E-mail: michelle.mossa@aintree.nhs.uk

Research & Development Directorate
University Hospital Aintree
Room 2.06, Clinical Sciences Centre
Liverpool
L9 7AL

Do I have to take part?

It is up to you to decide on whether to join the study once you understand what it involves. If you agree to take part we will ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive. You will be able to choose if samples and information collected up to that point may be used or should be destroyed.

What will I have to do?

This research study involves gathering information about you and your symptoms and collecting clinical samples such as blood, sputum and a salt-water mouthwash. If you agree to take part in this study, you will be seen by a member of the research team on several occasions during your treatment in hospital and recovery at home.

What will happen to me if I take part?

The research study will run alongside your routine hospital care and you will not need to spend any longer in hospital than normal. Soon after your diagnosis of pneumonia a member of the research team will review your medical notes and ask you about your symptoms and your past medical history. They will ask to take some clinical samples and **table 1** has details of when the various samples will be taken. You will be seen again approximately 48 hours later for another review and further samples and then again on the day of your discharge.

Following discharge from hospital you will be invited to attend appointments 1, 6 months and a year later in the out-patient department on convenient dates. At these appointments, a member of the research team may take further samples, will question you about your symptoms and may ask you to have some breathing tests and an x-ray. These assessments are designed to monitor your recovery in greater detail than is current practice. We would like to phone your GP and then you one year after your admission to hospital to review your symptoms and find out about any other medical events that have occurred. If during that phone call you tell us you do not want to attend the one year follow-up visit we will offer you the option of going through the symptom questionnaire over the phone.

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
E-mail: michelle.mossa@aintree.nhs.uk

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Liverpool
L9 7AL

Expenses and payments

You will receive a modest financial reimbursement for your time and inconvenience from participation in the study. This will be calculated in the following way:

Participation with study procedures during the in-hospital stay.	£20
Participation with the 1 month follow up-visit	£20
Participation with the 6 month follow up visit	£20
Participation with the 12 month follow up visit	£10

Table 1 – Research study samples

	Within 24 hours of admission	48 hours after 1 st assessment	1 month after discharge	6 months after discharge
Blood samples	✓	✓	✓	✓
Sputum (phlegm) sample	✓		✓	✓
Urine sample	✓			
Mouthwash	✓		✓	✓
Urine	✓			

What are the possible disadvantages of taking part?

You will be asked to have additional tests (blood, sputum, mouthwash, urine, x-rays and blowing tests) as part of this study. When you are in hospital we will not repeat tests already requested by your team of doctors.

What are the possible benefits of taking part?

The study is unlikely to benefit you directly but you will have the reassurance of being monitored by pneumonia experts more closely than would normally be the case. A proportion of people who get pneumonia do so because of a previously unrecognised abnormality in their lungs and it is possible our in-depth tests may detect this.

What will happen if I don't want to join or carry on with the study?

Taking part in the study is entirely voluntary. You are free to withdraw at any time and do not have to give a reason for this. This will have no effect on your medical care now or in the future. You will be able to choose if samples and information collected up to that point may be used or should be destroyed.

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R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
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What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you wish to complain formally, you can do this by contacting the Research Governance office at Aintree University Hospital. The contact person is Mrs Michelle Mossa 0151 529 5871. In the unlikely event that you are harmed during the research due to someone's negligence then you may have grounds for a legal action against Aintree University Hospitals NHS Foundation Trust. The normal NHS complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you will be kept strictly confidential. It will be stored securely within the Aintree University Hospital with anonymised samples also being stored at Liverpool School of Tropical Medicine. Access to your personal information collected in the study will be restricted to authorised research staff.

Involvement of the General Practitioner/Family doctor (GP)

We will write to your GP to tell them that you have been involved in the project and will be seen for follow up in our pneumonia clinic and we will keep them informed of your recovery.

What will happen to the samples that I give?

The samples that you give during this study will be used to find out more about the bacteria and viruses that cause pneumonia and how your body responds to them. The samples will be labeled with a code number only. It will not be possible for persons outside of the research study to trace these samples back to you. We would also like to keep any excess sample material to use in future ethically approved studies that are related to the aims of this project. You could ask for these samples to be destroyed at any time now or in the future.

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
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E-mail: michelle.mossa@aintree.nhs.uk

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What will happen to the results of the research study?

At the end of the study, we will send you a short report of our findings. This will be a summary of all participants' results and it will not be possible to derive any specific information about your tests from this. The results of this research study will be presented at scientific meetings and published in medical journals.

Who is funding the research?

This study is jointly funded by the Royal Liverpool and Broadgreen University Hospitals NHS Trust and the National Institute of Health Research.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by North Wales Research Ethics Committee (Central & East).

Further information and contact details

If you have any further questions please contact either of the following members of the research team during normal working hours.

Dr Dan Wootton (Principle Investigator)

Tel: 0151 529 5932 Email: dwootton@liverpool.ac.uk

Dr Stephen Gordon (Chief Investigator)

Tel: 0151 705 2579 Email: sbgordon@liverpool.ac.uk

Pneumonia Aetiology and Severity Study (PASS)

Additional Information Sheet for Research Bronchoscopy

As part of the study we would like to invite you to undergo a test called a bronchoscopy. Before you decide whether to have this test please take time to read the following information carefully. One of our team will go through the information sheet with you and answer any questions. This information sheet should be read alongside the Pneumonia Aetiology and Severity Study (PASS) Participant Information Sheet.

What is the purpose of this research bronchoscopy?

After having pneumonia, some patients are fully back to normal quickly whilst others take longer to recover. The removal of infected material and damaged lung cells is an important part of recovery from pneumonia and is achieved by the lungs' immune system. We want to investigate if there is any difference in these immune functions between people who recover from pneumonia quickly and those who recover more slowly. As part of the research study we plan to collect lung samples from a group of patients who have recently been admitted to hospital with pneumonia and the best way to do this is via bronchoscopy. Knowing the reasons why recovery from pneumonia is delayed in some people will help us to develop new treatments to improve recovery.

Why have I been invited to take part?

Previously you agreed to take part in a research study about pneumonia. During the study some patients will be classified as having 'rapid recovery' and others as 'delayed recovery'. In order to understand the difference in recovery times we are inviting all study participants to have a bronchoscopy so we can investigate differences between the two groups.

What are the possible benefits to me of taking part?

In a few people the research bronchoscopy may identify an unexpected abnormality in the lungs. This is more likely if you smoke. If this occurs you will be referred to the Respiratory Medicine Department of Aintree University Hospital.

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
E-mail: michelle.mossa@aintree.nhs.uk

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Will I get paid for taking part?

You will be offered £100 as reimbursement and for any inconvenience as a result of the bronchoscopy.

Do I have to take part?

It is up to you to decide whether to join this part of the study. If you agree to take part we will then ask you to sign a consent form but after signing you remain free to change your mind at any point before the bronchoscopy without giving a reason. Declining to have a bronchoscopy does not affect your participation in any other aspect of the study or the standard of medical care you receive.

What is a bronchoscopy?

A bronchoscopy involves passing a thin, flexible tube called a bronchoscope through the nose or mouth and into the breathing tubes (bronchi). We will take samples from the lungs to investigate their functions and the bronchoscope contains a video camera so we can look at the breathing tubes. One of the samples we take is called a 'lavage' and involves passing some salty water into the breathing tubes. The water is then gently sucked back up and is sent for analysis. Another sample is obtained using a tiny brush which is passed down the bronchoscope to remove a small number of cells for analysis. If we see an unexpected abnormality in your breathing tubes we will take samples (biopsies) using tiny forceps which are passed through the bronchoscope. You can't feel biopsies which are standard medical practice when abnormalities are seen during any bronchoscopy.

How long is the test and is there anything I need to do beforehand?

The test is an out-patient procedure. You must not eat for 4 hours before the bronchoscopy but can have water to drink up to 2 hours prior to the bronchoscopy then nothing by mouth for two hours before the bronchoscopy. It takes around 7 minutes to complete the bronchoscopy. You then have 2 hours in our recovery area for monitoring.

What time commitments are involved in this part of the study?

If you decide to join this part of the study, in addition to the appointments described in the main study information sheet, we will ask you to attend hospital for the bronchoscopy and we will book a time with you to receive a phone call to get your feedback on the procedure.

What will happen to the samples that I give?

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
E-mail: michelle.mossa@aintree.nhs.uk

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The samples that you give will be labelled with an anonymous code and stored in secure facilities at Aintree Hospital and the Liverpool School of Tropical Medicine. The bronchoscopy sample will be used to identify lung cells and to investigate their function. They will also be used to look for bacteria in the lungs. Some of the sample volume will also be stored anonymously for possible use in future ethically approved studies

Are there any risks from a bronchoscopy?

Before the bronchoscopy begins we will place a small needle in the back of the hand and will take a blood sample. This needle can be tender initially, like having a blood test, but it is removed soon after the bronchoscopy is over. You will be monitored closely during the bronchoscopy. A bronchoscopy is not painful but can make some people cough while it is being carried out. To reduce the tendency to cough we will use local anaesthetic in the nose, the back of the throat and the breathing tubes. People are not put to sleep for a bronchoscopy but we will offer you some sedation. The sedation makes some people more relaxed and you may want a short sleep when the bronchoscopy is over. Some people who have this sedation can't recall the procedure afterwards.

If you have the sedative you will not be able to drive home and will need another form of transport or someone to pick you up. We will be happy to arrange and pay for a taxi. If you have had the sedative you should not sign legally binding documents or handle heavy machinery until 24 hours after the sedative was given. All patients are monitored for 2 hours after the bronchoscopy while the effects of the local anaesthetic and sedation wear off and during this time you will not be able to eat or drink.

The British Lung Foundation describes bronchoscopy as a safe procedure (The British Lung Foundation patient information sheet is available on request). Afterwards, most people do not have side-effects but a few describe sore throat or hoarseness for a few hours, nasal discomfort or rarely a minor nose bleed after the test. Some get mild discomfort in the chest as they breathe but this is easily treated using paracetamol for 24 hours after the test.

A member of the study team is available 24 hours a day in the very unlikely event of serious problems after the bronchoscopy. The contact details are at the end of this document.

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
Tel: 0151 529 5870/1 Fax: 0151 529 5875
E-mail: michelle.mossa@aintree.nhs.uk

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University Hospital Aintree
Room 2.06, Clinical Sciences Centre
Liverpool
L9 7AL

Further information and contact details

If there is anything that is not clear or if you would like more information please ask when you meet the study team or by using the contacts at the end of this document during normal working hours.

Dr Sarah Wilks (Clinical Fellow)

Tel: 0151 529 5886

Dr Dan Wootton (Principle Investigator).

Tel: 0151 529 5886 Email: dwootton@liverpool.ac.uk

Dr Stephen Gordon (Study Chief Investigator)

Tel: 0151 705 3172 Email: sbgordon@liverpool.ac.uk

R&D Director: Prof Robert Moots
R&D Deputy Director: Michelle Mossa
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Research & Development Directorate
University Hospital Aintree
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Pneumonia Aetiology and Severity Study (PASS)

Consultee Information Sheet

We feel your relative / friend is unable to decide for him/herself whether to participate in this research.

To help decide if he/she should join the study, we'd like to ask your opinion whether or not they would want to be involved. We'd ask you to consider what you know of their wishes and feelings, and to consider their interests. Please let us know of any advance decisions they may have made about participating in research. These should take precedence.

If you decide your friend/relative would have no objection to taking part we will ask you to read and sign the consultee declaration on the last page of this information leaflet. We'll then give you a copy to keep. We will keep you fully informed during the study so you can let us know if you have any concerns or you think you relative / friend should be withdrawn.

If you decide that your friend / relative would not wish to take part it will not affect the standard of care they receive in any way.

If you are unsure about taking the role of consultee you may seek independent advice.

We will understand if you do not want to take on this responsibility.

The following information is the same as would have been provided to your relative/friend.

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What is the purpose of the study?

Pneumonia is an infection of the lungs which can be caused by a number of different bacteria and viruses. Traditionally patients have been told by their doctor that it takes 6 weeks to fully recover from pneumonia. It is now clear that some patients recover more quickly than this while others take much longer. In this study we want to investigate a group of patients who are admitted to hospital with pneumonia and follow their progress over 12 months to investigate reasons for different patterns of illness.

It is possible that particular bacteria or viruses or combinations of both lead to more severe pneumonia and a more prolonged recovery. In our study we will perform state of the art tests to try to find out if there are differences in the organisms present in the lungs of people with different patterns of pneumonia illness. We will investigate ways of predicting which patients become severely unwell and who takes longer to recover. For example it may be that some people's genetic make-up predisposes them to slow recovery from pneumonia if they have certain bacteria in their lungs. Investigating this will involve taking samples and recording clinical information at several points during admission and following discharge from hospital. We hope that the information generated by this study will lead to the development of new therapies to treat and improve recovery from pneumonia in the future.

Why have I been invited to take part?

We aim to recruit 400 patients with pneumonia for this study. Our research team works with doctors and a specialist nurse to identify suitable patients for the study. You have been invited to take part because the doctors looking after you think you have pneumonia.

Do I have to take part?

It is up to you to decide on whether to join the study once you understand what it involves. If you agree to take part we will ask you to sign a consent form. You are free to withdraw at any time without giving a reason. This would not affect the standard of care you receive. You will be able to choose if samples and information collected up to that point may be used or should be destroyed.

What will I have to do?

This research study involves gathering information about you and your symptoms and collecting clinical samples such as blood, sputum and a salt-water mouthwash. If you agree to take part in this study, you will be seen by

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a member of the research team on several occasions during your treatment in hospital and recovery at home.

What will happen to me if I take part?

The research study will run alongside your routine hospital care and you will not need to spend any longer in hospital than normal. Soon after your diagnosis of pneumonia a member of the research team will review your medical notes and ask you about your symptoms and your past medical history. They will ask to take some clinical samples and **table 1** has details of when the various samples will be taken. You will be seen again approximately 48 hours later for another review and further samples and then again on the day of your discharge.

Following discharge from hospital you will be invited to attend appointments 1 and 6 months and a year later in the out-patient department on convenient dates. At these appointments, a member of the research team may take further samples, will question you about your symptoms and may ask you to have some breathing tests and an x-ray. These assessments are designed to monitor your recovery in greater detail than is current practice. Finally we would like to phone your GP and then you one year after your admission to hospital to review your symptoms and find out about any other medical events that have occurred. If during that phone call you tell us you do not want to attend the one year follow-up visit we will offer you the option of going through the symptom questionnaire over the phone.

Expenses and payments

You will receive a modest financial reimbursement for your time and inconvenience from participation in the study. This will be calculated in the following way:

Participation with study procedures during the in-hospital stay.	£20
Participation with the 1 month follow up-visit	£20
Participation with the 6 month follow up visit	£20
Participation with the 12 month follow up visit	£10

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Table 1 – Research study samples

	Within 24 hours of admission	48 hours after 1 st assessment	1 month after discharge	6 months after discharge
Blood samples	✓	✓	✓	✓
Sputum (phlegm) sample	✓		✓	✓
Urine sample	✓			
Mouthwash	✓		✓	✓
Urine	✓			

What are the possible disadvantages of taking part?

You will be asked to have additional tests (blood, sputum, mouthwash, urine, x-rays and blowing tests) as part of this study. When you are in hospital we will not repeat tests already requested by your team of doctors.

What are the possible benefits of taking part?

The study is unlikely to benefit you directly but you will have the reassurance of being monitored by pneumonia experts more closely than would normally be the case. A proportion of people who get pneumonia do so because of a previously unrecognised abnormality in their lungs and it is possible our in-depth tests may detect this.

What will happen if I don't want to join or carry on with the study?

Taking part in the study is entirely voluntary. You are free to withdraw at any time and do not have to give a reason for this. This will have no effect on your medical care now or in the future. You will be able to choose if samples and information collected up to that point may be used or should be destroyed.

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What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you wish to complain formally, you can do this by contacting the Research Governance office at Aintree University Hospital. The contact person is Mrs Michelle Mossa 0151 529 5871. In the unlikely event that you are harmed during the research due to someone's negligence then you may have grounds for a legal action against Aintree University Hospitals NHS Foundation Trust. The normal NHS complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

All information which is collected about you will be kept strictly confidential. It will be stored securely within the Aintree University Hospital with anonymised samples also being stored at Liverpool School of Tropical Medicine. Access to your personal information collected in the study will be restricted to authorised research staff.

Involvement of the General Practitioner/Family doctor (GP)

We will write to your GP to tell them that you have been involved in the project and will be seen for follow up in our pneumonia clinic and we will keep them informed of your recovery.

What will happen to the samples that I give?

The samples that you give during this study will be used to find out more about the bacteria and viruses that cause pneumonia and how your body responds to them. The samples will be labeled with a code number only. It will not be possible for persons outside of the research study to trace these samples back to you. We would also like to keep any excess sample material to use in future ethically approved studies that are related to the aims of this project. You could ask for these samples to be destroyed at any time now or in the future.

What will happen to the results of the research study?

At the end of the study, we will send you a short report of our findings. This will be a summary of all participants' results and it will not be possible to derive any specific information about your tests from this. The results of this research study will be presented at scientific meetings and published in medical journals.

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Who is funding the research?

This study is jointly funded by the Royal Liverpool and Broadgreen University Hospitals NHS Trust and the National Institute of Health Research.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by North Wales Research Ethics Committee (Central & East).

Further information and contact details

If you have any further questions please contact either of the following members of the research team during normal working hours.

Dr Dan Wootton (Principle Investigator)

Tel: 0151 529 5886

Email: dwootton@liverpool.ac.uk

Dr Stephen Gordon (Chief Investigator)

Tel: 0151 705 2579

Email: sbgordon@liverpool.ac.uk

Consultee Declaration Form

Pneumonia Aetiology and Severity Study (PASS)

Principal Investigator: Dr Dan Wootton

<i>Use patient label</i>
Patient name:
Date of Birth:
Hospital number:

PASS participant identification number:

Please initial each box

1. I have been consulted about’s participation in this research project. I have had the opportunity to ask questions about the study and understand what is involved.
2. In my opinion he/she would have no objection to taking part in the above study.
3. I understand that I can request he/she is withdrawn from the study at any time, without giving any reason and without his/her care or legal rights being affected.
4. I understand that relevant sections of his/her care record and data collected during the study may be looked at by responsible individuals from University Hospital Aintree, Liverpool School of Tropical Medicine or from regulatory authorities, where it is relevant to their taking part in this research.
5. I agree to their GP being informed of their participation in the study.

Name of Consultee	Signature	
Date		
Relationship to participant	Signature	
Date		
Person undertaking consultation	Signature	
Date (designation e.g. researcher)		

When completed: 1 copy for participant; 1 copy for study case report file; 1 copy to be kept in medical notes.

**9 APPENDIX 2: EFFEROCYTOSIS EXPERIMENT
STANDARD OPERATING PROCEDURE (SOP)**

SOP: Tuesday Lab procedures.

- **Non-stock reagents:-**

- 15ml media (= IMDM with 10% human AB serum)

<http://products.invitrogen.com/ivgn/product/21980032>

<http://products.invitrogen.com/ivgn/product/34005100?ICID=search-product>

- HEPES buffered saline

<http://www.sigmaaldrich.com/catalog/product/fluka/51558?lang=en®ion=GB>

- Polymorphprep™ <http://www.axis-shield-density-gradient-media.com/Leaflet%20Polymorphprep.pdf>

- Lysis buffer <http://www.biolegend.com/rbc-lysis-buffer-10x-1498.html>

- APC labelled annexin V, 7AAD, annexin binding buffer

http://www.bdbiosciences.com/documents/BD_Reagents_AnnexinV_Product_InfoSheet.pdf

- Nunc upcell 48 well plates

<http://www.sigmaaldrich.com/catalog/product/sigma/z688770?lang=en®ion=GB>

Day before use study numbers to label the following for each subject:

- (PEN) 5x orange sealed sputum pots numbered 1-5 with study number
 - (PEN) 3x 50 ml falcons with study number
 - (PEN) four 0.5ml eppendorfs V / 7 / V7 / U for fresh neuts
 - (PEN) four 0.5ml eppendorfs V / 7 / V7 / U for fresh HAMs
 - (cryo-labels) One 3ml cryo-vial labelled WB
 - (Cryo-labels) Three 50ml falcons SN
 - (cryo-labels) Ten 1.5ml cryo-vials SN
 - 2 Cyto-spin slides – fresh HAMs and fresh neuts
1. Prepare two falcons containing 40ml $\frac{1}{2}$ x Hepes buffered saline (= 30ml H₂O and 10ml 2x Hepes buffered saline.
 2. Prepare 10ml 1x lysis buffer

= 9ml H₂O and 1mL 10x lysis buffer
 3. Morning of Bronch - put water bath on and warm the saline for BAL
 - ✓ Place UpCell 48 well plate in incubator
 - ✓ Get ice in Yeti and place 5 labelled orange pots on ice.
 - ✓ Place the 5 pre-labelled 50ml falcons on ice with four 50ml falcons for every subject
 - ✓ Turn on a refrigerated centrifuge and refrigerate to 4°C

- **BAL Processing**

Processing BAL should be cold throughout - preserve phenotype/prevent HAMs adhering to plastic

Please complete the BAL record sheet as you go along

1. Filter contents of BAL pots through separate 100 micron filters into 3 cold, labelled, 50ml falcons.
2. Take 3ml of the whole BAL and place in the 3.5ml cryo-vial labelled WB.
3. Place WB cryo-vials on ice prior to transfer to -80
4. Pool rest of BAL, record volume (plus 3mls), divide into 3 cold 50ml Falcons.
5. Spin the BAL at 2000rpm for 10 mins at **2-8 degrees** (brake on).
6. Divide supernatant between 3 50ml falcons labelled SN (supernatant).
7. Remove ten 1ml aliquots of SN (supernatant) and place in cryo-vials labelled SN
8. Place falcons and cryo-vials of SN on ice prior to transfer to -80
9. Re-suspend the cell pellets in 1ml **cool** complete media, **combine** and make up to 10ml.
10. Count cells using haemocytometer (10µl cells + 10µ trypan blue); count trypan stained cells.
11. Pellet and re-suspend in **warm** CM @ 1×10^6 cells/ml

12. Take 100µL aliquot of cells into an eppendorf, add 150µl PBS/EDTA and run cyto-spin
13. Take four x 100µL aliquots and place in the four epindorfs (V/7/V7/U) and add 500µl PBS.
14. Pellet in micro-fuge (hinges out) at 450rcf for 4mins
15. Meanwhile add 500µl cell suspension to 13 wells of the 48 well UpCell plate and label then place plate in incubator – **note the time it goes into incubator**
16. Remove supernatant from eppendorfs, re-suspend in 500µl PBS and re-pellet
17. Repeat above then re-suspend in 100µl binding buffer to each
18. Add 5µl annexin V APC and or 5µl 7aad to relevant samples
19. Vortex and place in dark (room temp) for 15 mins
20. Meanwhile open up flow database
21. create new / disc D / dan wootton /databases / name = “today’s date HAMS”)
22. load protocol (file /protocol / load / disc D / dan wootton / protocols / fresh HAM apoptosis)
23. add 400µl **binding buffer** then analyse

- **Venesection**

1. Using a green needle with a monovette adaptor, 36 ml blood is drawn into 4 x 9ml EDTA monovettes (BD Vacutainer®, BD Becton Dickinson UK Limited, Oxford, UK) in endoscopy recovery post bronchoscopy.
2. The blood should then be used as soon as possible – within two hours.
3. Allow blood to settle at 18-22 degrees (i.e. the time it takes to get it to the lab)
4. If there is a likely delay in analysis then neutrophils are best left to wait as whole blood in the EDTA tube rather than being separated then waiting.

- **Neutrophil separation**

This protocol is derived and adapted from local protocols and with reference to the polymorph-prep manufacturers SOP. Other than centrifugation, all aspects of this protocol are carried out in a class II bio-safety cabinet and, unless otherwise specified, all substrates and reagents are used at room temperature.

Warm the ½ x Hepes Buffered Saline (Serum free IMDM, IMDM with 5% AB serum

1. Place 9ml polymorphprep (used 1:1 with blood @ room temp) into each of four plain 20 ml **universal tubes**. 30ml, polystyrene, screw-top, universal containers - henceforth referred to as ‘universals’
2. With a 10ml serological pipette, take blood from monovettes and layer over polymorphprep Spin @ 2000rpm for 35 mins, room temp, **break off** (takes 50 mins).

This was a slightly faster spin than some methods report and was optimised following our groups anecdotal finding that the blood of patients with symptoms suggesting active inflammation would fail to separate with lower rcf.

3. Take neutrophil layer (down to the RBC layer) and add the cells from 2 tubes to each falcon containing HBS (This step represents returning the neutrophils to physiological osmolality).
4. Pellet cells by spinning at 2000 rpm at room temp. for 10 mins (brake on).
5. Carefully remove supernatant from pelleted neutrophils.
6. Add 1ml lysis buffer) to each tube, combine and add another 4ml.
7. Place on ice for 3 mins with a quick vortex every minute.
8. Make up to 50ml with serum free IMDM and spin at 2000 rpm to pellet.
9. Remove supernatant, re-suspend in 1ml serum free IMDM and make up to 10ml.
10. Count with haemocytometer.
11. Divide the 10ml of cell suspension between two falcons labelled stained and unstained. Into the unstained falcon place the minimum volume of cell suspension to ensure there will be at least 10 million cells left after recovering from the culture flask.
12. Make each falcon up to 20ml with IMDM and pellet.
13. Take off supernatant from the cells in the tube labelled stained and re-suspend cells in 5ml **serum free** IMDM
14. Add 10 μ L DMSO to 50 μ g Cell Tracker (= 10mM)
15. Add the 10 μ L of Cell Tracker solution to the 5ml of cells (= 20 μ M)
16. Incubate in the falcon for 15mins at 37 degrees

17. Meanwhile take off the supernatant from the unstained cells and re-suspend in 1ml serum free IMDM then add 4 ml Serum free IMDM then add 35 ml IMDM with 5% serum – place in a labelled 75cm² culture flask and transfer to the incubator.
18. Add 35ml IMDM **with 5% serum to the stained cells and count using a haemocytometer**
19. Take four appropriate 1×10^5 aliquots into four 1.5ml eppendorfs labelled V, 7, V7, U
20. Add 500 μ L PBS to each eppendorf and pellet at 450rcf in microfuge for 4 mins
21. Meanwhile take 1×10^5 cells for a cytospin.
22. Transfer the remaining cell suspension in a 75cm² culture flask and culture for 20 hours.
23. Meanwhile wash the cells in the eppendorfs twice with 500 μ L PBS
24. Resuspend in 100 μ L 1x binding buffer
25. Add 5 μ L **annexin V APC** and 5 μ L 7AAD to the relevant tubes, vortex and place in dark for 15mins
26. Meanwhile stain the cytospin
24. Open flow database: create new / disc D / dan wootton /databases / name = "today's date neuts"
27. Load protocol: file /protocol/ load /disc D /dan wootton /protocols /neutrophils
28. Add 400 μ L 1x binding buffer to each eppendorf and analyse on flow

SOP: Wednesday lab procedures.

1. First make Crystal Violet Quenching Solution

This is derived from the method published in:

Hallden G et al. Quenching of intracellular autofluorescence in alveolar macrophages permits analysis of fluorochrome labelled surface antigens by flow cytometry. *Journal of Immunological Methods*. 1991 142;207-214

The idea is to create a super-saturated solution of crystal violet by heating and then to remove any precipitate that emerges on cooling by filtration.

The solution should be created on the day of use as the crystal violet will precipitate out over time leaving the remaining solution in a less potent quenching state.

1. Take a standard bijou and place on scales
2. Zero scales and add approx 10mg crystal violet (Sigma-Aldrich 61135-25G)
3. Add an appropriate volume of PBS to create a solution of 1mg/ml
4. Place the bijou in a heated sonication bath set at 65 degrees
5. Sonicate and heat until the crystal violet has completely dissolved
6. Allow the solution to cool before use
7. Just before use – pass through a filter

2. Efferocytosis

Warm serum free IMDM, warm IMDM with 10% AB serum

- Harvest the neutrophil suspension from flask to a 50ml falcon (pipette up and down gently).
- To remove more neutrophils from culture flask spray bottom with 10ml IMDM (**no serum**), remove and add to the falcon.

- Wash twice with 50ml warm (37 degrees) IMDM (**no serum**), 1500rpm 5 mins – each time re-suspend in 1ml IMDM gently to avoid clumping the cells.
 - Count final suspension using haemocytometer.
 - Re-suspend at 5×10^6 /mL in **serum free** IMDM.
 - Take four 20 μ L into four epindorfs labelled V/7/V7/U add 500 μ L PBS to each and spin at 450rcf in the microfuge for 4 mins
 - Take 20 μ L and place in cytospin with 180 μ L PBS EDTA and start
 - Meanwhile remove media from macrophages and rinse macrophages with 500 μ L **warm** IMDM (**serum free**) to remove non-adherent cells.
 - Add 0.5ml (2.5×10^6) neutrophils to each well of macrophages and incubate for 90 mins at 37 degrees.
 - Wash the cells in the epindorfs twice with 500 μ L PBS
 - Add 100 μ L 1x binding buffer to each
 - Add 5 μ L annexin V or 7AAD to each as appropriate and place in dark for 15 mins
 - Stain cytospin
25. Open flow database: create new / disc D / dan wootton /databases / name = “today’s date efferocytosis”
- Take neutrophil suspension from each well of macrophages and wash twice with 1ml warm Ca Mg free HBSS
 - Add 500 μ L Ca Mg free HBSS and place plate on water ice for 15 mins.
 - Label flow tubes

- Remove all the fluid from each well by forceful pipetting and transfer to 1.5ml epindorfs
- Pellet
- Add 100 μ L PBS to each epindorf
- Add 100 μ L crystal violet to each epindorf, vortex and after 30 seconds add 1ml PBS
- Pellet and wash three times with 1ml PBS
- Resuspend in 500 μ L PBS/BSA and analyse

10 APPENDIX 3: TOP 20 OTU TABLE

The following pages contain counts for each of the top 20 (by abundance) OTUs for each sequenced PASS sputum sample.

In the final column of the final table are the sums for each OTU in these 76 samples and these values are those used in figure 5.3

A note on interpretation: the data presented in these tables are from the rarefied data-set (see 5.3.13.2). That is, they do not represent the true number of reads of each OTU sequenced in each sample, but rather the number of reads that were obtained by using the software package Phyloseq to randomly choose 549 (rarefaction level) reads from each sample. Moreover, because only 20 OTUs (from a total of 774 OTUs) are displayed here extreme caution must be taken making comparisons between samples; for the reasons explained in 5.3.13.3 it would be statistically inadmissible to attempt meaningful inferences of relative abundance from these tables.

OTU_name	1323.t1.s	9385.t1.s	7905.t1.s	3466.t1.s	7401.t1.s	5902.t1.s	1249.t1.s	4661.t1.s	9012.t1.s	9934.t1.s
<i>Lactobacillus_2187</i>	32	0	0	0	0	0	0	0	0	0
<i>Prevotella_2758</i>	2	37	7	4	1	1	0	2	1	1
<i>Aggregatibacter_3926</i>	0	2	1	0	0	0	0	3	18	3
<i>Leptotrichia_2233</i>	0	3	1	15	0	4	5	2	12	0
<i>Haemophilus_4739</i>	4	0	0	8	65	0	1	20	39	35
<i>Prevotella_433</i>	9	0	2	3	3	2	5	20	0	2
<i>Escherichia-shigella_1216</i>	0	0	0	0	0	0	0	0	0	0
<i>Klebsiella_1954</i>	0	0	0	0	0	0	0	0	0	0
<i>Moraxella_2510</i>	0	0	20	0	0	0	0	0	0	0
<i>Pseudomonas_3976</i>	0	0	0	0	0	0	0	0	0	0
<i>Granulicatella_740</i>	0	0	1	3	3	2	55	45	7	14
<i>Neisseria_4683</i>	1	37	18	18	2	0	1	3	56	22
<i>Prevotella_956</i>	21	82	37	47	11	14	12	17	40	10
<i>Fusobacterium_1252</i>	20	10	2	56	51	27	16	74	64	63
<i>Actinobacillus_419</i>	9	39	38	0	0	46	0	74	34	4
<i>Streptococcus_1024</i>	57	20	11	19	1	76	14	50	3	29
<i>Streptococcus_3600</i>	131	2	6	108	17	16	3	0	3	1
<i>Streptococcus_4318</i>	12	0	39	13	11	9	31	8	28	34
<i>Veillonella_1328</i>	27	176	307	37	37	262	141	48	89	16
<i>Haemophilus_617</i>	11	1	0	1	0	4	1	45	5	116

OTU_name	5031.t1.s	1197.t1.s	1673.t1.s	7094.t1.s	6056.t1.s	7646.t1.s	4462.t1.s	9571.t1.s	3107.tuk.s	5898.t1.s
<i>Lactobacillus_2187</i>	0	0	0	0	1	0	65	0	4	0
<i>Prevotella_2758</i>	0	3	2	5	3	0	0	71	0	41
<i>Aggregatibacter_3926</i>	0	0	0	17	0	0	0	0	0	0
<i>Leptotrichia_2233</i>	0	6	27	1	0	2	0	2	0	28
<i>Haemophilus_4739</i>	0	0	0	20	0	0	0	45	0	0
<i>Prevotella_433</i>	0	0	21	0	2	4	0	0	0	5
<i>Escherichia-shigella_1216</i>	0	0	0	0	0	0	0	0	0	0
<i>Klebsiella_1954</i>	0	0	0	0	0	0	0	0	0	0
<i>Moraxella_2510</i>	528	6	0	0	0	0	2	0	0	0
<i>Pseudomonas_3976</i>	0	0	0	0	0	0	0	2	0	0
<i>Granulicatella_740</i>	0	1	1	0	0	1	0	42	0	0
<i>Neisseria_4683</i>	0	0	182	35	123	0	0	1	0	0
<i>Prevotella_956</i>	1	6	7	19	28	3	0	0	0	63
<i>Fusobacterium_1252</i>	0	0	57	98	31	27	16	21	0	16
<i>Actinobacillus_419</i>	0	0	0	0	0	0	0	78	0	10
<i>Streptococcus_1024</i>	4	2	2	0	16	8	0	55	0	52
<i>Streptococcus_3600</i>	0	5	2	5	17	38	158	18	7	0
<i>Streptococcus_4318</i>	0	0	7	26	160	6	69	5	15	22
<i>Veillonella_1328</i>	1	91	13	66	39	217	5	89	2	204
<i>Haemophilus_617</i>	14	403	0	40	0	0	2	8	484	0

OTU_name	7290.t1.s	7556.t1.s	9969.t1.s	3556.t1.s	1387.t1.s	6636.t1.s	5621.t1.s	8163.t1.s	8228.t1.s	1603.t1.s
Lactobacillus_2187	0	0	0	0	0	22	100	1	0	0
Prevotella_2758	13	0	0	2	12	0	0	35	1	1
Aggregatibacter_3926	0	5	0	0	20	1	0	0	1	115
Leptotrichia_2233	3	18	0	32	3	0	0	5	0	2
Haemophilus_4739	0	0	0	22	21	0	0	1	0	5
Prevotella_433	14	18	23	3	0	26	0	4	1	0
Escherichia-shigella_1216	0	0	0	0	0	0	0	0	0	0
Klebsiella_1954	0	0	34	0	0	0	0	0	0	0
Moraxella_2510	0	0	0	25	9	0	0	0	0	0
Pseudomonas_3976	0	0	0	0	0	0	0	0	0	0
Granulicatella_740	66	0	0	66	2	0	2	0	6	4
Neisseria_4683	0	106	0	22	0	0	0	0	112	8
Prevotella_956	47	6	1	3	0	1	0	57	22	16
Fusobacterium_1252	114	7	0	7	0	4	0	13	176	43
Actinobacillus_419	0	0	0	0	54	0	0	2	1	25
Streptococcus_1024	20	112	3	18	83	0	111	29	5	5
Streptococcus_3600	9	93	0	12	7	19	0	81	0	2
Streptococcus_4318	2	5	38	75	142	275	16	6	17	2
Veillonella_1328	2	9	197	40	35	42	145	131	95	23
Haemophilus_617	0	5	46	3	82	1	1	2	0	102

OTU_name	2123.t1.s	8420.t1.s	6835.t1.s.A	7554.t1.s	6406.t1.s.A	4897.t1.s	0414.t1.s	5974.t1.s	5600.t1.s	2671.t1.s
Lactobacillus_2187	0	0	0	0	0	0	0	0	0	0
Prevotella_2758	2	4	4	0	0	3	0	5	0	0
Aggregatibacter_3926	0	22	0	0	0	1	0	0	0	0
Leptotrichia_2233	1	0	1	81	0	2	1	5	0	0
Haemophilus_4739	0	13	0	0	0	4	3	2	0	0
Prevotella_433	1	9	4	2	0	42	6	10	0	151
Escherichia-shigella_1216	9	0	0	0	0	0	0	0	0	0
Klebsiella_1954	0	0	0	0	0	0	0	1	0	0
Moraxella_2510	0	0	0	0	0	0	0	0	0	0
Pseudomonas_3976	0	0	0	0	499	0	1	0	0	0
Granulicatella_740	3	10	0	0	0	3	6	58	0	48
Neisseria_4683	0	1	0	0	0	6	33	0	0	0
Prevotella_956	31	5	14	0	0	12	1	24	0	2
Fusobacterium_1252	1	60	5	50	0	127	1	22	0	0
Actinobacillus_419	61	25	0	0	0	0	0	80	0	0
Streptococcus_1024	24	0	0	0	5	16	11	25	1	20
Streptococcus_3600	44	5	1	3	34	0	2	8	0	13
Streptococcus_4318	184	27	5	0	0	9	91	50	6	132
Veillonella_1328	18	38	101	4	0	19	3	129	0	0
Haemophilus_617	0	0	380	215	0	0	358	1	539	0

OTU_name	3149.t1.s	9120.t1.s	2738.t1.s	9219.t1.s	6874.t1.s	9622.t1.s	3371.t1.s.A	9988.t1.s	1571.t1.s	1172.t1.s
Lactobacillus_2187	0	0	0	44	16	0	0	0	0	0
Prevotella_2758	1	0	0	0	0	2	0	5	0	0
Aggregatibacter_3926	0	0	0	0	0	0	0	0	0	0
Leptotrichia_2233	0	0	0	0	0	1	0	16	0	0
Haemophilus_4739	0	0	0	0	0	0	0	6	0	1
Prevotella_433	0	1	0	0	0	3	47	1	0	0
Escherichia-shigella_1216	0	0	0	2	2	0	0	7	0	0
Klebsiella_1954	0	0	0	10	0	399	0	0	0	0
Moraxella_2510	0	1	0	1	0	0	1	0	40	0
Pseudomonas_3976	0	0	0	0	0	0	0	0	0	216
Granulicatella_740	0	0	4	11	1	0	0	20	17	0
Neisseria_4683	0	0	0	0	0	0	0	0	9	0
Prevotella_956	1	0	0	27	13	1	27	117	0	0
Fusobacterium_1252	0	0	0	0	1	33	23	0	0	0
Actinobacillus_419	0	0	0	0	0	0	133	1	217	0
Streptococcus_1024	4	0	6	1	0	0	4	148	1	0
Streptococcus_3600	0	0	7	130	229	1	2	8	1	0
Streptococcus_4318	199	72	4	15	145	0	139	28	175	329
Veillonella_1328	2	2	17	119	96	1	27	66	0	0
Haemophilus_617	334	464	500	3	0	2	52	1	36	0

OTU_name	5432.t1.s	0001.t1.s	6257.t1.s	8902.t1.s	5318.t1.s	5813.t1.s	8305.t1.s	5316.t1.s	2780.t1.s	6016.t1.s
Lactobacillus_2187	0	1	0	0	0	2	0	8	0	0
Prevotella_2758	7	3	0	2	29	19	2	9	0	0
Aggregatibacter_3926	8	0	0	26	0	0	0	0	2	0
Leptotrichia_2233	0	0	0	19	17	15	0	3	2	5
Haemophilus_4739	0	0	0	9	3	1	40	0	19	0
Prevotella_433	4	20	0	0	2	3	2	0	0	0
Escherichia-shigella_1216	0	3	0	0	0	0	1	0	0	0
Klebsiella_1954	0	0	0	0	0	0	0	0	0	0
Moraxella_2510	0	0	0	0	0	0	0	0	19	0
Pseudomonas_3976	0	0	0	0	0	0	0	0	0	0
Granulicatella_740	0	0	0	18	1	1	66	0	54	0
Neisseria_4683	27	0	0	5	42	8	1	13	46	22
Prevotella_956	66	0	0	7	145	71	3	55	2	0
Fusobacterium_1252	16	11	2	22	5	1	6	0	0	0
Actinobacillus_419	0	0	0	9	3	0	258	0	0	0
Streptococcus_1024	127	2	3	41	3	14	3	58	41	9
Streptococcus_3600	2	1	1	0	12	23	0	47	14	15
Streptococcus_4318	5	9	0	193	2	7	103	90	100	137
Veillonella_1328	55	38	1	111	167	209	2	56	39	18
Haemophilus_617	13	396	513	3	22	2	1	0	13	324

OTU_name	1664.t1.s	5700.t1.s	3925.t1.s	4238.t1.s	0657.t1.s	3824.t1.s	4254.t1.s	8560.t1.s	6076.t1.s	1913.t1.s
Lactobacillus_2187	0	1	7	0	0	0	37	0	0	0
Prevotella_2758	0	0	0	0	0	1	0	0	1	0
Aggregatibacter_3926	0	0	0	0	0	0	0	0	0	2
Leptotrichia_2233	0	0	0	0	0	0	0	1	5	35
Haemophilus_4739	0	0	31	0	0	0	0	6	0	2
Prevotella_433	0	20	0	0	0	2	11	0	0	0
Escherichia-shigella_1216	428	0	0	34	0	0	25	0	0	0
Klebsiella_1954	1	0	0	5	0	0	50	0	0	0
Moraxella_2510	0	0	3	0	0	4	0	0	0	0
Pseudomonas_3976	0	0	0	0	0	0	0	0	0	0
Granulicatella_740	58	36	0	3	49	8	0	0	0	1
Neisseria_4683	0	2	0	49	64	0	0	0	1	61
Prevotella_956	0	1	0	1	24	74	0	0	0	0
Fusobacterium_1252	0	4	0	0	1	1	66	0	0	5
Actinobacillus_419	0	0	0	0	106	0	0	5	0	13
Streptococcus_1024	0	10	90	8	177	0	0	0	76	19
Streptococcus_3600	26	26	140	73	2	223	1	8	396	0
Streptococcus_4318	0	0	32	3	26	10	2	1	0	84
Veillonella_1328	20	17	51	0	56	185	4	114	1	107
Haemophilus_617	0	331	155	362	0	3	0	394	38	56

OTU_name	1357.t1.s	4454.t1.s	1420.t1.s	5276.t1.s	8041.t1.s	6867.tuk.s	Row_sum
<i>Lactobacillus_2187</i>	0	1	0	0	0	0	272
<i>Prevotella_2758</i>	0	0	0	4	0	0	223
<i>Aggregatibacter_3926</i>	0	0	5	3	0	113	351
<i>Leptotrichia_2233</i>	0	0	2	5	2	0	324
<i>Haemophilus_4739</i>	0	0	17	0	0	0	378
<i>Prevotella_433</i>	0	0	0	0	0	0	476
<i>Escherichia-shigella_1216</i>	0	0	0	0	1	0	512
<i>Klebsiella_1954</i>	0	0	0	0	0	93	593
<i>Moraxella_2510</i>	0	1	0	0	0	0	124
<i>Pseudomonas_3976</i>	0	0	0	0	0	4	720
<i>Granulicatella_740</i>	0	0	32	1	0	2	787
<i>Neisseria_4683</i>	0	0	22	0	1	0	819
<i>Prevotella_956</i>	0	0	31	1	5	0	1205
<i>Fusobacterium_1252</i>	0	0	0	9	2	0	1221
<i>Actinobacillus_419</i>	0	0	169	0	0	1	1407
<i>Streptococcus_1024</i>	0	0	10	20	6	19	1668
<i>Streptococcus_3600</i>	0	4	38	0	0	0	2050
<i>Streptococcus_4318</i>	0	0	56	15	58	14	3320
<i>Veillonella_1328</i>	0	2	34	6	3	0	3797
<i>Haemophilus_617</i>	547	541	0	0	461	0	7486

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