The use of soluble and surface TREM-1 as markers of Ventilator-associated pneumonia in Intensive Care

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Declaration

This thesis is entirely my own work, except for the areas that I have acknowledged.

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Vimal Grover
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Finally, I would like to dedicate my thesis to Linsey, my fiancée, who has inspired me and who has unflinchingly supported me through all the joys and frustrations of this work.
For Linsey
Abstract

Ventilator-associated pneumonia (VAP) is the commonest nosocomial infection in Intensive Care and is associated with significant morbidity and mortality. Biomarkers offer the potential to speed up diagnosis and differentiate pulmonary from non-pulmonary infection. We postulated that measurement of cell surface receptors in addition to soluble proteins, in dual sites (blood and BAL) and calculation of an index ratio of BAL / blood would increase the discriminative utility and differentiate pulmonary from non-pulmonary infection.

Our body of work included paired blood and BALF obtained from 91 patients in a pilot study: 27 with VAP, 15 ventilated patients with non-pulmonary sepsis, 18 ventilated patients with no evidence of infection and 31 non-ventilated non-infected patients. In each sample, the monocytic and neutrophilic surface proteins TREM-1, CD11b and CD62L were assessed using flow cytometry. Soluble proteins (IL-1β, IL-6, IL-8) were assayed using ELISA in addition to Procalcitonin, CRP and white cell count.

The levels of soluble TREM-1, IL-1β and IL-8 were significantly raised in the BAL of patients with VAP. BAL monocytic surface TREM-1 was also significantly higher in VAP. The BAL / blood ratio increased the discrimination of patients with VAP from non-VAP. Furthermore, the BAL / blood ratio of patients differentiated VAP from non-pulmonary infection. Monocytic and neutrophilic TREM-1 were assessed during the development and resolution phases of VAP. Monocytic surface TREM-1 and its BAL / blood ratio accurately mirrored the changes with infection, indicating them to be putative biomarkers of infection.

Finally, we constructed and validated a biomarker panel to discriminate patients with VAP from non-VAP. The panel comprised the BAL / blood ratios of monocytic TREM-1 and CD11b, the BAL levels of soluble TREM-1, IL-8 and IL-1β together with the blood levels of IL-6 and CRP. It had high utility in identifying patients with VAP.
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<th>Description</th>
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<td>AGNB</td>
<td>Aerobic Gram Negative Bacilli</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>APACHE II</td>
<td>Acute physiology and chronic health evaluation score II</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome</td>
</tr>
<tr>
<td>AUROC</td>
<td>Area under ROC curve</td>
</tr>
<tr>
<td>BAL (F)</td>
<td>Bronchoalveolar Lavage (Fluid)</td>
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<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
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<tr>
<td>CAP</td>
<td>Community-acquired pneumonia</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
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<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CPIS</td>
<td>Clinical Pulmonary Infection Score</td>
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CRP C-reactive Protein
CXR Chest X-ray
DAP-12 DNA-X adaptor protein of 12 kDa
DAMP Danger Associated Molecular Patterns
DC Dendritic cell
DNA Deoxyribonucleic acid
EDTA Ethylenediaminetetraacetic acid
EIA Enzyme immunoassay
ELF Epithelial lining fluid
ELISA Enzyme-linked immunosorbent assay
EPIC II Extended prevalence of infection in Intensive Care 2 study
EQA External Quality Assessment
ETT Endotracheal tube
EU Endotoxin Unit
EVC Exhaled Ventilator Condensate
FAMA Fluorescent antibody to membrane antigen
FCS Fetal calf serum
FDA Fisher discriminant analysis
GCS Glasgow Coma Score
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>G-CSF</td>
<td>Granulocyte-Colony Stimulating Factor</td>
</tr>
<tr>
<td>HELICS</td>
<td>Hospitals in Europe Link for Infection Control and Surveillance</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>HV</td>
<td>Healthy volunteers</td>
</tr>
<tr>
<td>ICO</td>
<td>Intra-Cellular Organisms</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>Ig-SF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>IL-x</td>
<td>Interleukin-x</td>
</tr>
<tr>
<td>IL-ra</td>
<td>Interleukin receptor antagonist</td>
</tr>
<tr>
<td>IPS-1</td>
<td>Interferon-β promoter stimulator</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>IVAC</td>
<td>Infective ventilator-associated condition</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinases</td>
</tr>
<tr>
<td>LIR</td>
<td>Leukocyte immunoglobulin receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LR</td>
<td>Likelihood ratio</td>
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LTA  Lipoteichoic acid

MALDI-TOF  Matrix Assisted Laser Desorption / Ionization – Time of Flight mass spectrometry

mDC  Myeloid dendritic cell

MFI  Mean fluorescence intensity

MHC  Major Histocompatibility Complex

MIP-1α  Macrophage Inflammatory Protein-1α

MMP  Matrix metalloproteinase

MRSA  Methicillin resistant Staphylococcus Aureus

MSSA  Methicillin sensitive Staphylococcus Aureus

mTREM-1  Surface TREM-1 on monocytes

mTREM-2  Surface TREM-2 on monocytes

NBL  Non-directed bronchial lavage

NF-κB  Nuclear factor kappa light chain enhancer of activated B cells

NHSN  National Healthcare Safety Network

NICE  National Institute for Health and Care Excellence

NK  Natural Killer cell

NLR  NOD-like receptor

NOD  Nucleotide oligomerisation domain
<table>
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<td>NProCT</td>
<td>N-terminal fragment of Procalcitonin</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>NRES</td>
<td>National Research and Ethics Service</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>Surface TREM-1 on neutrophils</td>
</tr>
<tr>
<td>nTREM-2</td>
<td>Surface TREM-2 on neutrophils</td>
</tr>
<tr>
<td>NVC</td>
<td>Non-ventilated control. No infection present</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end-expiratory pressure</td>
</tr>
<tr>
<td>PPR</td>
<td>Pathogen pattern recognition receptor</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
</tr>
<tr>
<td>PSB</td>
<td>Protected Specimen Brush</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid inducible gene</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-1 like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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</table>
ROC  Receiver-Operator Characteristic
SIRP  Signal regulatory protein
SIRS  Systemic Inflammatory Response Syndrome
STAT  Signal transducer and activation of transcription
s-TF  Soluble tissue factor
sTREM-1 Soluble TREM-1
TB  Tuberculosis
TIMP  Tissue inhibitors of matrix metalloproteinases
TLR  Toll-like receptor
TNF-α  Tumour Necrosis Factor-α
TBA  Tracheobronchial aspirate
TREM-1  Triggering Receptor Expressed on Myeloid Cells-1
TREM-2  Triggering Receptor Expressed on Myeloid Cells-2
VAC  Ventilator-associated condition
VAP  Ventilator-Associated Pneumonia
VC  Ventilated control. Ventilated patient free of infection
VSE  Ventilated with sepsis elsewhere. Ventilated patient with non-pulmonary sepsis
Chapter 1

Introduction

1.1 Pneumonia

Pneumonias are infective inflammatory conditions of the lower respiratory tract, caused by bacterial, fungal or viral organisms. They may be subdivided on aetiology into a number of types – Community-acquired pneumonia, aspiration pneumonia, pneumonia in the immunocompromised and nosocomial pneumonia (Table 1.1). In the large EPIC II study of ICU patient infection prevalence (2007), 4503 of 13796 ICU patients were considered to have a respiratory tract infection, indicating its high prevalence.

Table 1.1 Types of pneumonia

<table>
<thead>
<tr>
<th>Pneumonia type</th>
<th>Aetiology</th>
</tr>
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<tbody>
<tr>
<td>Community acquired (CAP)</td>
<td>Contracted outside of healthcare setting</td>
</tr>
<tr>
<td>Aspiration</td>
<td>Aspiration of upper gastrointestinal bacteria into lower respiratory tract</td>
</tr>
<tr>
<td>Pneumonia in immunocompromised</td>
<td>Pneumonia in immunocompromised individuals caused by organisms not normally pathogenic</td>
</tr>
<tr>
<td>patients</td>
<td></td>
</tr>
<tr>
<td>Nosocomial</td>
<td>Contracted in a healthcare setting</td>
</tr>
</tbody>
</table>

Table 1.1. Pneumonias may be broadly divided into community-acquired pneumonia (CAP), aspiration, nosocomial and infections in the immunocompromised patient.
1.2 Community-acquired pneumonia (CAP)

Pneumonia refers to infection of the lung parenchyma; where this is acquired outside of hospital or an allied healthcare facility (eg nursing home) this is referred to as CAP. The British Thoracic Society (BTS) has defined CAP on the basis of clinical and radiological criteria: symptoms of an acute lower respiratory tract illness (cough plus at least one other symptom), new focal chest signs on examination, symptoms/signs of systemic illness (fever greater than 38°C and/or symptoms of fever, chills, aches and pains), new radiographic shadowing, no other explanation for the illness (eg pulmonary oedema) and which is treated as CAP with antibiotics. Microbiological confirmation consists of sputum culture, blood culture of an appropriate organism (eg Streptococcus pneumoniae), urinary Legionella and pneumococcal antigen and tests for atypical pathogens (eg mycoplasma pneumoniae and Chlamydia psittaci)[2]. The CURB-65 score is used to assess mortality risk from pneumonia. It comprises (C) Confusion of new onset, (U) Urea of >7 mmol/l, (R) respiratory rate >30 / min, (B) systolic BP < 90 mmHg or diastolic BP < 60 mmHg and (65) Age > 65 years. The greater the number of features present, the higher the mortality risk. Microbial causes of CAP are described in Table 1.2. CAP is mainly caused by Streptococcus pneumoniae, Haemophilus Influenzae, Staphylococcus and Legionella.
### Table 1.2 Prevalence of causative organisms for CAP in the ICU

<table>
<thead>
<tr>
<th>Organisms responsible for CAP in the ICU</th>
<th>Prevalence (mean %, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae (pneumococcus)</td>
<td>21.6 (15.9-28.3)</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>3.8 (1.5-7.6)</td>
</tr>
<tr>
<td>Legionella spp</td>
<td>17.8 (12.6-24.1)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8.7 (5.0-13.7)</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>Unknown</td>
</tr>
<tr>
<td>Gram negative enteric bacilli</td>
<td>1.6 (0.3-4.7)</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>2.7 (0.9-6.2)</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>Unknown</td>
</tr>
<tr>
<td>Chlamydia psittaci</td>
<td>2.2 (0.6-5.4)</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>0 (0-2.0)</td>
</tr>
<tr>
<td>All viruses</td>
<td>9.7 (5.9-14.9)</td>
</tr>
<tr>
<td>Influenza A and B</td>
<td>5.4 (2.6-9.7)</td>
</tr>
<tr>
<td>Mixed bacterial</td>
<td>6.0 (3.0-10.4)</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>4.9 (2.3-9.0)</td>
</tr>
<tr>
<td>None</td>
<td>32.4 (25.7-39.7)</td>
</tr>
</tbody>
</table>

Table 1.2. Organisms may be single or mixed, bacterial, viral or others. Values are given as the mean percentage with the 95% confidence interval. Adapted from the British Thoracic Society Community-acquired pneumonia in Adults Group [2].
1.3 Nosocomial / healthcare associated pneumonia

This occurs in people from the following groups [3]:

(a) Patients hospitalized in an acute care facility for 2 or more days within 90 days of the infection

(b) Residents of a nursing home or long-term care facility

(c) Patients who received wound care, intravenous antibiotic or chemotherapy within the last 30 days of this current infection episode

(d) Patients in any setting receiving haemodialysis

1.4 Ventilator-associated pneumonia (VAP)

VAP is the commonest nosocomial infection in Intensive Care, affecting between 10-20% of intubated patients. It causes significant morbidity, increased length of mechanical ventilation, length of hospital stay, healthcare costs and potentially increased mortality [3-6]. It has been defined by the National Institute of Health and Clinical Excellence (NICE) as ‘a pneumonia that develops 48 hours or more after intubation with an endotracheal or tracheostomy tube, and that was not present before intubation.’ [7] There remains debate as to whether VAP increases mortality, because of the risk of confounding; if a patient dies, was it due to the VAP or due to the condition requiring intubation and ventilation? In 2009, Melsen and colleagues concluded that in trauma and ARDS patients there was no attributable mortality to VAP, but that there may be mortality in other patient groups [8]. More recently, the same group have analysed original patient data from randomised clinical trials involving VAP and concluded that it has a mortality rate of approximately 13%, principally due to the increased length of stay in ICU [9].

1.4.1 Aetiology and pathogenesis of VAP

The presence of an endotracheal tube (ETT) is central to the pathogenesis of VAP. During critical illness, the upper airway (oropharynx) becomes colonised
with aerobic gram-negative bacteria. The ETT inhibits coughing and the normal protective upper airway reflexes; it also allows pooling of oropharyngeal secretions in the upper airway. Such secretions micro-aspirate beyond the low-pressure, high volume cuff of the ETT (by very small channels that develop between the cuff and the tracheal mucosa); with ventilator cycling, the bacteria in the secretions enter the lower respiratory tract; they also form a biofilm on the internal surface of the ETT; over time this may lead to the development of parenchymal infection (VAP) [10].

The diagnostic criterion for VAP to occur 48 hours after intubation with an endotracheal tube excludes patients with pathogens in the lower respiratory tract incubating at the time of onset of mechanical ventilation. VAP may be caused by a variety of micro-organisms. Ibrahim et al identified different organisms in patients who had early onset VAP (within 96 hours of ICU admission) versus late onset VAP (>96 hours of admission) [11]. Overall, in the cases of early VAP the oropharynx is colonised with largely sensitive organisms; later on, antibiotic-resistant bacteria acquired in hospital predominate.

### 1.4.2 VAP microbiology

Table 1.3 highlights the difference in microbiology between early and late onset of VAP, table 1.4 demonstrates the data for all VAP cases. Causative micro-organisms include bacteria, viruses and fungi. The proportion of cases of sensitive Staphylococcus Aureus decreases in the late-onset VAP. This is associated with an increase in the proportion of MRSA, pseudomonas, acinetobacter and stenotrophomonas which are associated with antibiotic resistance.
<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Early-onset NP (n=235)</th>
<th>Late-onset NP (n=185)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas Aeruginosa</td>
<td>59 (25.1)</td>
<td>71 (38.4)</td>
<td>0.003</td>
</tr>
<tr>
<td>Methicillin Sensitive</td>
<td>42 (17.9)</td>
<td>20 (10.8)</td>
<td>0.043</td>
</tr>
<tr>
<td>Staphylococcus Aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin Resistant</td>
<td>42 (17.9)</td>
<td>39 (21.1)</td>
<td>0.408</td>
</tr>
<tr>
<td>Enterobacter SPP</td>
<td>24 (10.2)</td>
<td>19 (10.3)</td>
<td>0.985</td>
</tr>
<tr>
<td>No growth</td>
<td>22 (9.4)</td>
<td>7 (3.8)</td>
<td>0.025</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>17 (7.2)</td>
<td>21 (11.4)</td>
<td>0.144</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>14 (6.0)</td>
<td>5 (2.7)</td>
<td>0.111</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>13 (5.5)</td>
<td>12 (6.5)</td>
<td>0.681</td>
</tr>
<tr>
<td>Candida spp</td>
<td>12 (5.1)</td>
<td>7 (3.8)</td>
<td>0.517</td>
</tr>
<tr>
<td>Acinetobacter spp</td>
<td>6 (2.6)</td>
<td>10 (5.4)</td>
<td>0.130</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>6 (2.6)</td>
<td>3 (1.6)</td>
<td>0.513</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>6 (2.6)</td>
<td>7 (3.8)</td>
<td>0.470</td>
</tr>
<tr>
<td>Aspergillus spp</td>
<td>5 (2.1)</td>
<td>1 (0.5)</td>
<td>0.174</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>4 (1.7)</td>
<td>5 (2.7)</td>
<td>0.482</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>4 (1.7)</td>
<td>2 (1.1)</td>
<td>0.594</td>
</tr>
<tr>
<td>Citrobacter spp</td>
<td>3 (1.3)</td>
<td>3 (1.6)</td>
<td>0.767</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>3 (1.3)</td>
<td>1 (0.5)</td>
<td>0.441</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>3 (1.3)</td>
<td>5 (2.7)</td>
<td>0.288</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>1 (0.4)</td>
<td>4 (2.2)</td>
<td>0.103</td>
</tr>
<tr>
<td>Pneumocystis carinii</td>
<td>1 (0.4)</td>
<td>2 (1.1)</td>
<td>0.428</td>
</tr>
<tr>
<td>Alcaligenes xylosoxidans</td>
<td>4 (1.7)</td>
<td>5 (2.7)</td>
<td>0.482</td>
</tr>
<tr>
<td>Multiple pathogens</td>
<td>49 (20.9)</td>
<td>51 (27.6)</td>
<td>0.109</td>
</tr>
</tbody>
</table>

**Table 1.3**. The organisms were identified in patients with nosocomial pneumonia. Greater than 90% of these patients were ventilated. Values are given as numbers and percentages of bacteria, viruses or other. In 20-9-27.6% of cases, mixed organisms were identified. Spp=species. Adapted from Ibrahim et al (2000) [11].
**Table 1.4 Organisms isolated from VAP cases**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>24.4</td>
</tr>
<tr>
<td>Acinetobacter spp</td>
<td>7.9</td>
</tr>
<tr>
<td>Stenotrophomonas malophilia</td>
<td>1.7</td>
</tr>
<tr>
<td>Enterobacteriaceae*</td>
<td>14.1</td>
</tr>
<tr>
<td>Haemophilus spp.</td>
<td>9.8</td>
</tr>
<tr>
<td>Staphylococcus aureus**</td>
<td>20.4</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>8.0</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>4.1</td>
</tr>
<tr>
<td>Coagulase negative staphylococci</td>
<td>1.4</td>
</tr>
<tr>
<td>Neisseria spp.</td>
<td>2.6</td>
</tr>
<tr>
<td>Anaerobes</td>
<td>0.9</td>
</tr>
<tr>
<td>Fungi</td>
<td>0.9</td>
</tr>
<tr>
<td>Other (&lt;1% each)***</td>
<td>3.8</td>
</tr>
</tbody>
</table>

**Table 1.4.** 24 studies were pooled with 1689 episodes, using BAL culture. The majority of species were bacterial, with only a minor proportion of fungal organisms.


** Methicillin-resistant S. aureus 55.7%, Methicillin-sensitive S. aureus 44.3%


Other includes rarer bacteria (Table 1.3) and viruses [12].
1.4.2a Viral and fungal VAP

The role of viruses in contributing to VAP has been relatively under-explored due to a greater difficulty in isolation and identification in comparison to bacterial pneumonias. Luyt et al (2011) discussed that Herpes Simplex Virus (HSV) and possibly Cytomegalovirus (CMV) contributed to viral VAP and secondary bacterial infection but that their contribution was probably under-represented in previous studies. Indeed, the H1N1 influenza outbreak may also have been associated with an increased risk for VAP [13].

The contribution of fungi to VAP remains uncertain. Candida species are frequently isolated in BAL cultures but are clinically felt to be due to colonisation rather than infection. Aspergillus species may colonise too but may be pathogenic in immunosuppressed patients. In a recent study by Conway-Morris [14] patients with BAL containing candida species alone were seen to have elevated levels of pulmonary cytokines, consistent with activation of inflammation. This may be suggestive of fungal infection rather than colonisation and may indicate that the prevalence of such infections are wider than clinically thought.

1.4.3 Risk factors for VAP

In a review by Cook et al (1998) many risk factors for VAP were identified [15] – table 1.5. These risk factors may further be subdivided into fixed and modifiable. Antacid therapy increases gastric pH and reduces the incidence of gastrointestinal bleeding associated with critical illness, but encourages the growth of aerobic gram-negative bacilli (AGNB). Reintubation of patients increases the risk of aspiration of oropharyngeal secretions. In neurological cases of critical illness, upper airway reflexes and coughing are already inhibited and therefore the patient is at risk of VAP. With supine positioning, there is a gravitational risk of aspiration. With enteral feeding, nasogastric tubes increase the risk of sinusitis and colonisation of the upper airway with AGNB, but overall the benefits of enteral feeding outweigh these risks. Finally,
Factors such as increased sedation increase the duration of mechanical ventilation and the risks of developing VAP.

**Table 1.5 Risk factors for VAP development**

<table>
<thead>
<tr>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixed</strong></td>
</tr>
<tr>
<td>COPD</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Increased illness severity</td>
</tr>
<tr>
<td>Neurosurgery</td>
</tr>
<tr>
<td>Head injury or low GCS</td>
</tr>
<tr>
<td>Age &gt; 60 years</td>
</tr>
<tr>
<td><strong>Modifiable</strong></td>
</tr>
<tr>
<td>Antacid therapy (H2 antagonists or proton pump inhibitors)</td>
</tr>
<tr>
<td>Supine positioning</td>
</tr>
<tr>
<td>Prolonged mechanical ventilation</td>
</tr>
<tr>
<td>Reintubation</td>
</tr>
<tr>
<td>Enteral / NG feeding</td>
</tr>
<tr>
<td>Aspiration</td>
</tr>
<tr>
<td>Low cuff pressure &lt;20cm H20</td>
</tr>
<tr>
<td>Sinusitis</td>
</tr>
<tr>
<td>ARDS (Acute respiratory distress syndrome)</td>
</tr>
</tbody>
</table>

*Table 1.5.* From Cook et al (1998). These are divided into fixed and potentially modifiable factors [15].
1.4.4 Diagnosis of VAP

Given that VAP is the most common nosocomial infection in ICU, it is essential to correctly diagnose the condition. Prompt treatment with antibiotics is life-saving in infected patients [16]. However, over-treatment of non-infected patients increases the risk of Clostridium difficile associated colitis and the development of multi-resistant organisms. Timely and accurate diagnosis is therefore required. On both these counts there are problems; microbiological identification of organisms may take 48-72 hours (if positive), although a gram-stain may yield information more quickly. False negative results occur due to sampling errors, infection with culture-negative organisms or prior antibiotic treatment. False positive results can occur with proximal lower-respiratory tract colonisation (positive microbiology without evidence of infection, e.g. candida). This is a particular risk with tracheal aspirate sampling where sampling of distal airways is less likely to occur.

A major problem with VAP that hampers research is that there is no gold standard for its diagnosis. Histopathological specimens which show alveolar pus can be considered a gold standard, but this technique is not only impractical, but relies on post-mortem verification, biasing towards severe cases and underestimating sensitivity [17]. Furthermore, histopathological findings demonstrate VAP to be a multifocal disease. Therefore, in addition to method of sampling and method of analysis, site of sample acquisition is clearly important too [18]. If a pathology sample is taken from an area of unaffected lung, false negative results can be reported. Moreover, variability in reporting of specimens can occur between pathologists, decreasing the diagnostic accuracy [19].

There is no consensus for the practical gold standard. Clinical assessment employs clinical, radiological and microbiological criteria to determine VAP probability. Diagnosis may either be refuted, ‘possible’, ‘probable’, or definite. Clinical features include cough, dyspnoea, fever, auscultatory changes, raised or reduced white cell count, worsening gas exchange, presence of purulent secretions, roentgenographic changes and positive cultures. An issue with these features are that they may be present in non-pneumonic illness; trauma
induced pulmonary contusions, pulmonary oedema, pulmonary embolism can mimic all these changes. Using histopathology as a gold standard, single clinical signs were assessed for their ability to identify VAP. Fever as expected had a sensitivity of 67% and specificity of 65% [20]. Purulent secretions had 83% sensitivity but poor specificity (42%) compared with WCC increase having 77% sensitivity and 58% specificity. CXR infiltrates had high sensitivity (92%) but poor specificity (33%) too, suggesting them to be useful as a rule-out test [21].

The radiological features seen on CXR have been compared with histopathological specimens by Wunderink et al (1992). Alveolar infiltrates and air bronchograms had the best sensitivity (88% and 83% respectively) but poorer specificities (27% and 58% respectively). Compared with multiple air bronchograms, single ones were less sensitive (17% versus 67%) but highly specific (96% versus 62%). Fissure abutment had poor sensitivity (6%) but high specificity (96%) [22].

The Johanson criteria initially encompassed a number of these features; new or developing changes on chest radiography, together with two or more of: fever >38°C, high or low white cell count and purulent secretions [23]. The Johanson criteria had a sensitivity of 69% and specificity of <75% [21].

Considering microbiology, the method of sampling and the culture technique are important.

Specimens may be acquired in a variety of ways:

(i) Tracheobronchial aspirate (TBA) – where secretions are suctioned from an in-line suction catheter introduced via the endotracheal tube

(ii) Non-directed bronchial lavage (NBL) – a suction catheter is lodged via the endotracheal tube into a distal airway (likely in the right lung), 20ml of saline is instilled and 2-3ml sample aspirated back.

(iii) Protected specimen brush (PSB) – a catheter with a brush is kept within a sheath and this is introduced via the endotracheal tube. The sheath protects
the inner catheter from organisms that colonise the upper airways; sample is then acquired by means of the brush.

(iv) Mini-BAL – this is similar to the PSB in that the sheath protects the inner catheter, however there is no brush. Saline is instilled and sample aspirated.

(iv) Bronchoalveolar lavage (BAL) – formal bronchoscopy of a defined airway, instillation of saline and aspiration of sample.

Each of these vary in the ease of sampling, the ability to direct sampling to a focused site and the utility in sampling distal airways [18]. Compared with a histopathological gold standard, TBA had 69% sensitivity and 92% specificity for VAP diagnosis [21]; PSB had 22-36% sensitivity and 50-77% specificity [24, 25]. Mini-BAL had up to 56% sensitivity and 89% specificity when used for gram stain [26]. The sensitivity and specificity of BAL compared with post-mortem varies depending on the culture method (≥ 10⁴ cfu/ml or presence of intracellular organisms/ BAL neutrophil level) from 11-100% and 45-100% [21, 24-26]. Overall, BAL offers the ability to target a defined lobe/segment but can be associated with temporary reduction in oxygenation of the patient when PEEP is lost during the procedure. The role of bronchoscopy and other invasive techniques versus non-invasive sampling remains under debate. Two meta-analyses showed similar rates of mortality, duration of ventilation and ICU length of stay between the groups. However, since then Conway –Morris et al (2009) showed that use of BAL rather than non-invasive techniques resulted in a 21% reduction in the use of antibiotics [27]. Once the sample has been obtained, cultures are performed. This may be assessed by quantitative or semi-quantitative means. In the former, growth of >10⁴ cfu/ml is considered significant for BAL and mini-BAL, >10³ cfu/ml for PSB and >10⁵ cfu/ml for TBA. With semi-quantitative culture, growth is given as none, light, moderate and heavy. A positive culture is considered to have moderate or heavy growth. Overall there is much debate as to the optimal means of diagnosing VAP microbiologically. In the recent guidance from the CDC, quantitative (≥ 10⁴ cfu/ml) and semi-quantitative microbiology were both accepted as equal means of diagnosing VAP [28].
1.4.5 VAP scoring systems

Attempts have been made to combine the clinical, radiological and microbiological features into a score to predict VAP. Three systems have been utilised most in VAP research, all employing subtly different combinations of these criteria.

The Center for Disease Control / National Healthcare Safety Network (CDC/NHSN) criteria apply to nosocomial pneumonias, not exclusively VAP [29]. The Clinical Pulmonary Infection Score (CPIS) and Hospitals in Europe Link for Infection Control though Surveillance (HELICS) criteria apply only to VAP [30-32]. In 2013, after completion of our study, the CDC published new guidance on VAP detection and definition, to improve surveillance amongst ICU’s. Whilst not a scoring system for VAP, it details consensus guidelines for VAP diagnosis and is therefore included in this review [28].

1.4.5a Clinical Pulmonary Infection Score (CPIS)

The CPIS score is relatively easy to calculate (Table 1.6). A score of over 6 was suggestive of pneumonia. The original sensitivity of 93% and specificity of 100% obtained by Pugin was based on 28 patients and a quantitative microbiological gold standard [30]. Using a histological gold standard rather than a bacteriological one, the sensitivity dropped to 72-77% and specificity to 42-85% depending on the cutoff of the ROC curve [18]. If compared with a gold standard obtained by BAL, the sensitivity was 30-89% and specificity (17-80%). As Rea-Nato has pointed out, if the cutoff level of 6 for the CPIS score is changed and the gold standard to which it is compared is altered as above, the diagnostic performance can change. Furthermore, as with all the scoring systems for VAP, there is inter-observer variation in the scores determined, reducing overall accuracy. Schurink et al (2004) found a kappa score of 0.14-0.18 in their series of 52 patients [33].
Comparing studies assessing the utility of CPIS is difficult as the gold standard varies (histopathology or quantitative culture). Moreover, some studies recruited all mechanically ventilated patients and others patients with suspected VAP. The sensitivities varied from 60-89% and specificities 42-85% [21, 26, 33, 34]. Overall, the limitations are not unique to CPIS and it remains a useful tool in the research setting.
Table 1.6 The Clinical Pulmonary Infection Score

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>≥36.5 and ≤38.4</td>
<td>0</td>
</tr>
<tr>
<td>≥38.5 and ≤38.9</td>
<td>1</td>
</tr>
<tr>
<td>≥39 and ≤36.5</td>
<td>2</td>
</tr>
<tr>
<td>Blood leukocytes (mm$^3$)</td>
<td></td>
</tr>
<tr>
<td>≥4000 and ≤11000</td>
<td>0</td>
</tr>
<tr>
<td>&lt;4000 and &gt;11000</td>
<td>1</td>
</tr>
<tr>
<td>+ band forms ≥50%</td>
<td>Add 1</td>
</tr>
<tr>
<td>Tracheal secretions</td>
<td></td>
</tr>
<tr>
<td>Absence of tracheal secretions</td>
<td>0</td>
</tr>
<tr>
<td>Presence of nonpurulent tracheal secretions</td>
<td>1</td>
</tr>
<tr>
<td>Presence of purulent tracheal secretions</td>
<td>2</td>
</tr>
<tr>
<td>Oxygenation: PaO$_2$ / FiO$_2$ (mmHg)</td>
<td></td>
</tr>
<tr>
<td>&gt;240 or ARDS</td>
<td>0</td>
</tr>
<tr>
<td>≤240 and no ARDS</td>
<td>2</td>
</tr>
<tr>
<td>Pulmonary radiography</td>
<td></td>
</tr>
<tr>
<td>No infiltrate</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse (or patchy infiltrate)</td>
<td>1</td>
</tr>
<tr>
<td>Localized infiltrate</td>
<td>2</td>
</tr>
<tr>
<td>Progression of pulmonary infiltrate</td>
<td></td>
</tr>
<tr>
<td>No radiographic progression</td>
<td>0</td>
</tr>
<tr>
<td>Radiographic progression (after CHF and ARDS excluded)</td>
<td>2</td>
</tr>
<tr>
<td>Culture of tracheal aspirate</td>
<td></td>
</tr>
<tr>
<td>Pathogenic bacteria cultured in rare or light quantity or no growth</td>
<td>0</td>
</tr>
<tr>
<td>Pathogenic bacteria cultured in moderate or heavy quantity</td>
<td>1</td>
</tr>
<tr>
<td>Same pathogenic bacteria seen in Gram stain</td>
<td>Add 1</td>
</tr>
</tbody>
</table>
The Clinical Pulmonary Infection Score (CPIS) combines clinical, radiological and microbiological criteria to determine whether or not a patient has VAP. ARDS = acute respiratory distress syndrome. CHF = congestive heart failure. \( \text{PaO}_2 / \text{FiO}_2 \) = ratio of arterial oxygen pressure to fraction of inspired oxygen. Pathogenic bacteria cultured = predominant organism in the culture. Overall: CPIS at baseline was assessed on the first five variables. CPIS at 72 hours was calculated based on all seven variables. A score over six at baseline or at 72 hours was considered suggestive of pneumonia. From Pugin et al (1991) [30].
### 1.4.5b HELICS criteria for diagnosis of ventilator-associated pneumonia

The HELICS criteria create 5 categories of VAP depending on the microbiological technique used to diagnose the pneumonia (Table 1.7). This makes comparing one with another difficult. In one study, using quantitative BAL culture rather than endotracheal aspirate resulted in a four-fold reduction in the diagnosis of VAP [27]. Therefore comparison of VAP rates between hospitals requires knowledge of the culture technique used.
Table 1.7 The HELICS criteria for VAP diagnosis

<table>
<thead>
<tr>
<th>X-ray</th>
<th>And at least one of the following</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fever &gt; 38°C with no other cause</td>
</tr>
<tr>
<td></td>
<td>Leucopaenia ($\geq$4000 WBC/mm$^3$) or leucocytosis ($\geq$12 WBC/mm$^3$)</td>
</tr>
<tr>
<td></td>
<td>And at least 1 of the following (or at least 2 if clinical pneumonia only = PN4 and PN5)</td>
</tr>
<tr>
<td></td>
<td>New onset of purulent sputum or change in character of sputum (colour, odour, quantity, consistency),</td>
</tr>
<tr>
<td></td>
<td>Cough or dyspnoea or tachypnoea</td>
</tr>
<tr>
<td></td>
<td>Suggestive auscultation (rales or bronchial breath sounds), rhonchi, wheezing</td>
</tr>
<tr>
<td></td>
<td>Worsening gas exchange</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>And according to the used diagnostic method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteriologic diagnostic performed by:</td>
</tr>
<tr>
<td></td>
<td>PN1 Positive quantitative culture from minimally contaminated lower respiratory tract specimens</td>
</tr>
<tr>
<td></td>
<td>BAL $\geq$ $10^4$ CFU/ml or $\geq$5% of BAL obtained cells contain intracellular bacteria on direct microscopic exam (classified on diagnostic category BAL)</td>
</tr>
<tr>
<td></td>
<td>Protected brush with a threshold of $\geq$10$^5$ CFU/ml</td>
</tr>
<tr>
<td></td>
<td>Distal protected aspirate with a threshold of $\geq$10$^3$ CFU/ml</td>
</tr>
<tr>
<td></td>
<td>PN2 Positive quantitative culture from possibly contaminated lower respiratory tract specimen - Quantitative culture of lower respiratory tract specimen (e.g. endotracheal aspirate) with a threshold of $\geq$ 10$^6$ CFU/ml</td>
</tr>
<tr>
<td></td>
<td>Alternative microbiology methods</td>
</tr>
<tr>
<td></td>
<td>PN3</td>
</tr>
<tr>
<td></td>
<td>Positive blood culture not related to another source of infection</td>
</tr>
<tr>
<td></td>
<td>Positive growth in culture of pleural fluid</td>
</tr>
<tr>
<td></td>
<td>Pleural or pulmonary abscess with positive needle aspiration</td>
</tr>
<tr>
<td></td>
<td>Histologic pulmonary exam shows evidence of pneumonia</td>
</tr>
<tr>
<td></td>
<td>Positive exams for pneumonia with virus or particular germs (Legionella, Aspergillus, mycobacteria, mycoplasma, Pneumocystis carinii)</td>
</tr>
<tr>
<td></td>
<td>positive detection of viral antigen or antibody from respiratory secretions (e.g. EIA, FAMA, shell viral assay, PCR), positive direct exam or positive culture from bronchial secretions or tissue, seroconversion (e.g. influenza viruses, Legionella, Chlamydia) and detection of antigens in urine (Legionella)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microbiology</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PN4 Positive sputum culture or non-quantitative lower respiratory tract culture</td>
</tr>
<tr>
<td></td>
<td>PN5 No positive microbiology</td>
</tr>
</tbody>
</table>

Table 1.7. HELICS criteria for VAP diagnosis. Of the 5 methods, PN1 and PN2 were validated without previous antimicrobial therapy [32].
1.4.5c CDC / National Healthcare Safety Network (NHSN) definition for clinical diagnosis of hospital-acquired pneumonia

This definition of HAP does not specifically refer to VAP, which is one of its limitations. Although straightforward to understand, it does not include any form of microbiology (Table 1.8). When compared with BAL in approximately 300 trauma patients, its sensitivity was 84% and specificity 69% [35]. Microbiological culture ($\geq 10^5$ cfu/ml) of BAL was used as the gold standard, which differs from the conventional use of $\geq 10^4$ cfu/ml in most other studies.

**Table 1.8 CDC / NHSN definition of hospital-acquired pneumonia**

<table>
<thead>
<tr>
<th>Radiological signs</th>
<th>Two serial chest radiographs with at least one of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- New or progressive and persistent infiltrate</td>
</tr>
<tr>
<td></td>
<td>- Consolidation</td>
</tr>
<tr>
<td></td>
<td>- Cavitation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>At least one of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Fever (temperature $&gt; 38^\circ$C) with no other recognised cause</td>
</tr>
<tr>
<td></td>
<td>- Leucopaenia ($&lt; 4.0 \times 10^9$ cells/L) or leucocytosis ($&gt; 12.0 \times 10^9$ cells/L)</td>
</tr>
<tr>
<td></td>
<td>- For adults $&gt; 70$ years of age, altered mental status with no other recognised cause</td>
</tr>
<tr>
<td>And $\geq$ two of the following:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- New onset of purulent sputum, change in character of sputum</td>
</tr>
<tr>
<td></td>
<td>- Increased respiratory secretions, or increased suctioning requirements</td>
</tr>
<tr>
<td></td>
<td>- New-onset or worsening cough, or dyspnoea, or tachypnoea</td>
</tr>
<tr>
<td></td>
<td>- Rales or bronchial breath sounds</td>
</tr>
<tr>
<td></td>
<td>- Worsening gas exchange, e.g. oxygenation index ratio ($\text{PaO}<em>2/\text{F}</em>\text{O}_2$) $\leq 240$, increased oxygen requirement, or increased ventilation demand</td>
</tr>
</tbody>
</table>

**Table 1.8.** Hospital-acquired pneumonia diagnosed by clinical and radiological criteria, in the absence of microbiological confirmation. Of note, it does not specifically apply to VAP [29].
1.4.5d The new CDC paradigm for VAP surveillance

In 2013, the CDC published new definitions for VAP for surveillance purposes. Their aim was to reduce variability between ICUs in VAP definition rates due to observer bias in diagnosis. Such a change was required because VAP rates are now becoming used as a benchmark of quality in healthcare institutions with funding penalties for Units that have high rates [28].

The concept of Ventilator-associated events was created, where mechanical ventilation in patients is present and stable or improving but then deteriorates. If this deterioration continues for 2 or more days then a ventilator-associated condition (VAC) is present. Causes may be pulmonary or non-pulmonary but are mainly due to pneumonia, pulmonary oedema, atelectasis and ARDS. Any of these potentially setback a patient’s progress and increase length of stay. Infection related VACs (IVAC) comprise patients with VAC and an abnormal temperature or white-cell count and are given new antibiotics for at least four days. Possible and probable pneumonias require the presence of purulent respiratory secretions, pathogenic bacteria or both. If either of these is present, the pneumonia is ‘possible.’ If the purulent secretions and positive cultures are present then the pneumonia is ‘probable.’ Patients with ‘probable’ pneumonias can also have positive tests for histopathology, pleural-fluid cultures or Legionella. Of interest, positive cultures may be either quantitative or semi-quantitative to define a pneumonia and VAC. Table 1.9 summarises the diagnostic criteria for the CDC definitions.

Overall, the CPIS and the HELICS criteria are used for ongoing VAP research.
Table 1.9 CDC Surveillance Criteria for Ventilator-Associated Events

<table>
<thead>
<tr>
<th>Concept</th>
<th>Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>New respiratory deterioration</td>
<td>Ventilator-associated condition (VAC)</td>
<td>≥2 calendar days of stable or decreasing daily minimum positive end-expiratory pressure or daily minimum fraction of inspired oxygen, followed by a rise in daily minimum positive end-expiratory pressure of ≥3 cm of water or a rise in the daily minimum percentage of inspired oxygen by &gt; 20 points sustained for ≥ 2 calendar days</td>
</tr>
<tr>
<td>New respiratory deterioration</td>
<td>Infection-related ventilator-associated condition (IVAC)</td>
<td>VAC plus a temperature of &lt;36°C or &gt;38°C or a leucocyte count of ≤ 4000 or ≥ 12000 per cubic millimetre, plus one or more new antibiotics continued for at least 4 days within 2 calendar days before or after onset of a VAC, excluding the first 2 days of mechanical ventilation</td>
</tr>
<tr>
<td>New respiratory deterioration</td>
<td>Possible pneumonia</td>
<td>IVAC plus Gram’s staining of endotracheal aspirate or bronchoalveolar lavage showing ≥ 25 neutrophils and ≤ 10 epithelial cells per low-power field, or a positive culture for a potentially pathogenic organism, within 2 calendar days before or after onset of a VAC, excluding the first 2 days of mechanical ventilation</td>
</tr>
<tr>
<td>New respiratory deterioration</td>
<td>Probable pneumonia</td>
<td>IVAC plus Gram’s staining of endotracheal aspirate or bronchoalveolar lavage showing ≥ 25 neutrophils and ≤ 10 epithelial cells per low-power field, plus endotracheal aspirate with ≥ 10⁵ colony-forming units per millimetre or broncho-alveolar lavage culture with ≥ 10⁴ colony-forming units per millimetre, or endotracheal-aspirate or broncho-alveolar lavage semiquantitative equivalent, within 2 calendar days before or after onset of a VAC, excluding the first 2 days of mechanical ventilation</td>
</tr>
</tbody>
</table>

**Table 1.9.** The CDC defines ventilator-associated conditions (VAC), infection related VAC, as well as those due to possible or probable pneumonia [28]. The possible pneumonia has either gram stain or positive microbiology whereas the probable pneumonia has both.
1.5. The pulmonary Immune Response to Infection

1.5.1 Background

The distal airways of the lung are protected from external microbial challenges by a combination of innate and adaptive responses. The initial innate response can be considered first-line. It involves both anatomical and physiological mechanisms and interacts with the later adaptive response of lymphocytes and antibodies. Each will be considered separately.

1.5.2. Anatomical barriers

Air is filtered through mucus and hairs in the nose and turbinates. The large surface area of the intact lung epithelium acts as a mechanical barrier to the ingress of organisms. Mucus secreted by goblet cells effectively traps larger organisms, which are then wafted by cilia to the proximal respiratory tract to be expectorated or swallowed (the ‘mucociliary escalator’).

1.5.3. Physiological innate immunity

Innate immunity combines a plethora of soluble proteins and cells that act against evolutionarily-conserved microbial components. The soluble proteins involved include cytokines, chemokines, antimicrobial peptides, complement and the coagulation cascade. Cells include neutrophils, monocytes/macrophages, natural killer (NK) cells, eosinophils, basophils/mast cells and dendritic cells.

1.5.4. Pathogen pattern recognition receptors (PPR)

Danger signals are proteins associated with tissue damage and death and are perceived by the immune system as a threat. They include intracellular proteins such as heat shock proteins and nucleotides released with cell death, altered matrix proteins and nucleic acids from micro-organisms. They
constitute ‘Danger Associated Molecular Patterns’ (DAMPs) that are detected by the innate response.

‘Pathogen Associated Molecular Patterns’ (PAMPs) are the microbial components that are conserved throughout evolution and essential to microbial function. They include microbial envelope proteins as well as nucleic acids. They too are recognised by the innate response. Examples include bacterial flagellin, lipopolysaccharide (LPS), lipotechoic acid (LTA), viral envelope glycoproteins and RNA (single and double stranded), fungal mannoproteins and beta-glucans as well as parasitic glycolipids.

Both DAMPs and PAMPs are recognised by the innate response by a set of receptors known as the ‘Pathogen Pattern Recognition Receptors’ (PPR). These are encoded in the germline. Such receptors are present in large amounts across epithelial and innate immunity cells allowing a rapid response to external challenges. However, unlike the adaptive response, the innate response is amnestic – repeated challenged are not ‘remembered’ by this system and do not result in any more rapid or amplified response.

The innate response can either be activated by the detection of PAMPs, DAMPs or by detecting the ‘missing self.’ In the latter, healthy cells constitutively express MHC (major histocompatibility complex) Class I molecules. NK cells are normally inhibited from lysing cells by their inhibitory receptors which recognise Class I molecules and override the NK cell activation receptors. With virally infected cells, class I production can be attenuated (ie a ‘missing self’ protein), leading to a reduction of NK cell inhibition and thus activation and lysis of the infected cell.

**1.5.5. Receptors detecting PAMPs and DAMPs**

PAMPs may be detected by Toll-like receptors (TLR), NOD (nucleotide oligomerisation domain)-like receptors (NLR), C-type lectin receptors (CLR) and RIG (Retinoic acid inducible gene)-1 like receptors (RLR). The NLR may also recognise DAMPs.
1.5.5a. Toll-like receptors (TLR)

10 different TLR have been defined; they are transmembrane receptors with a leucine rich extracellular domain and a cytoplasmic portion for signalling that shares homology with the IL-1 receptor. TLR may be found in two main parts of the cell; on the surface of epithelial cells, macrophages and dendritic cells (TLR 2,4 and 6) and on the surface of the endoplasmic reticulum, phagosomes and endosomes (TLR 3,7 and 9). The former recognise PAMPs on the surface of microbes whereas the latter recognise intracellular nucleic acids. TLR 4 recognises lipopolysaccharide, TLR 2 lipoteichoic acid and the others recognise either other lipoproteins, nucleic acids or flagellin. Ligation of the TLR receptors results in pro-inflammatory cytokine secretion, interferon and immunoglobulin release as well as dendritic cell maturation.

1.5.5b. NOD-like receptors (NLR)

There are over 20 different NLR in humans, many of which are poorly characterised. NLR 1 and 2 recognise peptidoglycans and activate NF-κB; others form part of a multi-protein complex called the inflammasome – this recognises metabolites from cell damage and activates pro-inflammatory cytokines via caspase-1. The NLR are cytoplasmic receptors containing a leucine-rich repeating domain, a NOD domain and a signalling domain (eg. CARD – caspase recruitment domain).

1.5.5c. C-type lectin like receptors (CLR)

These receptors recognise microbial carbohydrates. They then activate pro-inflammatory immune responses, activate complement or opsonise bacteria. Examples include the mannose receptor, mannose binding lectin, pentraxins (such as C-reactive protein) and Dectin-1 (binding candidal β-glucan).
1.5.5d. RIG-1 like receptors (RLR)

RLR have a role in the innate response to viral infection. Double stranded RNA (not normally found in the cytoplasm) is produced during replication of some viruses. Both double and single stranded RNA from viruses are recognised by the RLR which then activate antiviral interferon responses by binding to the mitochondrial protein IPS-1 (interferon-β promoter stimulator).

1.5.6. Soluble proteins involved in the innate response

1.5.6a. Complement

Complement has a diverse set of roles that include the innate response, interaction with B-cells to facilitate the antibody response, together with the disposal of apoptotic cells and immune complexes. The topic of Complement is too large to be covered in detail in this review. However, here is a précis:

Complement consists of 25 cell surface and plasma proteins. Normally controlled by regulator proteins, complement may be activated by antigen-antibody complexes (classical activation), microbial products (alternate pathway) or by mannan-containing microbes (mannan binding lectin pathway). The resultant activation results in the formation of C3b which can opsonise microbes. c3a and c5a can be formed which are vasoactive – they increase vascular permeability and act as chemoattractants for mast cells and neutrophils to migrate into affected tissues. Several complement proteins can also assemble via a final common pathway into the membrane attack complex which can form pores in microbes and cause target cell lysis. Finally, in a similar way to MHC class I, the presence of surface complement can act as an inhibitor of cell lysis by NK cells (a variant of the ‘missing self’ hypothesis).
1.5.6b. Cytokines

Cytokines are secreted proteins which can regulate the immune response. Each cytokine can be secreted by more than one type of cell and their effector targets can be multiple cell types too. They can stimulate growth, differentiation and activate / inhibit immune responses. The cytokines secreted by the antigen presenting cells (APC) have function in regulating the innate response. The APC include monocytes/phagocytes and dendritic cells. The cytokines released from these cells include IL-1, IL-6, IL-8, IL-12 and TNF-α. Each of these cytokines bind to a surface receptor (which often share sequence homology with each other). The cytokine receptors tend to signal downstream processes via either the STAT protein (signal transducer and activation of transcription) or the Janus kinases (Jak). The former dimerise and migrate to the nucleus to bind with transcription factors such as NF-κB).

1.5.6c. IL-1 cytokine family

The family consists of IL-1α, IL-1β, IL-1 ra (receptor antagonist), IL-18 and IL-33. IL-1α and IL-1β stimulate leukocyte adherence to the endothelium, an effect antagonised by IL-1 ra. IL-1 induces fever, anorexia and sleep as well as causing the liver to decrease albumin production and synthesise acute phase reactants such as CRP. IL-1 can also activate T cells via an IL-2 effect.

1.5.6d. IL-6 cytokine family

The cytokine members in this group are IL-6, IL-11, IL-27, oncostatin M. The receptor for these proteins are a multi-subunit protein with a cytokine-specific binding site and a gp130 common subunit. Like IL-1, IL-6 induces fever, lethargy and anorexia. It also stimulates acute phase protein secretion to a greater extent than other cytokines. It helps CD4 Th17 regulatory T cell differentiation as well as promoting B cell differentiation into plasma cells. IL-6 preferentially signals through STAT-3.
1.5.6e. Tumour necrosis factor alpha (TNF-α)

This is the main mediator of septic shock. It may either exist as a soluble protein or a surface receptor. It can bind to two types of receptor: p55 and p75. It activates neutrophil function (chemotaxis, activation, degranulation and respiratory burst). It also increases vascular permeability and facilitates entry of neutrophils into inflamed tissues.

1.5.6f. Interferons

Type 1 interferons include IFN-α, IFN-β and IFN-ω. They are derived from B cells, plasmacytoid dendritic cells and monocytes/macrophages. They have antiviral properties, inhibiting viral replication and preventing transmission of virus to healthy cells. They promote NK and CD8 T cell activity to enhance the antiviral response. Type 3 interferons include IFN-λ, IL-28 and IL-29 also have similar anti-viral properties to type 1 interferons.

1.5.6g. Chemokines

These are 8-12 kDa proteins that are chemoattractants for immune cells. They recruit leukocytes and activate them to mount an immune response, regulate cell migration (T cells, B cells and dendritic cells), assist in angiogenesis and organ development and are markers for CD4 T cell subset maturation. Chemokines are subdivided by the positioning of conserved cysteine amino acid residues on the N-terminus: C-X-C chemokines have an interposing amino acid; C-C chemokines have adjacent cysteine residues. The C-X-C chemokines attract neutrophils and either activate or inhibit blood vessel formation. C-C chemokines attract T-cells, eosinophils and monocytes. IL-8 is a member of the C-X-C family and is chemoattractant to neutrophils and activates them to have a respiratory burst. IL-8 is released by cells of the innate system that have toll-like receptors.
1.5.6h. **Antimicrobial peptides**

Numerous small peptides exist that direct against evolutionarily-conserved microbial components. These peptides have significant antibiotic capability and include cathelicidins (such as LL-37) and defensins.

1.5.6i. **Other proteins**

Lactoferrin and ferritin bind iron, which is essential for bacterial replication. The coagulation cascade generates factors with antimicrobial properties in addition to limiting the spread of infection.

1.5.7. **Cellular innate immune response**

1.5.7a. **Neutrophils**

Neutrophils contain numerous granules to destroy bacteria and fungi. Once activated in the blood, they migrate into inflamed tissues along a chemoattractant gradient. They possess receptors such as L-selectin (CD62L) to allow migration. They activate a respiratory burst, which releases oxygen free radicals to destroy organisms.

1.5.7b. **Monocytes/macrophages**

Monocytes patrol the bloodstream and migrate into the tissues. In the tissues such as the lung, they are represented as macrophages. They phagocytose organisms, forming an endosome of membrane around them. This then merges with a lysosome (granule containing proteolytic enzymes) to digest the material. In the lung, alveolar macrophages are abundant. They also have a role in antigen presentation to the adaptive immune cells.

1.5.7c. **Dendritic cells (DCs)**

DCs are antigen presenting cells, linking the innate and adaptive immune responses. They are activated by microbes and pass to the lymph nodes to activate T and B cells. They may be divided into myeloid dendritic cells (mDC)
which secrete IL-12 and stimulate T-cells and plasmacytoid dendritic cells (pDC), which have the ability to secrete IFN-α.

1.5.7d. Eosinophils

Eosinophils have granules that are effective against parasitic infections.

1.5.7e. Basophils/mast cells

Mast cells release histamine following stimulation by IgE dependent pathways. Histamine causes bronchoconstriction, mucus production and mucosal oedema.

1.5.7f. NK cells

As previously mentioned, NK cells target virally infected cells and are activated by means of the 'missing-self' hypothesis.

1.5.8. Adaptive immune response

In contrast to the innate response, the adaptive response is anamnestic ie. there is immunological memory. Following a single challenge, subsequent microbial challenges result in an earlier and magnified response that complements the innate response. This is the principle of vaccination. The adaptive response consists of T-cell and B-cell activity. T-cells include CD4+ helper cells (which assist B-cells in production of antibodies), CD8+ cytotoxic T-cells involved in response to viral infection, Natural Killer T-cells and regulatory T-cells which have a role in immunological self tolerance. CD4+ T-cells are depleted in HIV and increase the risk of opportunistic infections of the lung. Pelekanou and colleagues (2009) commented that the levels of CD4+ lymphocytes were reduced in the blood of patients with VAP.

This thesis concentrates on the cells of the innate response (neutrophils and monocytes).
1.6 The TREM family

1.6.1 History

Neutrophils and monocytes are stimulated via a range of receptors that include CD14, Fc and complement receptors, chemokine receptors and Toll-like receptors (TLR's). Other activating receptors are present that belong to either the immunoglobulin superfamily (Ig-SF), sharing sequence homology with immunoglobulins and involved in cell-adhesion and protein binding, such as the leukocyte immunoglobulin-like receptors (LIR) and signal regulatory protein β1 (SIRPβ1), or to the C-type lectin superfamily, including the myeloid DAP-12-associating lectin-1. These receptors share sequence homology with activating NK cell receptors; the transmembrane domain possesses a negatively charged amino-acid and a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM). The receptors couple with either the gamma-chain of the Fc receptor or DAP-12 (DNA-X adaptor protein of 12 kDa) and activation leads to ITAM phosphorylation. This in turn leads to protein tyrosine kinase activation and cell activation [36].

In 1999, Cantoni et al discovered a new receptor (NKp44) on Natural Killer cells that was a member of the immunoglobulin superfamily and associated with DAP-12 [37]. In 2000, Bouchon and colleagues [36] postulated that other receptors similar to this may exist on other myeloid cells. Utilising a complementary DNA database, they identified a series of receptors sharing homology to NKp44 but present on myeloid cells. These were termed the ‘Triggering Receptors Expressed on Myeloid Cells’ (TREM). TREM-1, -2 and -3 were identified, all containing Lysine as the negatively charged transmembrane amino acid. TREM-1 and TREM-3 have roles in cell activation whereas TREM-2 inhibited cell responses.

1.6.2 TREM-1

TREM-1 exists in a variety of species (bovine, porcine, murine as well as human) demonstrating evolutionary conservation and an importance in immune functioning. Abrogation of TREM-1 activity in murine gram-negative
endotoxaemia protected from lethality [38-40]. TREM-1 exists as two isoforms, surface-TREM-1 and soluble TREM-1 (sTREM-1). Surface TREM-1 is a monomeric receptor present on the surface of neutrophils and monocytes/macrophages, herein described as neutrophilic TREM-1 (nTREM-1) and monocytic TREM-1 (mTREM-1). Dimerisation may occur [41]. Receptor activation signals via DAP-12 leading to increased synthesis of pro-inflammatory cytokines, including IL-8, monocyte chemotactic protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) as well as neutrophil degranulation [42]. The ligand for mTREM-1 and nTREM-1 still remains elusive, but may be present on platelets [43]. Initial research showed that surface TREM-1 is elevated in bacterial and fungal infections (extracellular) but not in viral, mycobacterial (intracellular) or other inflammatory conditions [36]. Soluble TREM-1 (sTREM-1) on the other hand, is not membrane bound. Two theories of its origin exist; the first is that it is synthesised separately from surface TREM-1 and secreted from the cell [44]; the second, more accepted hypothesis (figure 1.1) is that sTREM-1 is cleaved from the membrane (surface TREM-1) of the cell by matrix metalloproteinases [45]. In a human model of endotoxaemia created by injection of LPS to healthy volunteers, peripheral blood nTREM-1 levels decreased immediately. sTREM-1 levels increased over time, as did mTREM-1. This supports the hypothesis that sTREM-1 may be derived from surface TREM-1 (particularly nTREM-1) [46].

The role of sTREM-1 is thought to be as a decoy receptor for the surface TREM-1 ligand; in other receptor systems such as the TNF receptor, the soluble form acts as a decoy for the surface receptor. Ligand binding to the soluble form is not available to bind and activate the bound receptor, therefore acting as an antagonist. With regards to TREM-1, bacterial activation of the surface receptor amplifies immune responses; cleavage of the surface receptor to the soluble form then leads to reduced receptor activation and a natural form of negative feedback [47, 48].

Surface TREM-1 expression is increased with lipopolysaccharide and lipoteichoic acid stimulation, indicating a role in gram-negative and gram-positive infections respectively. Given the lack of a known TREM-1 ligand, research has stemmed from the use of antibodies to cross-link and activate
the receptor, or peptides (LP17) to block receptor activity. Studies have been conducted in animal models, human cell cultures and in vivo human sampling in septic states. Activation of TREM-1 in the presence of LPS results in a synergistic increase in pro-inflammatory cytokines indicating that TREM-1 may amplify immune responses to infection [42].

TREM-1 has gained attention in the field of infection diagnosis. The reasons for this are twofold: the perceived restriction of the surface receptors to neutrophils and monocytes, the two types of cells involved in innate response to infection and the amplification of cell responses to bacterial and fungal but not viral infections. nTREM-1 and mTREM-1 are measurable by immunohistochemistry or by flow cytometry; sTREM-1 by Enzyme-linked immunosorbent assay (ELISA). Regardless of technique, TREM-1 may be measured in a variety of body fluids.

**Figure 1.1 Surface and soluble TREM-1**

![Diagram of Surface and Soluble TREM-1](image)

**Figure 1.1.** Matrix metalloproteinases -8 and -9 may cleave surface receptor from monocytes and neutrophils to give soluble TREM-1. The ligand for soluble / surface TREM-1 is currently unknown.
1.6.3 TREM-2

TREM-2 has functions in the immune system but also in bone formation and neural development. Patients deficient in TREM-2 develop Nasu-Hakola disease, a condition characterised by the formation of bone cysts and central nervous system demyelination [49]. Immunologically, it functions to attenuate macrophage activation in response to ligands such as LPS [50]. Its function in the lung has not been well defined thus far. Hoogerwerf et al (2010) instilled LPS into the airways of healthy volunteers and then obtained immune cells from BAL. The surface expression of TREM-2 did not alter on alveolar macrophages [51]. However, Sun et al (2011) measured TREM-2 in the lung in experimental ALI. TREM-2 mRNA was decreased from baseline, in comparison to TREM-1 (which increased). The effect was reversed by administration of vasoactive intestinal polypeptide [52]. TREM-2 also functions as a phagocytic receptor for bacteria [53]. Overall, the putative role of surface TREM-2 and its soluble counterpart in pulmonary infection is unclear.

1.6.4 Summary of pulmonary immune response in VAP

The endotracheal tube bypasses the anatomical barriers of the innate immune system. Bacteria (or other micro-organisms) can spread to the lung as micro-aspiration of aerobic gram-negative bacilli around the cuff of the ETT, through the ETT or haematogenously.

In the lung, there are homeostatic mechanisms preventing inflammation. This includes proteins such as CD200 on the surface of macrophages, preventing activation [54]. In critical illness, there is dysregulation of T-regulatory cells, alveolar macrophages and neutrophils [55] that increases the risk of nosocomial infections. Sedation with agents such as benzodiazepines may also play a role.

Bacteria overwhelm the antibacterial peptides (such as cathelicidin) and stimulate TLR and other PAMP receptors on the surface of innate cells. Complement may be activated and the monocytes / neutrophils are stimulated to release pro-inflammatory cytokines. CD14 (on monocytes) and CD11b (a
protein involved in monocyte cell activation) interact with Toll-like receptors during stimulation with LPS [56]. Proteins such as TREM-1 and TREM-2 modify the immune response by amplifying / dampening cytokine responses and neutrophil degranulation. Soluble TREM-1 acts to regulate the surface TREM-1 on monocytes and neutrophils. Further immune cells are recruited from the blood and inflammation progresses. These neutrophils and monocytes use CD62L (L-selectin) to cross the endothelium into the alveolus.
1.7 Diagnostic biomarkers for VAP

A biomarker has been defined as ‘a characteristic [substance] that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ [57]. In the context of VAP, they have the potential to improve the speed and accuracy of diagnosis compared with current microbiological techniques, or to allow prompt cessation of antibiotic therapy when VAP resolves.

This review aims to understand the features of an ideal biomarker in VAP, appraise the biomarkers currently studied or in clinical practice and direct further research.

1.7.1 The ideal biomarker

Identification of a ‘footprint’ of an infectious agent that may be easily quantified and tracked so as to provide diagnostic, monitoring and prognostic information is the current focus of VAP biomarker research. However, such a biomarker does not yet exist for VAP. Distinguishing between infection and non-infective inflammation is difficult, as both processes lead to the same pathway of cell damage and release of pro-inflammatory cell contents via PAMPs and DAMPs. One approach is to screen levels of putative markers in the tissues and organs of those with infection, those without infection and in those whom it is suspected. Once identified, the validity of such markers may then be subsequently tested in blinded clinical trials of patients with suspected infection.

Morrow and de Lemos suggested three criteria that ought to be fulfilled by a putative biomarker:

(i) Ease of measurement by the clinician (including speed of testing)

(ii) Addition of new information compared to existing markers / tests (e.g. increased sensitivity / specificity of diagnosis)
(iii) Does the biomarker aid clinician decision making eg antibiotic stewardship [58].

Firstly, there needs to be an easily accessible and cost-effective validated assay. These issues are not straightforward. When assessing techniques in a research setting, analysers may be used that are difficult to employ clinically. Furthermore, reagent cost may be high whilst being researched, but reduce once in clinical use. Secondly, the test should be simple to perform and be rapid (over a few hours to influence clinical management). Once again, experimental tests may be slow to currently analyse but become faster over time. An accurate biomarker should be both sensitive and specific for the condition and provide information superior to that obtained in current practice. Ideally levels should follow disease progression, rising early in the disease process and falling promptly with resolution. Finally there should also be randomised controlled studies showing that biomarker-guided therapy improves patient outcomes.

Thus far, VAP biomarkers have failed in one or more of these hurdles. Most studies involve small numbers of VAP patients in a single ICU setting, making interpretation difficult and prone to the risk of outlier bias. A gold standard for the diagnosis of VAP is required to validate each biomarker against and this varies between studies. For the reason previously stated, most markers cannot distinguish between sepsis and non-specific inflammation (i.e., systemic inflammatory response syndrome, SIRS). This decreases accuracy in the complex environment of clinical care, where for example in the settings of trauma, surgery and burns, both co-exist.

Moreover, biomarkers are unlikely to be pathogen-specific given the likely different inflammatory profiles (“footprints”) elicited by the many causative organisms (bacterial, fungal and viral). Microbiological sampling will inevitably be required to tailor antimicrobial therapy but biomarkers can influence whether to commence or stop treatment [59]. The sampling site of the marker is also relevant. A blood biomarker may not necessarily be site-specific even though it is straightforward to measure [60]. For example, a raised serum C-reactive protein may indicate infection in the lung or elsewhere. Therefore
sampling of appropriate body fluid is necessary. Finally, in the respiratory tract, analysis of BALF soluble proteins requires correction for dilution. When bronchoscopy is performed, saline instillation dilutes the epithelial lining fluid and its constituent proteins to a variable and unknown extent. Measurement of an inert marker in both recovered BALF and serum and assuming their concentrations to be equal can allow soluble biomarker level correction. Urea, albumin and total protein levels have all been studied but most VAP studies correct using urea, according to Rennard’s method [61].

Despite these potential difficulties, research into VAP diagnostic biomarkers has accelerated over recent years. Sackett and Haynes (2002) outlined the methodological approaches to studying and validating biomarkers [62]:

(i) Determine whether test results in affected patients differ from those in healthy individuals. This can screen for putative biomarkers.

(ii) Determine whether patients with certain biomarker levels are more likely to have the target disorder.

(iii) Determine whether test results distinguish patients with and without a condition in a population of ‘at risk’ patients. This is the group where the test is likely to have the greatest impact.

(iv) Determine whether outcome is influenced; whether patients having a test fare better than similar patients who are untested. These studies have hitherto been rarely performed in VAP.

In studies based on the first and second phases, patients are selected on the basis of presence or absence of VAP, or healthy controls. This allows generation of cutoff levels together with summary ROCs, sensitivities, specificities, predictive values and likelihood ratios. The Youden index [63] allows the calculation of the optimal cutoff to obtain the highest combination of sensitivity and specificity for a marker.

In studies of the third phase, sequential patients ‘at risk’ of VAP are assessed to determine if the test (in comparison to a gold standard) predicts or refutes
disease correctly. Assessors of the markers are blinded to the final diagnostic category. Finally, fourth phase studies use biomarker-led treatment (initiation or withholding of antibiotic therapy) in a randomised controlled trial to test improvement in patient outcomes (such as reduction in ICU length of stay, mortality or reduction in use of antibiotics).

The following makers have been studied in VAP and will be reviewed against the Sackett and Haynes study hierarchy:

1. C-reactive protein
2. Procalcitonin
3. Cytokines
4. Triggering receptor expressed on myeloid cells-1 (TREM-1)
5. Other

1.7.2 C-reactive protein

C-reactive protein (CRP) is an acute-phase protein released from the liver in response to acute inflammation. Tillett and Francis discovered CRP [64] as a protein capable of precipitating part of a pneumococcus (Fraction C). Its half-life is 19 hours as its serum level is only production dependent [65]. Its role is believed to be in complement activation as CRP binds to a variety of receptors on necrotic and apoptotic cells (such as phosphocholine) in addition to bacteria [66].

Povoa et al [67] prospectively measured CRP levels in a mixed medical/surgical ICU. 112 Patients were classified into those who were infection-free and those who had proven infection. A third group of patients were excluded from analysis who cultured negative but in whom infection was suspected and treated accordingly. For a cutoff CRP level of 87 mg/l, the area under the ROC curve was high at 0.93 (sensitivity 93.4%, specificity 86.1%).
The positive predictive value (PPV) was 93.4% and the negative predictive value (NPV) was 86%. CRP had a sensitivity of 87.5% and specificity of 86.1% at a cutoff CRP level of >96 g/l for the subset of patients with VAP. Assessing pyrexia in addition to CRP improved the specificity but at the expense of reduced sensitivity.

A study by Matson et al showed a serum CRP increase of 25% from the previous day was a potential marker of secondary sepsis but was not seen in 6 of 49 episodes [68]. Considering VAP, Ramirez et al [69] assessed CRP levels in patients with suspected infection. Nine of twenty patients (45%) had microbiologically proven VAP. A CRP level of ≥ 196.9 mg/l had 56% sensitivity and 91% specificity for VAP diagnosis (AUROC curve of 0.714).

Serum CRP levels may be elevated in non-infective inflammatory conditions (such as surgery). Furthermore, levels are not site-specific for infection (for example VAP versus abdominal or line sepsis). Linssen et al therefore employed a high sensitivity assay to measure CRP (and procalcitonin) in the BAL fluid as a marker of tissue levels and compared it with microbiologically proven VAP as the gold standard. Despite ease of measurement, neither tissue biomarker was sufficiently accurate to differentiate VAP from non-VAP in 117 patients [70].

Overall for CRP, the first two criteria of Sackett and Haynes are met adequately. CRP measurement is only likely to be beneficial in facilitating VAP diagnosis in patients where other tissue sites of sepsis are unlikely.

1.7.3 Procalcitonin

Procalcitonin (PCT) is a 116 amino acid polypeptide, which under basal conditions in health, is synthesised by thyroidal C-cells. Cleavage there releases calcitonin (the active hormone), katacalcin and an N-terminal fragment (NProCT). Only minute amounts of the native PCT enters the blood (< 0.05 ng/ml), with a half-life of approximately 24 hours. During an episode of sepsis however, widespread PCT production occurs from parenchymal and differentiated cells. This enters the circulation at much higher levels (up to
1000 ng/ml) without cleavage [71]. The function of this PCT is unknown. Studies show increased mortality where supra-physiological levels of PCT are administered to animals with sepsis. Furthermore, antibodies directed at PCT abrogate experimental sepsis, suggesting it to be immunomodulatory [72]. In terms of ease of measurement, Procalcitonin assay is straightforward. The Brahms PCT Kryptor assay, a commercially available kit, measures PCT at levels from 0.02 – 5000 ng/ml in 20 minutes using 50 µL of blood (Brahms, Hennigsdorf, Germany).

Procalcitonin has been studied in critically ill patients. In a systematic review of adult post-operative and trauma patients, PCT fared better than CRP in differentiating sepsis from SIRS [73]. In a medical ICU, 47 septic patients (37 VAP and 10 bacteraemia) were compared with 23 culture negative patients. A single PCT cutoff level of 0.44 ng/ml on the day of suspected infection had a sensitivity of 65.2%, specificity of 83% and an AUROC of 0.8. Sequential PCT levels have been measured in critical care patients. A PCT increase of 0.26 ng/ml in one day had a positive predictive value of 100%, negative predictive value of 68% and an area under ROC curve of 0.89 for infection diagnosis. However, in some septic patients PCT was undetectable, a major limitation of the biomarker. Furthermore, the patients studied were neither post-surgery nor survivors of cardiac arrest [74]. Such conditions (in addition to renal failure, trauma, burns and rejection after transplantation) trigger SIRS and elevate PCT independently of sepsis [75-81]. Single cutoff levels may seem inappropriate for all clinical settings.

Gibot et al studied 50 critically ill patients and determined that the PCT was elevated (>0.15 ng/ml) in 88% of patients with proven nosocomial infection (31 VAP and 19 patients with extra-pulmonary infection). Absolute PCT levels could not differentiate pulmonary from non-pulmonary infection however [82]. Duflo et al measured serum PCT in 96 patients with and without VAP. PCT had 41% sensitivity and 100% specificity for VAP at a level of 3.9 ng/ml [83]. Pelosi et al measured PCT in patients with traumatic and non-traumatic brain injury. In this neurological setting, 25 out of 58 patients developed early VAP (within 72-96 hours of admission). At study entry, PCT, but not CRP or serum amyloid A protein, was elevated in this early VAP group. Brain injury per se
did not lead to an absolute increase of PCT levels. The sensitivity and specificity of PCT was 76% and 75% respectively. Moreover, PCT levels correlated with pneumonic severity [84]. Luyt et al measured serum PCT in patients with suspected VAP. 32 patients had proven VAP and 41 were non-VAP. Procalcitonin was measured on the day of VAP suspicion in addition to another day from the previous 5 days. A PCT level > 0.5 ng/ml on the day of suspicion had a sensitivity of 72% but poor specificity of 24% to predict VAP. A rise in PCT between the two time points had 41% sensitivity and 85% specificity. Several cases of infection would have therefore been falsely excluded if these criteria had been used to diagnose VAP [85]. Similarly, Ramirez et al found an elevated serum PCT in only 9/20 VAP patients, yielding an AUROC of 0.87, sensitivity of 78% and specificity of 97% for VAP diagnosis for a PCT cutoff of 2.99 ng/ml [69].

As a potential predictor of VAP following return of spontaneous circulation after cardiac arrest, Oppert et al measured serum PCT levels. In 28 patients, 12 developed VAP and Procalcitonin levels rose a median of 2 days prior to developing clinical VAP. A PCT level above 1 ng/ml had 100% sensitivity and 75% specificity to predict VAP [78]. In contrast Jung et al studied serum and BAL PCT levels during 86 VAP episodes in a medical ICU but found neither to accurately diagnose the condition [86]. BAL PCT levels have been performed in two other studies. Linssen et al assayed PCT and CRP but concluded that neither diagnosed VAP despite correcting for epithelial lining fluid dilution. The AUROC for CRP in BAL was 0.477 and the AUROC for PCT in BAL was 0.448 [70]. Duflo et al determined that alveolar PCT obtained by mini-BAL in 96 patients with and without VAP was not discriminative; no diagnostic performance figures were given but the similarity of alveolar PCT levels between the groups suggested an AUROC of approximately 0.5 [83]. Overall, PCT is not a useful diagnostic biomarker. One feature of an ideal VAP biomarker would be that it has high sensitivity. As has been discussed, PCT was undetectable in several microbiologically proven cases of infection. However PCT may have better utility in prognosticating and de-escalating antibiotic therapy for septic critically ill patients. Procalcitonin measurement may form part of a de-escalation protocol for antibiotics in VAP patients. In a
multicentre trial, Stolz et al [87] randomised 101 patients to conventional antibiotic therapy or to de-escalation guided by serum PCT values. Antibiotic use was significantly lower in the PCT group, with the number of antibiotic free-days alive 28 days after VAP onset 13 in the PCT group vs. 9.5 in the control arm, a 27% reduction. Other indicators (ICU length of stay, time on mechanical ventilator, 28-day and hospital mortality) did not differ though. One caveat is that such lengths of antibiotic duration are not consistent with current practice in many ICUs, where shorter courses are the norm.

1.7.4 Cytokines

Cytokine cascades in pulmonary and plasma compartments reflect the complex balance of pro- and anti-inflammatory immune responses to infectious agents. Dehoux et al measured dual compartment cytokine levels in healthy subjects and in patients with unilateral community-acquired pneumonia. In the CAP group, BAL fluid from the affected lung was compared with the normal side as an extra control. IL-1β, IL-6 and TNF-α concentrations were elevated in the affected lung of patients when compared with either their unaffected lung or healthy volunteers, but such changes were not seen in the plasma. Such compartmentalisation affirms the need to sample the respiratory tract and may be the key to a successful VAP diagnostic biomarker. IL-6 but not TNF-α or IL-1β was raised in the blood of patients compared with control [88].

Levels of pro-inflammatory cytokines are high in the lung of patients with ARDS, who may also develop secondary pneumonia. Kanangat et al hypothesised that bacterial intracellular growth may be increased by such inflammatory mediators [89]. Considering anti-inflammatory cytokines in addition, van der Poll et al administered intranasal IL-10 to a murine model of Streptococcal pneumonia and demonstrated it to be deleterious. Pneumonia worsened with IL-10 but abrogated by its blockade, suggesting it to have a key role [90]. In humans, the serum cytokines IL-1, -6, -8, -10 and TNF-α were assayed in 44 ICU patients by Ramirez et al [91]. 20 patients were
suspected of having VAP, of which 9 had microbiologically proven VAP. Only IL-6 levels were raised in VAP compared with non-VAP. Its sensitivity was 71% and specificity 89%, at a cut-off level of 620 pg/ml.

Millo and colleagues investigated the cytokines IL-1α, IL-1β, IL-6, IL-10 and TNF-α together with the cytokine inhibitors soluble TNF-α receptor I, IL-1 receptor antagonist and soluble IL-1 receptor II, in the lung and plasma of 9 patients with VAP. 19 non-VAP patients served as control. Samples were obtained via non-directed bronchial lavage (NBL). In temporal samples taken on alternate days, plasma cytokine levels were similar between groups. In the pulmonary compartment however, there were statistically significant increases in the levels of TNF-α, soluble TNF-α receptor I, IL-1α and IL-1β during VAP. Furthermore, the BAL/blood ratios of TNF-α, soluble TNF-α receptor, IL-1α, IL-1 receptor antagonist and IL-6 increased over time in patients subsequently developing VAP. Although this study was not designed to assess diagnostic biomarkers, it is tempting to speculate that measurement of compartmentalised cytokines may facilitate VAP diagnosis [92].

A prospective, observational study by Conway-Morris et al [14] highlighted the potential importance for BAL cytokines to diagnose/refute VAP, this time IL-1β and IL-8. In a mixed medical/surgical Scottish ICU, they compared 17 patients with microbiologically proven VAP, 55 non-VAP (but initially suspected) and 21 matched healthy controls. VAP was diagnosed by quantitative microbiology (BAL bacterial count above 10⁴ cfu/ml). The non-VAP group were stratified into 22 patients with sub-clinical growth (<10⁴ cfu/ml) and the remainder 33 with no growth. The serum levels of the cytokines TNF-α, IL-1β, IL-6, 8,10, G-CSF and MIP-1α were similar between the ventilated groups. However IL-1β, IL-8, G-CSF and MIP-1α (corrected for dilution) were significantly higher in the BALF of VAP patients compared with ventilated non-VAP patients. A cutoff IL-1β level of < 10 pg/ml in BALF had a high negative predictive value, providing a post-test probability of VAP of 2.8%. An IL-8 level of 2000 pg/ml had a positive likelihood ratio of 5.03. One caveat of this study was that the VAP case-mix did not include patients with Pseudomonal or Klebsiella infections which are common organisms elsewhere [93]. The
approach of using a biomarker panel of two or more cytokines (in this case IL-1β and IL-8) appears to fulfil the first three studies as described by Sackett and Haynes, albeit from a single centre. This research is now the subject of a prospective multicentre study (http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=12129).

In sepsis, the inflammatory and coagulation cascades interact, with fibrin deposition in the pneumonic lung. During VAP development, pulmonary protein C levels fall, thrombin levels rise and fibrinolysis decreases [94, 95]. Determann et al measured lung coagulation factors by non-directed lavage in patients developing VAP. The levels of PAI-1 (plasminogen activator inhibitor-1) and sTF (soluble tissue factor) were raised two days prior to overt VAP. PAI-1 and sTF had sensitivities of 0.89 and 0.78 and specificities of 0.95 and 0.74 respectively for detecting VAP [96].

In summary, dual compartment cytokine sampling offers the potential to diagnose VAP.

**1.7.5 TREM-1**

Surface TREM-1 is predominantly located on monocytes and neutrophils, the cells responsible for the innate immune response to infection. TREM-1 has therefore been investigated in a number of infectious states including sepsis [97], empyema [98] and meningitis [99]. Such studies have proposed surface and soluble TREM-1 as putative diagnostic biomarkers for infection.

Soluble TREM-1 has been measured in blood, BAL fluid, NBL and the exhaled ventilator condensate (EVC). Its exact role in diagnosis and prognostication of VAP remains debated. In a landmark study in a medical ICU setting, Gibot and colleagues measured soluble TREM-1 in 148 patients using mini-BAL sampling and immunoblotting. At a cut-off level of 5 pg/ml sTREM-1 had an AUROC curve of 0.93 and a positive likelihood ratio of 10.38 for diagnosing VAP (as assessed by quantitative culture). The overall sensitivity was 98% and specificity 90%. Dilutional correction was not
performed, unlike in more recent studies [100]. Determann et al studied temporal changes of sTREM-1 obtained via NBL. In a small cohort of ventilated mixed medical and surgical patients, 9/19 patients had VAP, as judged by clinico-microbiological criteria. sTREM-1 was assayed on alternate day samples using ELISA. sTREM-1 was higher in VAP than controls, with a sensitivity of 75% and specificity 84% at a 200 pg/ml cut-off level. The authors commented that monitoring serial TREM-1 levels further enhanced diagnostic accuracy. In particular, lung sTREM-1 levels fell with antibiotic treatment and disease resolution, an important requirement of an ideal VAP biomarker. Once again, sTREM-1 was not corrected for possible dilutional effects. Interestingly, plasma levels were not discriminative for VAP. This suggests that tissue levels of sTREM-1 are of greater relevance than plasma levels [101].

**Negative studies**

The diagnostic utility of TREM-1 has fared less well in other studies. Song et al [102] measured sTREM-1 in addition to PAI-1 in their study using samples obtained by NBL. VAP due only to the gram-negative bacterium Pseudomonas aeruginosa was diagnosed by quantitative culture in 33 ICU patients. sTREM-1 was assessed by ELISA but levels but were non-discriminative for VAP. Contrary to this, sTREM-1 levels have previously been shown to be elevated in gram-negative infections, albeit from the blood [36]. In 23 medical ICU patients, Horonenko et al measured sTREM-1 in BAL as well as in the exhaled ventilator condensate (EVC). In this case BAL sTREM-1 was not diagnostic of VAP. sTREM-1 in EVC had higher specificity, especially when indexed against the total protein content of the EVC. The chosen method of VAP diagnosis, beyond clinicopathological standards is clearly important; comparison of positive microbiological culture with negative, rather than using the CPIS score, resulted in all differences between groups disappearing [103]. Anand measured sTREM-1 using ELISA in a study of 105 medical ICU patients. sTREM-1 was non-significantly increased in the VAP patients. The sensitivity was low at 42.1% and specificity 75.6% for a
200 pg/ml cut-off level, figures not useful clinically [104]. Oudhuis and colleagues also reported a negative result for sTREM-1 to diagnose VAP [105]. BAL samples from 240 patients were assayed by ELISA and corrected for dilution using urea. In both medical and surgical patients, the AUC for sTREM-1 to classify VAP cases was 0.58. VAP was diagnosed microbiologically by quantitative means (10^4 cfu/ml) or ≥2% intra-cellular organisms present on gram stain (ICO). Gibot speculated that the results seen may have been confounded in part due to sample contamination or perhaps by repeated freeze/thawing of stored samples [106]. In contrast, Huh et al measured sTREM-1 in 80 medical ICU patients with bilateral lung infiltrates, a patient group in which there is significant risk of co-infection and present a diagnostic dilemma. VAP was determined by quantitative culture of NBL samples. sTREM-1 levels (assayed using ELISA) at a cutoff of 184 pg/ml had a sensitivity of 86% and specificity of 90% for VAP diagnosis. In particular sTREM-1 levels were elevated in patients with bacterial and fungal infections, but not viral or intracellular bacteria, a finding that correlated with the initial bench research into TREM-1 [36, 42]. The authors speculated that some cases of alveolar haemorrhage may have confounded the results [107].

In a study by Conway-Morris et al [14] BAL sTREM-1 measurement was not discriminative for VAP. They compared 17 patients with VAP with 55 with non-VAP and 21 matched volunteers (see previously). Palazzo et al studied BAL and EVC sTREM-1 levels in patients with clinically suspected VAP [108]. Using quantitative cultures (>10^4 cfu/ml) 19/45 patients had VAP. sTREM-1 BALF levels had a sensitivity of 79% but low specificity of 23%, using a cutoff level of 204 pg/ml. EVC fared worse, with a cutoff level of 10 pg/ml yielding a poor sensitivity of 42% and specificity of 50%. In a paediatric study of 33 patients, 16 with VAP (see below in ‘other’) Srinivasan et al [109] measured sTREM-1 levels in BAL but found them non-discriminative. Finally, a Chinese study on 32/92 patients with VAP (diagnosed with quantitative culture of BALF) demonstrated peripheral blood sTREM-1 to have 75% sensitivity and 64% specificity for diagnosis, with a cutoff level of 189 pg/ml (AUC 0.732) [110].
Overall, there is uncertainty regarding the utility of soluble TREM-1 to diagnose VAP and whether BAL, NBL or EVC is the best method of sample acquisition. The reasons for this may be manifold; sampling differences, differences in VAP diagnostic methodology, assay methods, prior antibiotic usage and sample sizes. The utility of surface TREM-1 to diagnose VAP has never been determined. Further study is necessary in groups suspected to have VAP, with comparison to other putative cytokine biomarkers. Table 1.10 shows a summary of the data on TREM-1 for VAP diagnosis.
<table>
<thead>
<tr>
<th>Study / Year</th>
<th>Number of VAP patients / total</th>
<th>Method of measuring soluble TREM-1 and diagnosing VAP</th>
<th>Diagnostic performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gibot et al (2004)</td>
<td>46 / 148</td>
<td>Immunoblotting. No dilutional correction Mini-BAL (20ml). Quantitative culture with &gt;10^3 cfu/ml</td>
<td>At 5pg/ml AUC 0.93 LR 10.38, Sens 98%, Spec 90% Mean TREM-1 (VAP)= 34 pg/ml</td>
</tr>
<tr>
<td>Determann et al (2005)</td>
<td>9 / 28 Note a</td>
<td>ELISA. No dilutional correction. Kinetic TREM-1 changes in BALF Non-directed BAL on alternate days (20ml of saline). Quantitative culture with &gt;10^4 cfu/ml.</td>
<td>Cutoff of 200 pg/ml Sens 75%, Spec 84% Mean TREM-1 (VAP) = 894 pg / ml TREM-1 levels fall with antibiotic therapy Only 9 VAP patients</td>
</tr>
<tr>
<td>Song et al (2007)</td>
<td>11 / 33 Note a-d</td>
<td>ELISA. No dilutional correction. Blind BAL, 20-60ml. Quantitative culture with &gt;10^4 cfu/ml. Pseud. Aeruginosa patients</td>
<td>TREM-1 not diagnostic of VAP nor does its level prognosticate Mean TREM-1 in group with ARDS patients (VAP) = 203 pg/ml Only 11 VAP patients</td>
</tr>
<tr>
<td>Horonenko et al (2007)</td>
<td>14 / 23</td>
<td>ELISA. BAL, 100ml saline and in EVC (exhaled ventilator condensate). CPIS score, Quantitative culture &gt;10^3 cfu/ml EVC corrected for protein</td>
<td>BAL TREM-1 not diagnostic of VAP EVC TREM-1 lower levels than BAL but diagnostic when correcting for protein Mean TREM-1 level (VAP) = 403 pg/ml (BAL) Only 14 VAP patients</td>
</tr>
<tr>
<td>Anand et al (2008)</td>
<td>19 / 105</td>
<td>ELISA. No dilutional correction. Quantitative culture &gt;10^3 cfu/ml. 150ml saline used for directed BAL</td>
<td>200pg/ml cutoff Sens 42.1%. Spec 75.6% Not diagnostic for VAP Mean TREM-1 (VAP) = 172 pg/ml</td>
</tr>
<tr>
<td>Study / Year</td>
<td>Number of VAP patients / total</td>
<td>Method of measuring soluble TREM-1 and diagnosing VAP</td>
<td>Diagnostic performance</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Huh et al (2008)</td>
<td>29 / 80 Note e</td>
<td>ELISA. No dilutional correction. Non-directed BAL. Quantitative culture &gt;10³ cfu/ml</td>
<td>Cutoff of 184 pg/ml. Sens 86%, Spec 90%. TREM-1 raised in bacterial/fungal not viral or intracellular. TREM-1 does not correlate with neutrophil count. Mean TREM-1 (VAP) = 521 pg/ml</td>
</tr>
<tr>
<td>Oudhuis et al (2009)</td>
<td>90 / 240 Note e</td>
<td>ELISA. Urea correction for dilution. BALF. Quantitative culture &gt;10⁴ cfu/ml or ≥2% ICO (intracellular organisms)</td>
<td>AUC for diagnosing VAP = 0.58. Mean TREM-1 (VAP) = 1849 pg/ml</td>
</tr>
<tr>
<td>Conway-Morris et al (2009)</td>
<td>17 / 72</td>
<td>ELISA with urea correction. BALF. Quantitative culture &gt;10⁴ cfu/ml</td>
<td>AUC for VAP diagnosis was 0.66 (0.48-0.83)</td>
</tr>
<tr>
<td>Srinivisan et al (2011)</td>
<td>16 / 33</td>
<td>CDC / NHSN criteria</td>
<td>No rise in VAP</td>
</tr>
<tr>
<td>Palazzo et al (2012)</td>
<td>19 / 45</td>
<td>ELISA. BALF and EVC. Quantitative culture &gt;10⁴ cfu/ml or PSB &gt;10³ cfu/ml</td>
<td>BAL – cutoff 204 pg/ml. Sens 79% / spec 23%. EVC – cutoff 10 pg/ml. Sens 42% / spec 50%</td>
</tr>
<tr>
<td>Su et al (2012)</td>
<td>32 / 92</td>
<td>ELISA. BALF and EVC. Quantitative culture &gt;10⁴ cfu/ml</td>
<td>Serum TREM-1 – 75% sens and 64% spec at cutoff of 189 pg/ml. AUC for diagnosis 0.732</td>
</tr>
</tbody>
</table>

Table 1.10. Summary Table of studies to diagnose VAP. Studies are in chronological order. The number of patients out of the total with VAP are given. The methods of diagnosing VAP and measuring TREM-1 are highlighted as well as the diagnostic performance. Notes: In addition to medical ICU patients, a= surgical ICU, b=neuro ICU, c=cardiac ICU, d=vascular ICU, e=bilateral lung infiltrates only. Cfu – colony forming units. AUC – area under ROC curve. LR – likelihood ratio. Sens – sensitivity. Spec - specificity.
1.7.6 Other markers

1.7.6a BAL endotoxin

Many VAP cases result from infection by gram-negative bacilli, which possess lipopolysaccharide in their cell membrane but lack the lipotechoic acid components of gram-positive bacteria. This LPS (endotoxin) can be secreted. If endotoxin could be reliably detected, it might be useful to diagnose gram-negative VAP. Its utility would depend on the prevalence of gram-positive and non-bacterial infections in a particular ICU (which varies worldwide). In ventilated patients with multiple trauma, BAL endotoxin was measured [111]. In 40 samples, gram-positive VAP or non-infective disease had low levels of endotoxin (<6 EU/ml) while all the patients with gram-negative pneumonia (as defined on CPIS) had levels above 6 EU/ml, a promising result. It was also suggested that endotoxin levels correlated with the overall gram-negative bacterial load in the respiratory tract. In 63 patients Kollef et al calculated that a cut-off value of 5 EU/ml rather than 6 EU/ml had a sensitivity of 100% and specificity of 75% for VAP diagnosis and also that the endotoxin assay was superior to simple gram staining [112]. Similarly, Flanagan et al found that BAL endotoxin assay in 64 patients had 81% sensitivity and 87% specificity for gram negative VAP. However, only when endotoxin was assayed from BAL fluid did it correlate with VAP; non-directed BAL fluid (and also serum endotoxin) levels did not have diagnostic accuracy [113]. In a general ICU population Nys et al [114] prospectively assayed BAL endotoxin in 93 patients. At a cut-off level of 4 EU/ml there was 82% sensitivity and 96% specificity for gram-negative VAP as assessed by quantitative microbiology.

In summary, BAL endotoxin measurement could diagnose gram-negative VAP, but requires a standardised assay. Its role may be to rapidly rationalise antibiotic targeting towards gram-negative bacteria as it cannot identify gram-positive organisms.

1.7.6b Elastin fibres

Elastin fibres form part of the lung interstitial scaffolding, thereby allowing expansion and elastic recoil of the lung (review by Starcher [115]). They can
be detected in bronchial secretions by simple microscopy as a marker of non-specific injury to the lung. Their use as a speedy diagnostic tool for VAP has been investigated. El-Ebiary et al looked at microbiologically confirmed VAP in non-ARDS patients. Unfortunately, the test sensitivity was only 32% and specificity 72%. This improved marginally to 43% sensitivity and 86% specificity if gram-positive infections were excluded [116]. Shepherd et al studied patients with pneumonia and ARDS but found a specificity of only 40% [117]. Further recent work highlighted the lack of utility of this marker. Boots et al analysed patients who were ventilated for more than 48 hours. Of almost 1000 samples taken from patients with either ARDS or VAP, elastin fibres were only detectable in 7 samples, a level far too low for diagnostic use [118]. Overall therefore, elastin fibres is not a suitable candidate for a VAP biomarker.

1.7.6c Other markers

Srinivasan et al [109] measured BALF PAI-1 (Plasminogen activation inhibitor-1) levels in 33 mechanically ventilated children. They distinguished 16 VAP cases from colonization using the Centers for Disease and Prevention / National Nosocomial Infections Surveillance criteria on suspected cases in addition to gram-staining the endotracheal aspirate. PAI-1 levels of ≥ 2.8 ng/ml had a sensitivity of 81.3% and specificity of 76.5% for VAP diagnosis.

Clara cell protein-10 (CC-10) is a low molecular weight immunosuppressive protein that is secreted into the alveoli. Vanspauwen and colleagues assessed its ability to diagnose VAP. In 79/196 patients with VAP (as defined by quantitative microbiology of BALF and/or the presence of intracellular organisms) the AUC for VAP diagnosis was 0.586, a poor result [119].

L-selectin (CD62L) is an adhesion molecule used by leukocytes to migrate through the endothelium into the tissues. It has a soluble and a surface form. Levels were measured in the blood of patients with VAP by Sasajima et al [120] There were changes in the levels of neutrophil L-selectin (surface form) in the blood of patients with VAP. In non-infected patients, expression rose, peaked on day 2 and then fell. In some patients with VAP, the peak was on a later day or the level was biphasic – rising, falling and then rising again.
CD11b is a leukocyte activation marker and has not been thus far been studied in VAP. Buhling et al measured CD11b on alveolar macrophages in patients with CAP [121]. CD11b expression increased in CAP compared with the negative disease control groups of COPD and interstitial lung disease. In contrast, Glynn et al were unable to distinguish CAP from control with blood neutrophil CD11b levels [122]. Of more interest, however, Hoogerwerf et al instilled lipopolysaccharide (LPS) and lipotechoic acid (LTA) into the airways of healthy volunteers and obtained BAL fluid [51]. LPS, but not LTA induced a significant increase in neutrophilic CD11b expression. It is possible that the discrepant results seen were due to inadequate dosing of LTA. However, the authors speculated that the effect seen may reflect differences in the pulmonary immune response to gram-positive and gram-negative organisms. CD11b may be a putative biomarker in VAP but remains poorly studied.

Emerging techniques that may offer rapid methods of diagnosing VAP include proteomics (studying proteins of interest) and metabolomics (studying metabolic products). They are being researched to diagnose MRSA, other staphylococci, streptococci, Legionella as well as resistance genes. They include the ‘AccuProbe’ (Gen-Probe, San Diego, USA), ‘GeneOhm’ (Becton-Dickinson, New Jersey, USA) and the MALDI-TOF (matrix assisted laser desorption / ionization – time of flight mass spectrometry (Bruker Daltonic, Coventry, UK). The Accuprobe uses a chemiluminescent DNA probe targeted to a micro-organism nucleic acid. GeneOhm uses real-time PCR to identify staphylococci. MALDI-TOF identifies proteins and peptides associated with the pathogen of interest in blood [123]. Proteomic profiles of BAL specimens were examined in patients with VAP by Lu et al (2008). Their assay demonstrated around 200 proteins whose levels differed between VAP and non-VAP patients. These included those related to cell-structure, metabolism and immunity. The latter included complement proteins and immunoglobulin fragments, but not cytokines [124].

It is clear that numerous proteins have been investigated to diagnose VAP but few have shown promise in the small studies conducted thus far.
1.8 Discussion of biomarkers in VAP

Cardiac troponins are an example of an ideal diagnostic biomarker in clinical use today. From a negligible basal level, cardiac injury releases troponins into the blood allowing rapid identification. The biomarker also reflects the pathophysiological process of myocardial damage. In the analogous state of VAP, to search for new biomarkers, ideally the marker of interest would highlight part of the pathophysiological process causing VAP such as the immune response to pulmonary infection. As has been described, endotoxin measurement, cytokine analysis and TREM-1 would fit with this principle. Elastin fibres however indicate damage per se, not specifically due to infection. CRP and PCT are systemic markers of inflammation/infection and are not specific for VAP. Overall, in searching for new biomarkers, a marker linked to the pathophysiological process is probably the most fruitful way to proceed, although it may not identify all prospective candidates.

Earlier the Morrow and de Lemos criteria of a clinically relevant biomarker were described [58]. First was ease of measurement. It is obvious that blood tests are simpler to obtain than BAL. However, this review has shown that cytokine levels in the blood appear non-specific due to compartmentalization and PCT and CRP are not site-specific. Therefore despite the increased difficulty, respiratory tract sampling is needed. The means of acquiring respiratory samples is still under debate. Some studies highlight that NBL provides discordant results to BAL. VAP is a multi-focal disease and it is essential to sample the correct area. The relevant lung segment can be targeted accurately with bronchoscopy but is more technically difficult to perform than NBL.

The two other factors Morrow and de Lemos discussed were whether the biomarker provided new information and whether management dictated by biomarker measurement improved patient outcome. Endotoxin measurement may find use in restricting antibiotics targeted at gram-negative organisms. For PCT, its may facilitate antibiotic de-escalation. Finally, studies suggest inflammatory cytokine profiling in BAL may diagnose or refute VAP [14].
In complicated clinical entities such as VAP, standalone diagnostic biomarkers may be insufficient. Different organisms may elicit variable immune effects in the lung. It may be more appropriate to construct a biomarker panel and assess its ability to diagnose the condition. In this regard, our institution has recently validated a multiplex immune assay to assist in the diagnosis of patients with sarcoidosis and systemic sclerosis [125]. A further approach may be to index the ratio of non-specific markers of inflammation/infection in the lung to those in the blood (a BAL/blood ratio), to reduce inter-patient variability. Compartmentalisation as highlighted with the cytokines implies an increase in a marker in the lung and would yield an increased BAL/blood ratio. Such a result could also provide site-specificity as the BAL/blood ratio may be expected to be lower in patients with non-pulmonary as opposed to pulmonary infection. Finally, flow cytometric methods can measure cell surface proteins in the BAL and blood in order to study the immune response to pulmonary infection. This has hitherto been an under-researched area in the field of VAP.

1.9 Concluding remarks

An ideal biomarker could increase diagnostic accuracy in VAP and thereby improve patient care. Such a biomarker or panel of markers would represent a ‘footprint’ of infection and would complement clinical findings. Considerable challenges remain in translating laboratory based diagnostic work to the bedside. Nevertheless, the pursuit of early markers of infection, and subsequent resolution has great clinical importance. It would be useful to investigate further a dual compartment approach (i.e. lung-plasma), with cell and soluble marker based analysis as well as temporal changes. Furthermore biomarker panels incorporating putative cytokine markers (e.g. IL-1β and IL-8) have the potential to assist in the diagnosis of suspected VAP.
1.10 Hypothesis

Detection of soluble and surface TREM-1 (alone, or in combination with other inflammatory cytokines and surface proteins) in paired peripheral blood and BALF may improve the diagnostic classification of VAP and differentiate pulmonary from non-pulmonary infection.

1.11 Aims

1. To evaluate whether measurement of the standalone markers surface and soluble TREM-1 in blood and BAL can discriminate VAP from non-VAP cases.

2. To derive a BAL/blood ratio for surface and soluble proteins and determine whether it increases discrimination between VAP and non-VAP groups.

3. To evaluate whether an inflammatory biomarker panel derived from surface and soluble TREM-1, inflammatory cytokines (IL-1, IL-6 and IL-8), procalcitonin and expression of L-selectin and CD11b in peripheral blood and bronchoalveolar lavage fluid will accurately discriminate VAP from non-VAP patients.

4. To investigate the temporal changes of mTREM-1 and nTREM-1 during the development and resolution of VAP.
Chapter 2
Methodology

2.1 Study population

Patients were recruited at the Chelsea and Westminster Hospital Intensive Care Unit. The ICU comprises patients with general medical and surgical conditions as well as a specialised Burns Unit. The ICU does not include patients with trauma, neurosurgery or cardiothoracic disorders. The study was approved by the local Hospital Research and Development Department and by the National Research Ethics Service (NRES)

NRES number 08/H0702/61

Chelsea and Westminster R and D NHS Approval No: ANA09001CN

Please see the Appendix for

(i) Approval letter from NRES
(ii) Research and Development approval for Chelsea and Westminster Hospital
(iii) Consent form (ventilated patient)
(iv) Consent form (non-ventilated patient)

2.1.1 Inclusion criteria

Consenting patients / volunteers aged > 18 years. In ICU patients who were sedated and ventilated, assent from the family was sought in addition to approval from the Intensive Care Consultant in charge of the patient. When the patient regained mental capacity, their formal consent was then obtained.

2.1.2 Exclusion criteria

Patients were excluded if they had a bleeding diathesis, HIV or other immunodeficiency, tuberculosis, pregnancy, pneumothorax or hypoxia contraindicating bronchoscopy.
2.1.3 Recruitment groups

Patients were recruited into the following groups:

(i) ICU patients ventilated (through either a cuffed oral endotracheal or cuffed tracheostomy tube) with VAP; this was suspected according to standard clinical criteria (new CXR findings and at least one of the findings of pyrexia, raised WCC and purulent secretions) and defined as CPIS of 6 or more and positive microbiology (see below). Patients were ventilated either for respiratory failure or for major surgery. Paired blood and BALF samples were taken. Whilst the initial chest X-ray interpretation was that of the study investigators and the clinical intensive care team all presumed infiltrates in enrolled patients were independently confirmed by a radiologist.

(ii) ICU patients ventilated with no evidence of VAP but positive for non-chest sepsis on clinical, radiological and microbiological grounds (see below for microbiology). Paired blood and BALF samples were taken. The rationale for inclusion of such a group is that ICU patients may have infections at multiple sites and the ability of a biomarker to differentiate sites would be advantageous.

(iii) ICU patients ventilated with no evidence of VAP nor sepsis elsewhere (ie ventilated control). Such patients had a CPIS of below 6 and were negative for all cultures. Paired blood and BALF samples were taken.

(iv) Blood samples from patients admitted to hospital with community acquired pneumonia. (CAP) Such patients were defined clinically (productive cough, purulent sputum, pyrexia, shortness of breath), radiologically (chest x-ray changes of lobar or diffuse shadowing) and microbiology (sputum culture, pneumococcal and legionella antigen in urine). For those patients negative for microbiology, CAP was included if patients were clinically thought to have CAP and showed improvement with antibiotics. Bronchoscopy in the CAP patients was not feasible as it was too invasive and not part of their routine care.

(v) Given the need for ventilation is often influenced by the extent of chronic lung disease (COPD, interstitial lung disease), blood and BALF samples were
obtained from patients with these disorders who needed an outpatient bronchoscopy as part of their routine care or for assessment of solitary lung nodules.

(vi) Blood samples from healthy volunteers working in the Immunology laboratory.

In addition, a further analysis was performed where VAP was diagnosed using the HELICS PN4 and PN5 criteria. The overall agreement (kappa value) between the CPIS and HELICS was 0.95 for this study. Two patients with VAP would have been classified as non-VAP using HELICS and one patient with non-VAP could possibly have been placed into the VAP cohort. Of the 95 patients approached for the study, 1 declined. 3 patients had samples unsuitable for analysis and the remainder 91 were recruited (a 96% recruitment rate).

2.2 Microbiology

All samples were processed for culture and sensitivity by the Microbiology Laboratory at Imperial College Healthcare NHS trust (Chelsea and Westminster Hospital Laboratory) according to pre-defined Standard Operating Procedures. For BALF, samples were processed using semi-quantitative methods, with results stated as ‘none’, ‘mild’, ‘moderate’ and ‘heavy’ growth. Cultures were deemed positive if there was moderate or heavy growth but not if it was none / light. Quantitative processing to determine the number of colony forming units per ml were not available. In ICU patients, multi-site sampling was performed of patients with suspected sepsis. This could include blood cultures, urine, drain or other body fluid, relevant skin swabs and central venous catheter tips (following removal). In addition, urinary Legionella and pneumococcal antigen were assayed when deemed appropriate (Community-acquired pneumonia). Viral cultures were not routinely sent. Standard tests for tuberculosis (smear and growth) were conducted on BAL specimens when clinically indicated.
2.3 Data collection
For ventilated patients, the following data was collected: age, sex, diagnosis, smoking and antibiotic history, steroid use, chest x-ray findings, microbiology, CPIS, APACHE II score, CRP, white cell count and 28-day mortality. For non-ventilated (non-ICU) patients the same data was collected except for the APACHE II and CPIS.

2.4 Acquisition of samples
2.4.1 Blood samples
Peripheral venous blood and bronchoscopic samples were undertaken in consenting patients within 48 hours of meeting inclusion criteria. For ICU patients, two further blood samples were taken on alternate days to monitor temporal changes of the biomarker panel (2nd and 4th day following initial sampling).

Peripheral venous blood (10ml in EDTA) was collected for immediate analysis. Blood was centrifuged at 2000 rpm for 10 minutes at 20°C. Supernatant was collected and divided into aliquots for freezing at -70°C, for future ELISA analysis. The remaining cells were washed in phosphate buffered saline/1% fetal calf serum (FCS) to remove residual soluble TREM-1. 100 µl of cells were stained with the appropriate flow cytometry antibody for 30 minutes, after prior incubation with 10% FCS to block non-specific binding. Cells were then washed, red cells lysed and leukocytes fixed using a TQ-prep (Beckman Coulter, High Wycombe, UK). A 5 colour flow cytometer was used to analyse surface cell staining (Becton Dickinson, Oxford, UK).

CRP, urea and electrolytes, albumin, protein and white cell count were routinely measured as part of normal patient care by NHS Biochemistry and Haematology laboratories.
2.4.2 BAL samples

In consenting patients, bronchoscopy was performed once by either Dr. Vimal Grover, Dr. Suveer Singh or Dr. Pallav Shah (Consultant Respiratory Consultant at Chelsea and Westminster Hospital and The Royal Brompton Hospital, London UK). In Intensive Care, either a Pentax bronchoscope (Pentax Medical, Slough, UK) or Olympus bronchoscope (Olympus UK, Essex, UK) was used. In Bronchoscopy Clinic, Olympus bronchoscopes were utilised. Bronchoscopy was performed in an identical fashion. Sample site was chosen on clinical and X-ray findings; if CXR shadowing was absent or there was bilateral shadowing, the right middle lobe was sampled due to its ease of location and structure preventing bronchial collapse with gentle suction.

In ICU: The patients were sedated and ventilated with a FiO2 of 1.0. Atracurium 50mg (muscle relaxation) was given as a single bolus to facilitate bronchoscopy. 100ml of sterile normal saline 0.9% was instilled in aliquots into the chosen lung segment. The initial bronchial sample was discarded in accordance with other similar studies. The yield was generally about 30ml (range 16-43ml). Half the sample was sent to microbiology for culture and sensitivity, the other half were analysed for surface and soluble cytokines.

Bronchoscopy Clinic: Patients were sedated with midazolam (up to 5mg iv) and / or fentanyl (up to 50 micrograms iv). Topical lignocaine (4%) was administered to the oropharynx and 2% lignocaine instilled by a technique of ‘spray as you go’. 100ml of 0.9% normal saline was used. The initial bronchial sample was also discarded. The yield was typically 30ml (14-47ml).

Samples were collected on ice for immediate processing. BALF was filtered through sterile gauze to remove sputum. It was then centrifuged at 500g at 18°C for 10 minutes. Supernatant was collected in aliquots and frozen for subsequent ELISA analysis. Cells were washed in phosphate buffered saline/fetal calf serum to remove residual soluble TREM-1. Cells were resuspended to 1 x 10^6/ml prior to staining with the appropriate flow cytometry antibody for 30 minutes (see below), again after FCS use to prevent non-specific stain binding. Cells were washed and fixed as above.
2.5 Flow cytometry

2.5.1 Blood

Surface TREM-1 was assessed on monocytes and neutrophils using a mouse IgG1 anti-human fluorochrome-conjugated TREM-1 antibody (R&D systems, Abingdon, UK) compared with an appropriate IgG1 isotype control antibody.

The following other fluorochrome-conjugated antibodies were used:

(i) CD14-PC5 IgG2a (Beckman Coulter, High Wycombe, UK)

(ii) TREM-1-PE (R & D systems) or IgG1 isotype control antibody- PE (Pharmingen, Oxford, UK)

(iii) CD16-FITC IgG1(Beckman Coulter)

(iv) CD45-ECD IgG1(Beckman Coulter)

(v) CD11b-PE IgG1 or IgG1-isotype-PE control antibody (both Beckman Coulter)

(vi) CD62L-FITC IgG1 (L-selectin) or IgG1 isotype-FITC control antibody (both Beckman Coulter)

(vii) TREM-2-PE (R & D systems)

All the data (BAL and blood) for a particular patient were acquired using the flow cytometer for a single session. The flow cytometer is used in the Imperial College Healthcare NHS Trust Immunology Laboratory. As such, there are External Quality Assurance (EQA) procedures in place for quality control. Laser alignment occurs with ‘Flow-check’ beads (Beckman Coulter). These are fluorospheres of 10 µm that fluoresce with a spectrum wavelength of 525-700nm when excited by photons at 488nm. Furthermore, Immuno-trol cells (Beckman Coulter) are used as a positive control; these mimic whole blood in terms of scatter, population distribution of lymphocytes, monocytes and granulocytes as well as cell-specific antigen density. Finally, isotype control antibodies were used with each test antibody to determine the geometric
mean fluorescence intensity. Serial measurements of samples (n=3) determined that variability in MFI was 4-7%.

CD45 staining and side-scatter characteristics were initially used to select CD14 and CD16 positive cells as markers of monocytes and neutrophils respectively.
2.5.2 BAL

Once the supernatant had been removed (as above), an aliquot of cells was removed for analysis of the proportion of live and dead cells (see below). The remainder were washed with PBS/fetal calf serum 1% as for the peripheral blood flow cytometry. Incubation of cells and the order of antibodies was identical to that of peripheral blood.

2.5.3 Dead cell stain

In addition to trypan blue staining to confirm cell integrity, the LiveDead ViVid stain (Invitrogen, Paisley, UK) was used. This is an amine reactive dye that binds to intracellular free amines, but minimally to intact membranes. We used the ViVid-red dye and measured dead cells with the ECD detector on the flow cytometer. A peripheral blood sample of several days (containing numerous dead cells) was used as a positive control to confirm assay function. 5 fresh peripheral blood samples were tested for dead cells; none showed any present and therefore ViVid staining was not performed on any further patient blood samples. In BAL, cell viability ranged from 90-97%. Live cells were gated for further analysis. Therefore it is unlikely that results from the flow analysis were due to measurement on dead cells.

2.5.4 Acquisition and analysis

A minimum of 5000 monocytes were analysed from peripheral venous blood and 2000 alveolar macrophages from BAL. The mean fluorescent intensity of test antibody was compared with that of an isotype control antibody and the geometric mean calculated as an index of protein concentration expressed by a particular blood or BALF cell population. Serial analysis of BAL samples showed the variability in MFI (n=3) to be 5-7%. The MFI is the standard measure of fluorescence as measured by flow cytometry.
2.5.5 Dot plot expressions in blood

The following plots demonstrate the gating strategies and expression of TREM-1, CD11b and L-selectin in blood monocytes and neutrophils. Figure 2.1 shows the gating of monocytes and neutrophils in the blood.

**Figure 2.1 Monocyte and Neutrophil gating (blood)**

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Figure 2.1. The top left figure demonstrates Forward Scatter (FS) vs. Side Scatter (SS). In the top right picture, FS against FS area (Aux) allows gating out of couplets caused by antibody cross-linking. The bottom left picture demonstrates the immune cells gated on CD45 (region B). In the lower right diagram, neutrophils are region D (CD14 low), monocytes are region C (CD14 high).
Figure 2.2 demonstrates the expression of CD14 and CD16 on the monocytes and neutrophils.

**Figure 2.2 CD14 and CD16 expression (blood)**

Figure 2.2. The top left picture demonstrates the TREM-1 expression on CD14 positive cells (monocytes). The top right picture demonstrates the TREM-1 expression of neutrophils (CD16 positive). In the lower picture (above), CD14 and CD16 expression is highlighted, showing the neutrophils (yellow) as CD14 low CD16 high. Monocytes are CD14 high and CD16 low.
Figure 2.3 shows the expression of TREM-1 on monocytes and neutrophils (blood). It firstly shows the MFI for the isotype and then that for the TREM-1 antibody. The geometric mean is the ratio of the latter to the former.

**Figure 2.3 TREM-1 expression on monocytes and neutrophils (blood)**

In the above diagram, the TREM-1 expression of monocytes and neutrophils is demonstrated. On the left, the TREM-1 expression of monocytes (lower diagram) is compared with staining from an isotype control antibody (top left). On the right, neutrophil TREM-1 expression (lower diagram) is compared with isotype control antibody (top right).
Figure 2.4 illustrates the CD11b expression on monocytes and neutrophils in blood. The MFI for isotype and CD11b antibodies are shown.

**Figure 2.4 CD11b expression on monocytes and neutrophils (blood)**

![Histograms of CD11b expression](image)

Figure 2.4 The diagrams above represent typical histograms of expression of CD11b on monocytes (blue) and neutrophils (red). The respective cells are gated via CD45 and CD14 as previously. The monocytes (left) demonstrate expression of CD11b (lower diagram) compared with an isotype control antibody (top left). Similarly on the right, neutrophil CD11b expression (lower right) is compared with its isotype control antibody.
Figure 2.5 shows the expression of CD62L (L-selectin) on monocytes and neutrophils. Firstly the MFI for isotypes are shown and then that for CD62L.

**Figure 2.5 CD62L (L-selectin) expression on monocytes and neutrophils (blood)**

![Diagram showing CD62L expression on monocytes and neutrophils](image)

Fig 2.5 The diagrams above represent typical histograms of expression of L-selectin on monocytes (blue) and neutrophils (red). The respective cells are gated via CD45 and CD14 as previously. The monocytes (left) demonstrate expression of L-selectin (lower diagram) compared with an isotype control antibody (top left). Similarly on the right, neutrophil L-selectin expression (lower right) is compared with its isotype control antibody.
2.5.6 Dot plot expressions in BAL

The following plots demonstrate the gating strategies and expression of Live/Dead cells, TREM-1, CD11b and L-selectin BAL macrophages and neutrophils. Figure 2.6 shows the gating of macrophages and neutrophils.

**Figure 2.6 Macrophage and neutrophil gating (BAL)**

![Figure 2.6](image)

Figure 2.6. The top left figure demonstrates Forward Scatter (FS) vs. Side Scatter (SS). In the top right picture, FS against FS area (Aux) allows gating out of couplets caused by antibody cross-linking. The bottom left picture demonstrates the immune cells gated on CD45 (region F). In the lower right diagram, neutrophils are region G (CD14 low), macrophages are region H (CD14 high).
The staining of CD14 and CD16 in BAL are demonstrated in Figure 2.7

**Figure 2.7 CD14 and CD16 staining (BAL)**

Figure 2.7. The left diagram demonstrates neutrophils (red) as CD14 low and CD16 high. Macrophages are CD14 high. The right plot demonstrates TREM-1 expression on CD14 positive macrophages.
The expression of TREM-1 on BAL macrophages and neutrophils are highlighted in Figure 2.8. Firstly the MFI for isotype controls are shown and then that for the TREM-1 antibody.

**Figure 2.8 TREM-1 expression on macrophages and neutrophils (BAL)**

![Diagram showing TREM-1 expression on neutrophils and macrophages](image)

*Figure 2.8. In the above diagram, the TREM-1 expression of monocytes and neutrophils is demonstrated. On the left, the TREM-1 expression of neutrophils (lower diagram) is compared with staining from an isotype control antibody (top left). On the right, monocyctic TREM-1 expression (lower diagram) is compared with isotype control antibody (top right).*
Figure 2.9 demonstrates the expression of CD11b on BAL monocytes and neutrophils. The MFI for CD11b is compared with its isotype control antibody.

**Figure 2.9 CD11b expression on macrophages and neutrophils (BAL)**

Figure 2.9. The diagrams above represent typical histograms of expression of CD11b on macrophages (blue) and neutrophils (red). The respective cells are gated via CD45 and CD14 as previously. The macrophages (left) demonstrate expression of CD11b (lower diagram) compared with an isotype control antibody (top left). Similarly on the right, neutrophil CD11b expression (lower right) is compared with its isotype control antibody.
Figure 2.10 shows the expression of CD62L (L-selectin) on BAL macrophages and neutrophils. Firstly the MFI for the isotype is given and then that for the CD62L antibody.

**Figure 2.10 CD62L (L-selectin) expression on macrophages and neutrophils (BAL)**

![Diagram showing expression of CD62L on macrophages and neutrophils](image)

Figure 2.10. The diagrams above represent typical histograms of expression of L-selectin on macrophages (blue) and neutrophils (red). The respective cells are gated via CD45 and CD14 as previously. The macrophages (left) demonstrate expression of L-selectin (lower diagram) compared with an isotype control antibody (top left). Similarly on the right, neutrophil L-selectin expression (lower right) is compared with its isotype control antibody.
The levels of the live/dead cell stain incorporated into BAL cells are shown in figure 2.11.

**Figure 2.11. Live/Dead stain of BAL**

Figure 2.11. The above dot plot demonstrates cells gated according to the Live/Dead cell stain ViVid. The plot has previously been tested with peripheral blood cells with a large proportion of dead cells. The BAL cells to the left of the solid live stain negatively for ViVid and are therefore alive. The cells to the right of the solid line stain positively for ViVid and are therefore dead. In this case, the proportion of live cells is approximately 95%.
2.5.7 Temporal changes of mTREM-1, nTREM-1, mTREM-2 and nTREM-2

We studied the temporal changes of mTREM-1 and nTREM-1 to understand whether serial analysis may be effective in monitoring VAP development and resolution. 12 patients had sequential bronchoscopy (2-4 per patient) as clinical progress allowed; bronchoscopy was performed where VAP developed and also during resolution. Each bronchoscopy was performed approximately 48 hrs apart ie alternate days. For one patient in the Burns setting where bronchoscopy was being performed for therapeutic reasons (soot removal), BAL sampling was performed during VAP development and resolution. mTREM-1 and nTREM-1 levels in the BAL and blood were measured as previously stated. WCC and CRP levels were obtained at the same time as phlebotomy for blood markers. Neither sTREM-1 nor other soluble markers were measured. In addition, mTREM-2 and nTREM-2 were measured in six of the twelve patients in the blood and BAL.
2.6 ELISA

We utilised ELISA kits to measure human soluble TREM-1, IL-1 beta, IL-6, IL-8, Matrix-metalloproteinase-8 and -9 (MMP-8 and -9) and Tissue Inhibitors of Metalloproteinase-1 (TIMP-1) (Quantikine, R&D systems, Abingdon, UK) according to manufacturer’s instructions.

2.6.1 Soluble TREM-1

A sandwich ELISA was used. 96 well plates were pre-coated with an antibody specific for soluble TREM-1, together with a blocker to prevent non-specific protein binding. Supernatants from peripheral blood samples or BALF were assayed in duplicate. Serial dilutions of a known concentration of recombinant soluble TREM-1 were measured in duplicate in order to construct a calibration curve.

After incubation with sample / standard, wells were washed x4 with PBS / Tween (polysorbate detergent). A capture antibody with enzyme conjugate for soluble TREM-1 was added and further incubation allowed. After 4 further washes with PBS / Tween, substrate solution was added. Conjugated enzyme (as a result of captured soluble TREM-1) resulted in a colour change to blue. Stop solution was added and the resultant yellow colour was read by a plate reader at 450nm. Blank wells were used to correct results. Freezing did not have an effect on soluble TREM-1 concentrations. Soluble TREM-1 levels were similar in three separate samples assayed after 1 month and 9 months (coefficient of variation 7.4%).

2.6.2 IL-1-β, IL-6, IL-8, MMP-8, MMP-9 and TIMP-1

Similarly, commercially available kits (R and D systems, UK) were used according to manufacturer’s instructions. Table 2.1 details the performance of
each ELISA assay as detailed by the manufacturer. Dr. Ram Vyakernam had previously validated the cytokines in BAL using serial dilution.

**Table 2.1 Performance of the ELISA assay kits**

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Intra-plate coefficient of variation (%)</th>
<th>Inter-plate coefficient of variation (%)</th>
<th>Lower limit of detection (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble TREM-1</td>
<td>3.6-5.2</td>
<td>5.8-7.4</td>
<td>13.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2.8-8.5</td>
<td>4.1-8.4</td>
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</tr>
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<td>IL-6</td>
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<td>IL-8</td>
<td>5.4-6.5</td>
<td>6.1-9.7</td>
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<td>TIMP-1</td>
<td>3.9-5.0</td>
<td>3.9-4.9</td>
<td>156</td>
</tr>
</tbody>
</table>

**Table 2.1.** The manufacturer’s intra- and inter-plate coefficients of variation and the lower limit of detection are provided for soluble TREM-1, IL-1β, IL-6, IL-8, MMP-8, MMP-9 and TIMP-1.

**2.6.3 Correction for dilution using urea**

Rennard’s method was used to correct for dilution of the epithelial lining fluid (ELF) by saline in the BALF [61]. Briefly, it is assumed that urea, a small molecule, freely diffuses amongst the total body water, including the epithelial lining fluid. Its concentration in the ELF ought to be identical to that of the plasma. Measurement of the urea concentration in a known volume of BAL sample by a highly sensitive assay and comparison with a paired blood sample allows determination of the ELF dilution. An estimate of the native cytokine concentration may be calculated.
We employed a highly sensitive urea assay (Abcam, Cambridge, UK). It had a threshold of 10 µM urea. The assay uses urea as a substrate in the presence of enzymes to form a compound that reacts with an OxiRed probe to effect a colour change that is proportional to the urea concentration. The assay was used according to manufacturers instructions. A standard 96-well plate was used and a calibration curve created. Samples were assayed in duplicate.

2.7 Procalcitonin assay

Procalcitonin (PCT) was quantitatively measured in the paired blood and BALF samples in duplicate using a Brahms OCT mini-VIDAS machine (Biomerieux, Hampshire, UK) according to manufacturer's instructions. The limits of detection were 0.05 – 200 ng/ml. Procalcitonin has previously been measured in BALF (see introduction).

2.8 16S DNA levels

16S DNA levels were assessed in 2013 from samples frozen in 2009-2011 but not previously thawed. An in-house assay had been developed to quantify the DNA levels but not identify putative organisms. The levels were then corrected for dilution of BAL by urea. The plasma levels were assessed in the VAP, VC, VSE and NVC groups as well as healthy volunteers and the BAL assay was performed in the VAP, VC, VSE and NVC groups.

2.9 Statistics

2.9.1 Study design

Cross sectional cohort study.

2.9.2 Statistical analysis

A pilot study was performed to assess the ability of soluble and surface TREM-1 to discriminate VAP from non-VAP. Following this, a power calculation was performed. For a 90% power and a two-sided 5% significance
level, 25-30 patients in each previously indicated group were required. However, recruitment was significantly slowed by an outbreak of Acinetobacter Baumannii in the ICU. This curtailed admissions and meant that the final number of patients recruited who were ventilated but free of sepsis and those ventilated with non-pulmonary sepsis were 18 and 15 respectively.

Data was reported as medians and inter-quartile ranges. Differences in non-parametric variables were assessed firstly using the Kruskal-Wallis test and secondly the Mann-Whitney U-test with Dunn’s post-hoc correction if there were any statistical differences between the groups. Analysis of Receiver operator characteristics (ROC) was performed to assess the ability of individual components of the biomarker panel to diagnose bacterial VAP. Fisher discriminant function analysis was used to determine a combination of inflammatory biomarkers that optimally classified VAP in ventilated patients. With regards to Fisher analysis, a variable was entered into the “model” if the significance level of its F-value was <0.05 and was removed if the significance level was >0.1. A panel of biomarkers was identified and its accuracy to classify patients into the correct disease group was determined. Two techniques were used to validate the findings; the first (cross-validation) aimed to reflect the potential for outliers to bias the results of the sample. The phenotype of each case was classified by the functions derived from all cases other than that index case (‘leave one out classification’). A second technique would have involved applying the biomarker panel to new cohorts of patients and determining its accuracy in correctly classifying patients into each group. However, as previously described, recruitment was curtailed by the outbreak of a multi-resistant bacteria in the ICU. Therefore we validated our panel by randomly assigning original cases into a training cohort (60% of original cases) to obtain new classification function coefficients for the analytes derived from our original model and then applied the new parameters to a test cohort of the remaining 40% of cases. This was repeated ten times in total and a mean accuracy level determined to confirm the robustness of the panel.

We analysed the data using SPSS v19.0 software (SPSS Inc., Chicago, Illinois, US) and GraphPad Prism (GraphPad Software, California, USA).
Chapter 3

Patient characteristics

Patients and healthy volunteers were recruited into the following groups:

(a) Healthy volunteers (blood only) – 10 volunteers

(b) Non-ventilated Community-acquired pneumonia (blood only) – 10 patients

(c) Non-ventilated (non-infected) control patients from bronchoscopy clinic – 31 patients

(d) Ventilated (non-infected) control patients from ICU – 18 patients

(e) Ventilated with non-pulmonary sepsis from ICU – 15 patients

(f) Ventilated with VAP in ICU – 27 patients

The healthy volunteers were consenting members of the Immunology Department; seven were male and their median age was 31.5 years. None had any intercurrent illness and two had previously smoked (none now). Their blood was used for the assays but CRP and WCC were not measured (table 3.1).

The patients with CAP were those admitted to the Acute Medical Unit. They were not admitted to the ICU and were never invasively ventilated. Their median age was 59 years and 40% were male. 3 out of 10 patients were current smokers and 3/10 were ex-smokers. They all had CXR changes as part of their diagnosis. Microbiology was positive in half of the cases. In the other half, the clinical picture and resolution with antibiotic therapy was strongly suggestive of CAP. Half of the patients were receiving antibiotics (prescribed by their GP) at the time of admission. The median WCC was 12 x 10^9/L and the median CRP was 89 mg/L. Only one patient did not survive to discharge.
### Table 3.1 CAP patients and healthy volunteers

<table>
<thead>
<tr>
<th></th>
<th>Healthy volunteers</th>
<th>Non-ventilated CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers of patients</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age</td>
<td>31.5 (23-45)</td>
<td>59 (27-82)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>APACHE II</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CPIS</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CURB-65</td>
<td>N/A</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>Microbiology (% +ve)</td>
<td>N/A</td>
<td>50</td>
</tr>
<tr>
<td>Smoking (% current / ex)</td>
<td>0 / 20</td>
<td>30 / 30</td>
</tr>
<tr>
<td>Antibiotics (% receiving)</td>
<td>Nil</td>
<td>50</td>
</tr>
<tr>
<td>CXR (% with shadowing)</td>
<td>N/A</td>
<td>100</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>Nil</td>
<td>0</td>
</tr>
<tr>
<td>Hospital mortality (%)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Surgery (%)</td>
<td>Nil</td>
<td>0</td>
</tr>
<tr>
<td>Burns (% of cases)</td>
<td>Nil</td>
<td>0</td>
</tr>
<tr>
<td>WCC (x10^9/L)</td>
<td>Not tested</td>
<td>12 (6-21)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>Not tested</td>
<td>89 (22-465)</td>
</tr>
</tbody>
</table>

**Table 3.1.** For patients with CAP, microbiology was positive in five patients (50%). *Streptococcus pneumoniae* was present in sputum / urinary antigen in two patients, *Haemophilus influenzae* in sputum of one patient, *enterobacter* in one sputum sample and *MSSA* in the final sputum sample (methicillin sensitive *staphylococcus aureus*).
31 patients were recruited from the Bronchoscopy Clinic (Table 3.2). Their median age was 59, 61% were male. 35% were current smokers and 13% ex-smokers. Although microbiology was positive in twelve patients, these were likely colonisation as no patients were considered infected by the treating clinicians. CXR changes were present in the majority (81%) and 32% were receiving prescribed antibiotics at the time of presentation. Two patients were receiving steroid therapy (for sarcoidosis). Overall the median WCC was 7 x 10^9/L and the median CRP 6 mg/L. In terms of diagnosis, 7 patients had lung cancer, 4 sarcoidosis, 9 COPD (3 with no other diagnosis), 1 lung fibrosis and 6 with benign nodules.

For the ventilated control patients, there were 18 who were free of infection. Their median age was 60 and 50% males. Their median APACHE II score was 15 and their median CPIS was 4. 50% were receiving antibiotics, 78% had CXR changes. One third of patients were receiving steroids as part of their ICU stay. 39% of patients were post-operative and 17% burns patients. Microbiology was negative in all patients. Table 2 shows the initial data for individual groups.

15 patients were ventilated with non-pulmonary sepsis (‘sepsis-elsewhere’). Their median age was 62, 53% were male. Their median APACHE II score was 14 and the CPIS values were low (median 2). 27% had positive microbiology but clinical findings consistent with non-pulmonary infection (abdominal sepsis, cellulitis, infected burns, central venous catheter infection and urinary tract infection). 13% of patients had burns and 40% were post-operative. 93% of patients were receiving antibiotics as part of their clinical treatment. 27% had CXR shadowing. 27% of patients were receiving hydrocortisone as management of sepsis.

There were 27 patients with VAP, median age 68. 70% were male. The median APACHE II score was 18 and the median CPIS was 7. 100% of patients had positive microbiology. 89% were receiving antibiotics at the time of sampling. 96% had CXR shadowing and 30% were receiving low-dose steroids. 37% of patients were post-op and 15% were burns patients. Therefore the ventilated VAP and control patients were reasonably well-
matched. Cell counts did not differentiate between the groups that had BAL performed.

If the HELICS PN4 and PN5 definitions of VAP were used to diagnose or refute VAP then the classification of patients was highly concordant (kappa 95% for agreement). 2 patients with CPIS scores of 6 and VAP would be considered to be non-VAP. One patient in the ventilated control (non-VAP and non-VSE) had negative microbiology but could be considered to have had VAP because of progressive CXR shadowing (though this may also still be non-VAP).
### Table 3.2 Patient characteristics in the four groups

<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>VC</th>
<th>VSE</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>27</td>
<td>18</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>Age</td>
<td>68 (23-84)</td>
<td>60 (18-80)</td>
<td>62 (29-89)</td>
<td>59 (18-84)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>70</td>
<td>50</td>
<td>53</td>
<td>61</td>
</tr>
<tr>
<td>APACHE II</td>
<td>18 (5-45)</td>
<td>15 (2-23)</td>
<td>14 (3-24)</td>
<td>N/A</td>
</tr>
<tr>
<td>CPIS</td>
<td>7 (6-9)</td>
<td>4 (0-5)</td>
<td>2 (0-5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Microbiology (% +ve)</td>
<td>100</td>
<td>0</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Smoking (% current / ex)</td>
<td>44/15</td>
<td>28/22</td>
<td>33/20</td>
<td>35/13</td>
</tr>
<tr>
<td>Antibiotics (% receiving)</td>
<td>89</td>
<td>50</td>
<td>93</td>
<td>32</td>
</tr>
<tr>
<td>CXR (% with shadowing)</td>
<td>96</td>
<td>78</td>
<td>27</td>
<td>81</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>30</td>
<td>33</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>28-day mortality (%)</td>
<td>11</td>
<td>5</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Surgery (%)</td>
<td>37</td>
<td>39</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Burns (% of cases)</td>
<td>15</td>
<td>17</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>WCC (x10^9/l)</td>
<td>15 (4-24)</td>
<td>8 (3-27)</td>
<td>13 (7-24)</td>
<td>7 (3-18)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>84 (7-320)</td>
<td>88(2-193)</td>
<td>107 (69-341)</td>
<td>6 (1-296)</td>
</tr>
</tbody>
</table>

**Table 3.2.** The VC and VSE groups have not been combined. WCC levels were higher in VAP and VSE than VC (p=0.004 and p=0.003) and NVC (p=0.0001 and p=0.0002). Median CRP levels were significantly higher in VAP, VC and VSE than NVC (p<0.0001, p=0.0005 and p<0.0001). The data indicates the pre-BAL antibiotics and the microbiology following BAL. Steroids
were given according to Surviving Sepsis Campaign guidelines. In the VSE patients, positive microbiology was associated with pseudomonas, enterococcus, E. coli and MSSA. In the VAP patients, 1 patient had serratia, 4 Klebsiella spp, 6 pseudomonal infection, 2 MSSA, 2 MRSA, 3 E. coli, 5 Acinetobacter, 2 Stenotrophomonas, 2 Proteus spp and 4 candida spp. 4 patients had polymicrobial infection including candida spp.
Chapter 4

Expression of soluble proteins in VAP and disease controls

4.1. Introduction

Studies into biomarkers and VAP diagnosis have hitherto focused on soluble markers measured in BALF and blood. We wished to measure the levels of cytokines and sTREM-1 in these two compartments and derive a BAL/blood ratio. We then compared these with existing markers (CRP, WCC and PCT) to determine their discriminative utility as standalone markers. Given that sTREM-1 is cleaved from the surface of neutrophils and monocytes by matrix metalloproteinases, we measured levels of MMP-8, MMP-9 and their inhibitors (TIMP) in a subset of patients following conclusion of the main study. We also assessed 16S bacterial DNA levels in BAL and blood to compare with conventional microbiology.

4.2. Aims

1. Determine the utility of the blood biomarkers IL-1 beta, IL-8, IL-6 and sTREM-1 to discriminate patients with VAP and non-VAP in comparison with existing markers of inflammation (CRP, WCC and procalcitonin)

2. Assess the utility of the BAL biomarkers IL-1 beta, IL-6, IL-8, sTREM-1 and PCT to discriminate patients with VAP and non-VAP

3. Calculate a BAL / blood ratio for inflammatory cytokines and determine its utility to discriminate VAP from non-VAP patients.

4. Measure sTREM-1 and inflammatory cytokine levels in the blood of patients with CAP.

5. Determine the utility of 16S DNA to classify patients with and without VAP

6. Assess the quantitative levels of MMP-8, MMP-9 and TIMP in the BAL of patients with and without VAP.
4.3. Results

The summary data for the soluble biomarkers in blood, BAL, and the BAL / blood ratio in the four patient groups (VAP, ventilated non-infected control, ventilated with non-pulmonary infection and non-ventilated, non-infected control) are shown in Table 4.1. The BAL levels indicate ELF levels as they have been corrected for dilution using urea.

**Table 4.1. Soluble protein levels in VAP, VC, VSE and NVC**

<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>VC</th>
<th>VSE</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTREM-1 (µg/ml)</td>
<td>181(92-273)*</td>
<td>83(54-221)</td>
<td>105(61-316)</td>
<td>109(69-175)</td>
</tr>
<tr>
<td>IL-6 (µg/ml)</td>
<td>64(30-248)</td>
<td>22(4-84)**</td>
<td>62(18-251)***</td>
<td>10(7-21)</td>
</tr>
<tr>
<td>PCT (ng/ml)</td>
<td>1.3 (0.3-5.3) *</td>
<td>1.7 (0.4-5.5)**</td>
<td>4.7 (0.9-13.6)***</td>
<td>N/A</td>
</tr>
<tr>
<td>WCC (x10³/L)</td>
<td>15 (9-17)§*</td>
<td>7.5 (5-8.5)</td>
<td>13 (10-16)§§§</td>
<td>10 (6-15)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>84 (45-129)§</td>
<td>88 (31-117)</td>
<td>107 (93-189)***</td>
<td>5 (4-35)</td>
</tr>
<tr>
<td><strong>ELF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTREM-1 (µg/ml)</td>
<td>20143(8262-39104)§</td>
<td>5956(1512-14303)</td>
<td>6101(2820-12566)§</td>
<td>6727(2677-17938)</td>
</tr>
<tr>
<td>IL-1β (µg/ml)</td>
<td>2799(1229-7364)§</td>
<td>306(51-1322)</td>
<td>521(251-1591)°</td>
<td>530(193-2646)*</td>
</tr>
<tr>
<td>IL-6 (µg/ml)</td>
<td>3353(1321-15075)°</td>
<td>1176(612-3429)</td>
<td>1701(1150-3551)</td>
<td>1469(466-2546)</td>
</tr>
<tr>
<td>IL-8 (µg/ml)</td>
<td>45603(16770-104382)§</td>
<td>11572(4116-16290)</td>
<td>10293(5378-12576)°</td>
<td>17163(3740-84012)</td>
</tr>
<tr>
<td>PCT (ng/ml)</td>
<td>16.8 (9.7-51.7)</td>
<td>11.6 (6.2-31.5)</td>
<td>17.3 (8.8-27.2)</td>
<td>9.6 (4.1-18.2)</td>
</tr>
<tr>
<td><strong>ELF/blood ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTREM-1 (µg/ml)</td>
<td>190 (70-337)§</td>
<td>31 (16-85)</td>
<td>23 (7-65)§</td>
<td>84 (26-228)</td>
</tr>
<tr>
<td>IL-6 (µg/ml)</td>
<td>77 (20-145)</td>
<td>72 (15-272)</td>
<td>29 (21-104)</td>
<td>134 (30-355)</td>
</tr>
<tr>
<td>PCT (µg/ml)</td>
<td>28.9 (3.1-54.7)</td>
<td>5.1 (2.0-35.2)</td>
<td>3.2 (1.3-14.9)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4.1. PCT levels in blood for NVC were mainly below the detection limit of the assay. Median and IQR levels are shown. Key: * VAP > NVC, p<0.001. ** VC > NVC, p<0.001. *** VSE > NVC, p<0.0001. † VAP > NVC, p<0.0001. ++ VC > NVC, p<0.0001. § VAP > VC, p<0.01. §§ VSE > VC, p=0.003. $$$ VSE > NVC, p<0.001. ° VAP > VSE, p< 0.001. ° VAP > NVC, p<0.001. The data shows the levels of soluble protein levels in blood, ELF (corrected for dilution) and the BAL/blood ratio. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.1. Soluble proteins in blood

The soluble proteins sTREM-1, IL-1β, IL-6, IL-8 and PCT were measured in blood. In addition, CRP and WCC levels were obtained from patients via the NHS laboratories. IL-1β and IL-8 levels were below the limit of detection.

4.3.1a. sTREM-1 levels

The median levels of sTREM-1 in the NVC group were lower than in VAP (p<0.001). Separately, the VC and VSE groups did not differ significantly from the NVC. The ventilated groups however did not differ in their levels of sTREM-1. The CAP group (non-ventilated) were significantly higher than the NVC group (p=0.001) and HV groups (p=0.008). See figure 4.1.

**Figure 4.1. Peripheral blood sTREM-1 levels**

![Peripheral blood soluble TREM-1](image)

**Figure 4.1.** The data shows the levels of sTREM-1. The median and interquartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis), NVC (non-ventilated, non-infected control), CAP (community-acquired pneumonia) and HV (healthy volunteers). No differences between the groups were seen. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.1b. IL-6 levels

Median IL-6 levels were higher in VAP than NVC and HV (p<0.0001 for both). VC was higher than NVC (p<0.0001) and HV (p=0.001). VSE was higher than NVC and HV (p<0.0001 for both). See figure 4.2. In general, levels in CAP were higher than HV patients and higher still in ventilated patients. Therefore IL-6 levels were a general indicator of the severity of illness.

Figure 4.2. Peripheral blood IL-6 levels

![IL-6 in blood](image)

Figure 4.2. The data shows the levels of IL-6. The median and inter-quartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis), NVC (non-ventilated, non-infected control), CAP (community-acquired pneumonia) and HV (healthy volunteers). VAP > NVC and HC (p<0.0001 for both). VC > NVC (p<0.0001) and VC > HV (p=0.001). VSE > NVC and HV (p<0.0001 for both). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.1c. PCT levels

PCT levels were higher in VAP compared with NVC and HC. PCT levels were higher in VC than NVC and HC. They were higher in VSE than NVC and HC. Levels in CAP were higher than NVC and HC too (p<0.0001 for all comparisons except CAP vs. HC where p=0.0001). PCT did not discriminate the ventilated groups however. See figure 4.3. The majority of patients in the HC and NVC groups had undetectable levels of PCT.

**Figure 4.3. Peripheral blood PCT levels**

![PCT in blood](image)

**Figure 4.3.** The data shows the levels of PCT. The median and inter-quartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis), NVC (non-ventilated, non-infected control), CAP (community-acquired pneumonia) and HC (healthy controls). VAP, VC and VSE > NVC and HC (p<0.0001). CAP > HC (p=0.0001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.1d. CRP levels

Median CRP levels were significantly higher in VAP, VC and VSE than NVC (p<0.0001, p=0.0005 and p<0.0001). Levels in the ventilated groups were higher than the non-ventilated group indicating that ventilation independently may be associated with a rise in CRP. See figure 4.4.

**Figure 4.4 Peripheral blood CRP levels**

![Peripheral blood CRP level graph](image)

**Figure 4.4.** The data shows the levels of CRP. The median and inter-quartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis), NVC (non-ventilated, non-infected control) and CAP (community-acquired pneumonia). VAP > NVC (p<0.0001); VC > NVC (p=0.0005); VSE > NVC (p<0.0001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction. CRP was not measured in healthy volunteers.
4.3.2e. WCC levels

WCC levels were higher in VAP and VSE than VC (p=0.004 and p=0.003) and NVC (p=0.0001 and p=0.0002). The levels between NVC and CAP were not significantly different (figure 4.5). The NVC levels were higher than expected. Some patients were receiving steroid therapy, some had cancer and a small number may have had resolving infection.

Figure 4.5 Peripheral blood WCC levels

![Peripheral blood White Cell Count (WCC)](image)

Figure 4.5. The data shows the levels of WCC. The median and inter-quartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis), NVC (non-ventilated, non-infected control) and CAP (community-acquired pneumonia. VAP > VC (p=0.004) and NVC (p=0.0001); VSE > VC (p=0.003) and NVC (p=0.0002). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction. WCC was not measured in healthy volunteers.
4.3.2. Soluble proteins in BAL

4.3.2a. Cytokine levels

Median levels of sTREM-1 were higher in VAP than VC (p=0.001) and VSE (p=0.0009). See figure 4.6. BAL IL-1β levels were higher in VAP than VC (p=0.002), VSE (p=0.0009) and NVC (p=0.0002). The levels of IL-6 in VAP were higher than in NVC (p=0.0015). Levels of IL-8 were elevated in VAP compared with VC (p=0.0006) and VSE (p=0.0004)

**Figure 4.6. BAL Cytokine levels**

(a) BAL soluble TREM-1 levels after urea correction

(b) BAL IL-1β levels after urea correction

(c) BAL IL-6 levels after urea correction

(d) BAL IL-8 levels after urea correction

**Figure 4.6.** Urea corrected (a) BAL sTREM-1 levels. (b) BAL IL-1β levels. (c) BAL IL-6 levels. (d) BAL IL-8 levels. Medians and IQR are described. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.2b. PCT levels

PCT levels in BAL (corrected by urea to represent ELF) did not differ between any of the ventilated and non-ventilated groups. See figure 4.7.

**Figure 4.7. BAL PCT levels**

![Graph showing BAL PCT levels](image)

**Figure 4.7.** The data shows PCT levels. The median and inter-quartile range (IQR) are presented for VAP (ventilator-associated pneumonia), VC (ventilated non-infected control), VSE (ventilated with sepsis elsewhere, non-pulmonary sepsis) and NVC (non-ventilated, non-infected control). No differences between the groups were seen. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.

4.3.3. BAL/blood ratio of soluble proteins

The BAL/blood ratio of sTREM-1 is significantly higher for VAP than VC (p=0.001) or VSE (p=0.0008). The ratios are all above 1 due to the fact that the levels in the pulmonary compartment are significantly higher than the blood. The BAL/blood ratio of IL-6 did not discriminate any of the groups. The ratios are again above unity, indicating concentration of the cytokine in the BAL. For PCT, the majority of patients in the NVC group had undetectable levels of PCT. Therefore the BAL/blood ratio is artificially elevated and is meaningless. The BAL / blood ratio of the remaining groups did not significantly differ. See figure 4.8.
Figure 4.8 BAL / blood ratio of soluble proteins

(a) Soluble TREM-1 BAL/blood ratio after urea correction
(b) IL-6 BAL/blood ratio after urea correction
(c) BAL / blood ratio for PCT

Figure 4.8. (a) sTREM-1 (b) IL-6 (c) PCT. Medians and IQR are described. The undetectable levels of PCT in the blood of the NVC group render the BAL/blood ratio ineffective. For sTREM-1 VAP > VC (p=0.001) and VC (p=0.00008). There was no difference between the levels for PCT or IL-6. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction. BAL levels represent the ELF following dilutional correction using urea.
4.3.4 Summary of diagnostic performance of the soluble markers

In table 4.2, the diagnostic performance of the significant biomarkers has been presented with area under ROC curves, positive and negative predictive values as well as likelihood ratios.

The blood markers CRP, WCC, IL-6 and PCT have AUROCs of 0.64, 0.72, 0.73 and 0.67 respectively. The BAL markers fare better, with AUROCs of 0.74, 0.78, 0.75 and 0.68 for sTREM-1, IL-1β, IL-8 and IL-6 respectively. The BAL/blood ratio of sTREM-1 had an AUC of 0.71. The cutoff levels were chosen to optimise the sensitivity and specificity of the marker.
Table 4.2. Summary of diagnostic data for soluble proteins

<table>
<thead>
<tr>
<th></th>
<th>Optimal cut-off</th>
<th>AUC</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR+</th>
<th>LR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood CRP</td>
<td>70 mg/L</td>
<td>0.64</td>
<td>53</td>
<td>63</td>
<td>38</td>
<td>76</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.52-0.75)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood WCC</td>
<td>10.5 x10^9/l</td>
<td>0.72</td>
<td>69</td>
<td>70</td>
<td>49</td>
<td>84</td>
<td>2.3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.60-0.84)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Blood IL-6</td>
<td>55.5 µg/ml</td>
<td>0.73</td>
<td>66</td>
<td>63</td>
<td>43</td>
<td>81</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.62-0.83)</td>
<td></td>
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</tr>
<tr>
<td>Blood PCT</td>
<td>0.8 ng/ml</td>
<td>0.67</td>
<td>62</td>
<td>56</td>
<td>37</td>
<td>77</td>
<td>1.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.56-0.78)</td>
<td></td>
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</tr>
<tr>
<td>BAL sTREM-1</td>
<td>13.6 µg/ml</td>
<td>0.74</td>
<td>75</td>
<td>70</td>
<td>52</td>
<td>87</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.62-0.87)</td>
<td></td>
<td></td>
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<tr>
<td>BAL IL-1β</td>
<td>1.4 µg/ml</td>
<td>0.78</td>
<td>73</td>
<td>78</td>
<td>58</td>
<td>87</td>
<td>3.3</td>
<td>0.3</td>
</tr>
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<td></td>
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<td>(0.69-0.89)</td>
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<tr>
<td>BAL IL-8</td>
<td>22.9 µg/ml</td>
<td>0.75</td>
<td>72</td>
<td>74</td>
<td>54</td>
<td>86</td>
<td>2.8</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.64-0.86)</td>
<td></td>
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<tr>
<td>BAL IL-6</td>
<td>3.0 µg/ml</td>
<td>0.68</td>
<td>69</td>
<td>63</td>
<td>44</td>
<td>83</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.55-0.81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL PCT</td>
<td>13.3 ng/ml</td>
<td>0.63</td>
<td>60</td>
<td>63</td>
<td>41</td>
<td>79</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.50-0.76)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BAL/blood sTREM-1 ratio</td>
<td>92</td>
<td>0.71</td>
<td>69</td>
<td>74</td>
<td>53</td>
<td>85</td>
<td>2.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Table 4.2. This data includes diagnostic data for individual markers in Blood and BALF and the BALF/Blood ratios. The optimal cut-off for the Area under Curve (AUC), sensitivity (sens), specificity (spec), positive predictive value (PPV), negative predictive value (NPV) positive likelihood ratio (+LR) and negative likelihood ratio (-LR) are given for each individual analytes. The NPV and PPV are given for the analyte at the given cutoff level. The BAL levels represent ELF concentrations following correction for dilution. The Youden index was utilised to determine the optimal sensitivity and specificity of each analyte.
4.3.5. Matrix metalloproteinases

Matrix metalloproteinases and their inhibitors have been studied in VAP and pneumonias. MMPs (likely -8 and -9) shed soluble TREM-1 from the surface receptor [45]. Their pulmonary levels are elevated in hospital-acquired pneumonias [126] especially those with multi-resistant organisms including pseudomonas [127, 128]. Dyregulation of the proteases and their inhibitors may contribute to chronic lung diseases such as COPD and ILD [129].

The data we obtained from flow-cytometric analysis led to the hypothesis that MMP levels in VAP could contribute to differences in surface expression of mTREM-1 and nTREM-1 in the BAL and blood. We sought to assess the levels of MMP-8 and -9 together with their inhibitors in the BAL samples.

Following the conclusion of the main study, the levels of the matrix metalloproteinases MMP-8 and MMP-9 as well as TIMP-1 (tissue inhibitors of metalloproteinases) were assayed (2012) in a random selection of BAL samples that had been frozen at the time of the initial study (2009-2011) but not previously thawed. Their levels were corrected for dilution using the urea assay method. We did not measure MMP activity.

Figure 4.9 shows the levels of MMP-8, MMP-9 and TIMP-1. The levels of MMP-8 were not significantly different between the groups. The MMP-9 levels were significantly higher in the ventilated groups than the non-ventilated control; VAP > NVC (p=0.0002), VC > NVC (p<0.0001) and VSE > NVC (p=0.0002). There were no differences between the groups for TIMP levels.
Figure 4.9 BAL MMP and TIMP-1 levels

(a) MMP-8 levels

(b) MMP-9 levels

(c) TIMP levels

Figure 4.9. (a) BAL MMP-8 levels. (b) BAL MMP-9. (c) BAL TIMP-1 levels. Urea corrected cytokine levels with median and inter-quartile range highlighted. For MMP-9, VAP > NVC (p=0.0002); VC > NVC (p<0.0001) and VSE > NVC (p=0.0002). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.3.6. 16S DNA levels

Bacterial (prokaryotic) ribosomes comprise a 30S and 50S subunit. The 30S subunit contains ribonucleoproteins and ribosomal RNA, including the 16S ribosomal RNA (rRNA). The 16S gene DNA encodes for the rRNA. It has elements that are conserved between species, allowing binding of primers for PCR analysis. In addition, there are hypervariable regions, which differ between species and allow identification of individual bacteria. Measurement of 16S DNA has been studied in the BAL of ICU patients. Flanagan et al (2007) demonstrated that antibiotic treatment reduced the number of species of bacteria present in the airways that was associated with an increase in pseudomonal bacterial load [130]. Bousbia et al (2012) studied patients with VAP along with CAP, aspiration pneumonia and non-infected controls. They showed a large number of bacterial, viral and fungal species present even in control groups (non-infected) [131]. Hoedemaekers et al (2006) described a case of VAP associated with Dolosigranulum Pigrum. This was an extremely rare cause of VAP but was postulated to be pathogenic in this patient [132]. Such studies all illustrate a difficulty of using 16S measurements. Species identification is over-sensitive and may be prone to contamination. Furthermore, it cannot differentiate colonisation from infection. We measured total levels of 16S DNA without species identification, to determine whether differences existed between our patient groups. Furthermore, we wished to study those patients with negative BAL cultures to understand the bacterial load in their airways.

The plasma levels of a selection of patients were assessed in the VAP, VC, VSE and NVC groups as well as healthy volunteers and the BAL assay was performed in the VAP, VC, VSE and NVC groups (figure 4.10). There were no significant differences between the absolute levels of 16S DNA in the plasma between the groups, nor between the groups in the BAL.
Figure 4.10. Blood and BAL 16S bacterial DNA levels

(a) Blood and (b) BAL levels are highlighted with median and inter-quartile range. The BAL levels are urea-corrected for dilution occurring with bronchoscopy. No differences between the groups were seen. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
4.4. Discussion

4.4.1. Community-acquired pneumonia

Interestingly, the levels of sTREM-1 in the blood of CAP patients were higher than either volunteers or the non-ventilated bronchoscopy clinic patients. This was in accordance with a study by Porfyridis et al (2010) who compared patients with and without bacterial CAP [133]. In the blood of CAP patients, they found elevated CRP levels, sTREM-1, mTREM-1 and nTREM-1. sTREM-1 at a cutoff level of 19 pg/ml had a sensitivity of 82.6% and specificity of 63% to diagnose CAP in this setting. We did not measure sTREM-1 levels in acutely unwell medical patients without CAP and therefore cannot calculate a comparative sensitivity and specificity. Similarly, How et al (2011) assessed plasma sTREM-1 levels in patients with typical and atypical CAP. Median sTREM-1 levels in the former were 65.2 pg/ml and 25.9 pg/ml in the latter [134]. Our numbers of patients did not include patients with atypical CAP and therefore a comparison is difficult. Both studies are suggestive that sTREM-1 and mTREM-1 / nTREM-1 levels may be elevated in CAP. This warrants further study. With regards to PCT, we saw elevated levels in CAP compared with volunteers and outpatient bronchoscopy patients. PCT may have utility in CAP to diagnose it and also to assist in antibiotic cessation in patients with treated LRTI's [135].

4.4.2. Ventilator-associated pneumonia

The standalone markers have been investigated for their utility in discriminating VAP from the other ventilated and non-ventilated groups and comments have been made as each marker was discussed. The conventional markers CRP and WCC have limited utility in this regard as does PCT. The blood markers did not differentiate VAP from the other groups, suggesting VAP to involve compartmentalization. In this regard, sTREM-1, IL-1β and IL-8 are elevated in the BALF. This is in keeping with several studies previously discussed. The sTREM-1 data agrees with Gibot et al (2004) and Determann et al (2005) but differs from Song et al (2007), Oudhuis et al (2009), Anand et
al (2008) and Conway-Morris et al (2009) in being elevated in VAP [14, 100-102, 104, 105]. Reasons for this include assay differences, patient selection, prior freezing/thawing of samples in other studies, VAP definitions and method of sample acquisition. BAL PCT was not discriminative. This was in accordance with work by Linssen et al (2008) who showed no benefit to BAL measurement of PCT in the diagnosis of VAP [70].

The BAL/blood ratio of sTREM-1 increased the discrimination between the VAP and VC/VSE groups as compared with the BAL level. There may be variable levels of sTREM-1 between different individuals and the ratio may help correct for this. The data on the BAL/blood ratio will be discussed further in the main discussion of the thesis.

Considering the metalloproteinases, the results differ from those seen by Wilkinson et al (2012) who saw elevated levels of MMP-8 and MMP-9 in VAP [136]. Our sample size was smaller, due to the availability of BAL samples. Their samples did not include patients with non-pulmonary sepsis. Our data did not differ whether levels were corrected for dilution or not. As the data was acquired from relatively few samples (15 VAP, 9 VC, 8 VSE and 8 NVC), the data has not been included in the biomarker panel analysis nor the summary tables in this chapter. A caveat to the analysis was that the physiological activity of the enzymes was not assayed, just the absolute levels. It is possible that a qualitative difference may exist between our groups.

We found the 16S DNA data of interest. Both BAL and blood samples were positive for DNA in patients with VAP as well as non-infected non-ventilated controls. The assay used to measure DNA levels is in use by our Department across a variety of patient groups, including those with HIV. It is unlikely that the results are therefore due to assay error. The data likely reflects sensitivity of the technique and colonisation of airways. The results are in keeping with those presented by Conway-Morris et al (2011, State of the Art Intensive Care Society Winter meeting) and Bousbia et al (2012) where bacterial DNA was present even in healthy control patients [131]. It is also possible that some non-ventilated control patients had resolving infections that were negative for traditional culture methods. One caveat to our work is that we did not measure
quantitative bacterial levels for formal microbiology. Potentially, the DNA levels could have correlated with the quantitative bacterial burden and may have allowed the study of those patients with $<10^4$ cfu/ml BAL bacteria but high DNA levels. Such patients could have had subclinical VAP. Furthermore, we did not identify the dominant bacteria from the DNA samples and so could not compare with conventional microbiological results. Rapid bacterial identification by PCR could complement existing clinical methods. It could allow a better understanding of the role of bacteria not routinely grown in the laboratory that may be pathogenic.

4.5. Conclusions

BAL levels of cytokines (sTREM-1, IL-1$\beta$ and IL-8) can differentiate VAP from non-VAP patients. The BAL/blood ratio of sTREM-1 may increase the discrimination between the groups. The conventional markers such as CRP, WCC and PCT and other blood biomarkers have limited value as standalone markers in diagnosing VAP. Further studies could assess the utility of blood sTREM-1 to diagnose CAP. Quantitative analysis of matrix metalloproteinases and their inhibitors demonstrated no difference between our groups. 16S DNA analysis showed significant levels in non-ventilated control patients which may be a result of bacterial airway colonization.
Chapter 5

Expression of inflammatory proteins by monocytes and neutrophils in VAP and disease controls

5.1. Introduction

We have developed assays to measure surface expression of mTREM-1, CD11b and CD62L (L-selectin) in the blood and BAL. We sought to determine whether expression of such proteins differed in patients with VAP and non-VAP. Pilot results demonstrated mTREM-1 in the BAL and the BAL/blood ratio to be increased in VAP. We therefore studied the changes in the expression of these proteins on the surface of monocytes and neutrophils over time, during the development and resolution phases of VAP to inform us of their potential utilities as biomarkers. Towards the end of the study, we also measured TREM-2 levels in selected patients developing VAP following reports of changes in its levels in BAL with ALI [52].

5.2. Aims

1. To determine the utility of the blood monocytic and neutrophilic biomarkers TREM-1, CD11b and CD62L (L-selectin) to discriminate patients with VAP from non-VAP

2. To determine the utility of the BAL monocytic and neutrophilic biomarkers TREM-1, CD11b and CD62L (L-selectin) to discriminate patients with VAP from non-VAP

3. To determine if the BAL/blood ratio of the monocytic and neutrophilic biomarkers TREM-1, CD11b and CD62L increases the discrimination of patients with VAP from non-VAP

4. To determine the temporal changes of mTREM-1 and nTREM-1 in the blood and BAL of patients developing and resolving VAP in order to assess their utility as biomarkers

5. To assess the temporal changes of mTREM-2 and nTREM-2 in the blood and BAL of selected patients developing and resolving VAP.
5.3. Expression of surface markers in blood

In the six patient populations (four for BAL), TREM-1, CD11b and L-selectin (CD62L) were measured on the surface of monocytes and neutrophils in blood and BAL. Initially, blood levels will be discussed, then BAL levels and finally the BAL/blood ratios. The data is summarised in Table 5.1.

5.3.1. Blood mTREM-1 levels

Figure 5.1 illustrates the levels of mTREM-1 in blood. The level of mTREM-1 in the NVC was significantly higher than the healthy volunteers (p=0.002), as was the level in CAP (p=0.001). There was a spread in the levels of mTREM-1 in volunteers, indicating variation in health between individuals.

Figure 5.1. Blood monocytic TREM-1

![Blood monocytic TREM-1](image)

**Figure 5.1.** Levels of blood mTREM-1 in the six groups with median and interquartile range. VAP = ventilator-associated pneumonia; VC = ventilated control (non-infected); VSE = ventilated with sepsis elsewhere (non-pulmonary infection); NVC = non-ventilated control (non-infected); CAP = community-acquired pneumonia; HV = healthy volunteer. CAP > HC (p=0.001); NVC > HV (p=0.002). NVC and CAP > HV (p=0.002 and p=0.001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
Table 5.1. Expression of surface markers in the four patient groups

<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>VC</th>
<th>VSE</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTREM-1</td>
<td>5.1(3.1-8.5)</td>
<td>6.1(4.6-9.6)</td>
<td>4.8(4.0-7.0)</td>
<td>7.4(4.3-11.1)</td>
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<tr>
<td>nTREM-1</td>
<td>5.4(2.9-7.5)</td>
<td>2.9(2.0-4.6)</td>
<td>5.9(2.7-9.4)</td>
<td>4.7(3.1-8.5)</td>
</tr>
<tr>
<td>mCD11b</td>
<td>47.2(30.5-93.5)</td>
<td>34.0(23.8-51.8)</td>
<td>43.6(34.5-50.4)</td>
<td>36.6(21.6-50.7)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>42.0(30.7-87.7)</td>
<td>59.8(40.8-107.2)</td>
<td>61.7(56.0-92.5)</td>
<td>48.8(31.0-77.7)</td>
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<tr>
<td>mCD62L</td>
<td>9.4(7.3-16.9)*</td>
<td>9.5(5.5-14.4)**</td>
<td>10.1(7.2-13.7)</td>
<td>5.0(3.5-9.3)</td>
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<tr>
<td>nCD62L</td>
<td>9.6(6.9-17.2)</td>
<td>8.1(6.2-11.8)</td>
<td>9.0(7.1-11.2)</td>
<td>8.7(7.0-11.8)</td>
</tr>
<tr>
<td><strong>BAL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTREM-1</td>
<td>3.9 (2.4-6.5)</td>
<td>1.3(0.24-2.18)$</td>
<td>1.9 (1.40-2.41)$**</td>
<td>2.0(1.26-2.90)*</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>2.1(1.7-3.3)</td>
<td>1.2(1.0-1.8)</td>
<td>2.0(1.37-3.61)</td>
<td>1.5(1.18-3.10)</td>
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<td>mCD11b</td>
<td>24.0(8.0-82.0)</td>
<td>18.6(12.8-36.2)</td>
<td>18.2(9.0-26.4)</td>
<td>19.7(7.8-46.0)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>47.0(13.8-84.5)</td>
<td>70.3(34.2-168.0)</td>
<td>40.7(28.3-193.0)</td>
<td>53.0(23.1-136.0)</td>
</tr>
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<td>mCD62L</td>
<td>1.2(1.0-1.6)</td>
<td>1.2(1.1-1.4)</td>
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<td>1.4(1.0-2.1)</td>
<td>1.2(1.1-1.4)</td>
<td>1.3(1.0-1.8)</td>
</tr>
<tr>
<td><strong>BAL/blood</strong></td>
<td></td>
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</tr>
<tr>
<td>mTREM-1</td>
<td>0.9(0.6-1.1)</td>
<td>0.3(0.1-0.3)</td>
<td>0.4(0.2-0.5)</td>
<td>0.4(0.2-0.4)*</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>0.6(0.2-0.8)</td>
<td>1.0(0.6-1.6)</td>
<td>0.4(0.2-0.6)</td>
<td>0.4(0.2-0.8)</td>
</tr>
<tr>
<td>mCD11b</td>
<td>1.2(0.4-2.3)</td>
<td>0.5(0.2-0.8)$</td>
<td>0.2(0.1-0.5)$**</td>
<td>0.6(0.2-1.3)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>0.7(0.5-2.0)</td>
<td>0.5(0.2-1.3)</td>
<td>0.5(0.2-0.6)</td>
<td>0.5(0.1-1.4)</td>
</tr>
<tr>
<td>mCD62L</td>
<td>0.2(0.1-0.5)</td>
<td>0.1(0.1-0.2)</td>
<td>0.1(0.1-0.2)</td>
<td>0.2(0.1-0.3)</td>
</tr>
<tr>
<td>nCD62L</td>
<td>0.2(0.1-0.3)</td>
<td>0.2(0.1-0.4)</td>
<td>0.1(0.1-0.2)</td>
<td>0.2(0.1-0.2)</td>
</tr>
</tbody>
</table>
Table 5.1. Expression of surface markers in the four patient groups. The levels of the markers in the blood (MFI), BAL (MFI) and the BAL/blood ratio are described. MFI = mean fluorescent intensity. The median and interquartile range for each patient group is reported. Statistically significant differences between groups were determined using the Mann-Whitney U and Kruskal-Wallis tests with post hoc Dunn correction as follows: * VAP > NVC, p<0.001.
** VC > NVC, p<0.01. $ VAP > VC, p<0.0001. $$ VAP > VC, p=0.0001. + VAP > VC, VSE and NVC (p<0.0001 for all). ° VAP > VC, p=0.0085. °° VAP > VSE, p=0.0013.
5.3.2. Blood nTREM-1 levels

Figure 5.2 shows the levels of nTREM-1 in the six groups. There were no significant differences between patients who were ventilated and the non-ventilated and healthy controls.

Figure 5.2. Blood nTREM-1 levels

![Blood neutrophil TREM-1](image)

Figure 5.2. Blood nTREM-1 levels with medians and IQR. There were no significant differences between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.3.3. Blood mCD11b and nCD11b levels

mCD11b levels were measured in the six groups, but did not differ significantly between them. The levels in the healthy volunteers were spread over a narrow range in comparison with the other groups. This may be suggestive of a dimorphic pattern, where some patients have a raised mCD11b MFI and others do not. We were unable to discern a clinical difference between the groups even in those patients with raised MFI (fig 5.3a). nCD11b levels were assessed in the six groups and did not differ significantly (figure 5.3b). Once again, the levels in the healthy volunteers were within a lower range than the patients. Some patients in each group had elevated nCD11b levels but the majority had levels similar to healthy volunteers. Amongst those with raised levels (dimorphic pattern), no pattern emerged to distinguish the groups.

Figure 5.3. Blood mCD11b and nCD11b levels

(a) Blood monocytic CD11b and (b) Blood neutrophilic CD11b levels with medians and IQR. There were no significant differences between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.3.4. Blood mCD62L and nCD62L (L-selectin) levels

The levels of mCD62L were increased in VAP, VC and CAP compared with the NVC (p=0.0003, p=0.0054 and p=0.0041). Although the levels of mCD62L were increased in CAP compared with healthy volunteers, the difference was not significant when corrected for multiple comparisons (figure 5.4a). There was no significant difference between the levels of nCD62L between any of the groups. Once again, this differs from the monocyte levels and will be discussed later (figure 5.4b).

Figure 5.4. Blood mCD62L and nCD62L (L-selectin) levels

(a) Blood monocyte L-selectin
(b) Blood neutrophil L-selectin

Figure 5.4. Blood (a) mCD62L and (b) nCD62L (L-selectin) levels with medians and IQR. For mCD62L, VAP, VC and CAP > NVC (p=0.0003, p=0.0054 and p=0.0041 respectively). No differences were seen for nCD62L. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.4. Expression of surface markers in the BAL

5.4.1. BAL mTREM-1 levels

The data for the BAL mTREM-1 levels was significantly different (figure 5.5). It was higher in the VAP group than the VC (p<0.0001), VSE (p=0.0001) and NVC groups (p<0.0001). The data seen amongst the groups in the BAL is different to the blood and indicates that the changes in VAP may be compartmentalised to the lung.

**Figure 5.5. BAL mTREM-1 levels**

![Box-whisker plots](image)

**Figure 5.5.** BAL mTREM-1 levels in the four groups. Box-whisker plots (median and IQR with range) highlighted. Outliers are identified as dots. VAP levels were significantly higher than those in the other groups (p<0.001 for all). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.4.2. BAL nTREM-1 levels

nTREM-1 levels in BAL were assessed in the four groups. However, in comparison with mTREM-1, there was no significant difference between the groups. Once again, this highlights a difference between the results seen with the neutrophils and those seen with the macrophages (figure 5.6).

Figure 5.6. BAL nTREM-1 levels

Figure 5.6. BAL nTREM-1 MFI levels in the four groups with medians and IQR. No significant differences were seen between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.4.3. BAL mCD11b and nCD11b levels

Figure 5.7a illustrates the levels of mCD11b in the BAL. There were no significant differences between the groups. However, visual inspection suggests that there is a narrow spectrum of levels of mCD11b in patients with VSE and VC and that there are some patients with VAP (more than in the NVC) with higher levels, suggestive of a dimorphic picture. BAL levels of nCD11b did not differ between the four groups (figure 5.7b).

**Figure 5.7. BAL mCD11b and nCD11b levels**

(a) BAL monocyctic CD11b

(b) BAL neutrophilic CD11b

Figure 5.7. BAL (a) mCD11b and (b) nCD11b levels in the four groups with medians and IQR. No significant differences were seen between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.4.4. BAL mCD62L and nCD62L (L-selectin) levels

The BAL levels of mCD62L (L-selectin) did not differ between the four groups either (figure 5.8a). The BAL levels of nCD62L (L-selectin) were not different across the four patient groups (figure 5.8b).

Figure 5.8. BAL mCD62L and nCD62L (L-selectin) levels

(a) BAL monocytic L-selectin

(b) BAL neutrophilic L-selectin

Figure 5.8. BAL (a) mCD62L and (b) nCD62L (L-selectin) levels in the four groups with medians and IQR. No significant differences were seen between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.5. BAL / blood ratios for surface markers

5.5.1. mTREM-1 BAL/blood ratio

The ratio is higher for VAP than all other groups (p<0.0001 for all). Secondly, there is increased discrimination between the VAP and other groups compared with measurement of the BAL level alone (figure 5.9). This is in accordance with the ratio reducing the inter-patient variation. The BAL/blood ratio and the BAL level of mTREM-1 differentiate pulmonary from non-pulmonary infection, albeit the latter group only having 15 patients.

**Figure 5.9. BAL/blood mTREM-1 ratios**

![Box-whisker plot](image)

**Figure 5.9.** BAL/blood mTREM-1 ratios. Box-whisker plots (median and IQR with ranges) highlighted. Outliers are represented by dots. The ratio for VAP is higher than all groups (p<0.0001 for all). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.5.2. nTREM-1 BAL / blood ratio

The BAL / blood ratio of nTREM-1 was compared amongst the 4 groups but there was no significant difference between them. This is a further difference between the results seen for the macrophages and neutrophils (figure 5.10).

**Figure 5.10. BAL/blood nTREM-1 ratios**

![Graph showing BAL/blood nTREM-1 ratios for four patient groups (VAP, VC, VSE, NVC). The graph displays individual data points for each group with medians and IQRs. There is no significant difference between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn's post-hoc correction.]

**Figure 5.10.** BAL/blood nTREM-1 ratios for the four groups with medians and IQR. There was no significant difference between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.5.3. mCD11b and nCD11b BAL/blood ratios

The BAL / blood ratio for mCD11b were higher in VAP than VC (p=0.0085) and VSE (p=0.0013). There are several patients with ratios above 1.0 indicative of elevated pulmonary levels. However, large numbers of patients have ratios below 1.0 indicative of low pulmonary levels (figure 5.11a). The ratios for nCD11b were similar between the four groups (figure 5.11b) in comparison with the data for the monocytic CD11b. The majority of the VSE patients have ratios below 1. Visual inspection of the other groups is suggestive of a dimorphic picture, with ratios above and below 1.0.

Figure 5.11. BAL/blood mCD11b and nCD11b ratios

(a) BAL/blood monocytic CD11b ratio

(b) BAL/blood neutrophilic CD11b ratio

Figure 5.11. BAL/blood (a) mCD11b and (b) nCD11b ratios with medians and IQR. VAP > VC (p=0.0085) and VSE (p=0.0013). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn's post-hoc correction.
5.5.4. mCD62L and nCD62L (L-selectin) BAL / blood ratio

There were no significant differences between the four groups for mCD62L. The ratios are mainly below 1.0 (figure 5.12a). Similarly for nCD62L, the ratios were not significantly different between the groups and were all below 1.0 as well (figure 5.12b).

Figure 5.12. BAL/blood mCD62L and nCD62L (L-selectin) ratios

![Graphs showing BAL/blood ratios](image)

Figure 5.12. BAL/blood (a) mCD62L and (b) nCD62L (L-selectin) ratios for the four groups with medians and IQR. There were no significant differences between the groups. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.6 Diagnostic performance of the standalone surface markers

The summary data for the surface markers with significant differences between VAP and the other groups are highlighted in Table 5.2. The AUROCs compare favourably with the soluble proteins (Chapter 4). The BAL/blood ratio of mTREM-1 has a higher AUROC than the BAL mTREM-1 (0.93 compared with 0.85). The NPV of the BAL/blood ratio of mTREM-1 is high at 96% for a cutoff level of 0.50, as are the sensitivity and specificity (92% and 85% respectively). The BAL/blood ratio of mCD11b, BAL nTREM-1 and Blood mCD62L have intermediate AUROCs of between 0.64 and 0.73.

**Table 5.2. Summary of diagnostic data for selected surface proteins**

<table>
<thead>
<tr>
<th></th>
<th>Optimal cut-off</th>
<th>AUC (95% CI)</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR+</th>
<th>LR-</th>
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<td>85</td>
<td>72</td>
<td>96</td>
<td>6.2</td>
<td>0.1</td>
</tr>
<tr>
<td>BAL nTREM-1</td>
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<tr>
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<td>65</td>
<td>70</td>
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<td>0.5</td>
</tr>
<tr>
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<td>MFI 9.5</td>
<td>0.64 (0.52-0.76)</td>
<td>66</td>
<td>48</td>
<td>35</td>
<td>77</td>
<td>1.3</td>
<td>0.7</td>
</tr>
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</table>

**Table 5.2. Summary of diagnostic data for selected surface proteins.** The optimal cutoff for each marker is given to diagnose VAP. The sensitivities, specificities, area under ROC curve, PPV (positive predictive value), NPV (negative predictive value), LR+ (positive likelihood ratio) and LR- (negative likelihood ratio) are described.
5.7. HELICS analysis

The study was conducted with patients classified into VAP on the basis of CPIS and positive microbiology. However, given that there is no gold standard for VAP, we enrolled patients with a strong likelihood for, or the absence of VAP. This led to a cohort of 27 patients with VAP and 33 ventilated controls (15 VSE and 18 ventilated and non-infected). We re-analysed the groups using the HELICS definitions of VAP. We utilised semi-quantitative microbiological culture in our study. Therefore the HELICS definitions of VAP appropriate to our study are PN4 and PN5 (see introduction).

Of the 60 ventilated patients, 2 diagnosed with VAP according to CPIS may be non-VAP and 1 patient with non-VAP (negative microbiology) could possibly be re-classified as a VAP case. There was agreement on at least 57 cases therefore, a kappa value of 95%. If the 1 patient with non-VAP remained non-VAP, this would be 96.7%. Figure 5.13 shows the BAL/blood mTREM-1 data if patients were re-classified by HELICS. As can be seen, the pattern demonstrated is identical to that for the CPIS definition of VAP. The level of the BAL/blood ratio for VAP is significantly higher than that of the VC, VSE and NVC groups (p<0.001 for all).

Overall, for this preliminary study on VAP biomarkers, we are satisfied that our diagnostic criteria are valid.
Figure 5.13. The BAL/blood ratio of mTREM-1 in the four groups according to the HELICS definitions of VAP. The BAL/blood ratio is higher for VAP compared with VC, VSE and NVC (p<0.001 for all). The graph shows a box-(median and inter-quartile range)-whisker (range) with outliers in dots. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
5.8. Temporal changes of mTREM-1 and nTREM-1 with development and resolution of VAP

Characteristics of ideal biomarkers for VAP diagnosis include the ability to rise early with infection and fall with disease resolution. We have shown that mTREM-1 in BAL and the BAL/blood ratio are increased in VAP. It is important to understand whether these markers have utility in tracking infection formation and resolution.

BAL nTREM-1 and its BAL/blood ratio do not show the same pattern. There may be several reasons for the latter observations: firstly, nTREM-1 may not correlate with VAP. Secondly, nTREM-1 may rise with VAP but to a lesser extent than mTREM-1 making the results non-significant. Finally, nTREM-1 may display different temporal changes to mTREM-1 and sampling at a single point in time may have missed its rise. One study suggested that following activation of nTREM-1 and mTREM-1, nTREM-1 surface receptor was cleaved, leading to increased sTREM-1 and reduced nTREM-1 (whose level gradually recovered) and a slow rising mTREM-1 [48]. We therefore sought to explore surface TREM-1 changes during development of, and resolution of VAP. This would determine whether mTREM-1 in the BAL (or the BAL/blood ratio) rose with infection / fell with resolution; it could also determine the role of nTREM-1 over the same time period. The existing definitions of VAP used in the study were continued with regards to CPIS and microbiology.

From previous analysis of standalone markers, thresholds were determined for VAP diagnosis, albeit with variable sensitivity and specificity. BAL mTREM-1 had an MFI threshold of 2.25, BAL/blood mTREM-1 0.5, BAL nTREM-1 1.67 and BAL/blood nTREM-1 1.13. The sensitivities and specificities respectively at these thresholds were 76% and 81% for BAL mTREM-1, 94% and 85% for BAL/blood mTREM-1, 67% and 78% for BAL nTREM-1 and 52% and 45% for BAL/blood nTREM-1. For the 12 patients, the thresholds will be discussed in the summary.

The changes in mTREM-1 and nTREM-1 will be divided into four sections: firstly changes in the markers prior to the onset of VAP; secondly, changes in the markers with resolution of VAP; thirdly changes in the markers with
progressive and ongoing VAP; finally one patient with serial bronchoscopies showing the development, worsening and resolution of VAP will be used to illustrate the sequential changes in these markers.

5.8.1. Expression of mTREM-1 and nTREM-1 on monocytes and neutrophils prior to the onset of VAP

VAP was diagnosed at the time of the second or third bronchoscopies in the patients PS72, PS81, PS84, PS86 and PS87 and therefore biomarker levels are available prior to and during the development of VAP.

As a representative example, 3 bronchoscopies were performed for PS72 (figure 5.14). VAP was diagnosed at the time of the third bronchoscopy. The BAL mTREM-1 initially falls slightly before rising and the BAL/blood ratio shows a continual rise. BAL nTREM-1 falls over this period and the BAL/blood ratio shows the same pattern too. Both blood mTREM-1 and nTREM-1 initially fall, but the nTREM-1 subsequently rises and the mTREM-1 falls. Overall, the BAL/blood ratio mTREM-1 provides a better signal for the development of VAP than the BAL mTREM-1. The nTREM-1 signal is discordant. The CRP initially rises marginally then a larger rise at the time of the third bronchoscopy, akin to the changes in BAL mTREM-1. The WCC rises minimally during this period however.

Overall, BAL mTREM-1 and the BAL/blood ratio of mTREM-1 show evidence of rising prior to the development of clinical (overt) VAP in four out of five patients, highlighting potential for their use as diagnostic biomarkers.
Figure 5.14. (a) BAL and blood mTREM-1 and nTREM-1 for PS72. (b) BAL / blood ratios for mTREM-1 and nTREM-1. (c) CRP and WCC levels. Each bronchoscopy was 48 hours apart; VAP was diagnosed at the time of the third bronchoscopy.
5.8.2. Changes in mTREM-1 and nTREM-1 with resolution of VAP

For six patients (PS45, PS59, PS60, PS66, PS70 and PS75), VAP was diagnosed at the time of the first bronchoscopy and showed resolution over the course of the temporal analysis.

For PS45 (a representative patient), 2 bronchoscopies were performed, which show BAL mTREM-1 and nTREM-1 falling (figure 5.15). VAP was present at the time of the first bronchoscopy. The fall in mTREM-1 is greater than that of nTREM-1. There is a fall in the blood mTREM-1 and nTREM-1 too. The BAL/blood ratio of mTREM-1 mirrors the BAL mTREM-1 and is of greater magnitude than the change in nTREM-1. The CRP falls slowly whereas the WCC remains relatively steady.

Figure 5.15

(a) Blood and BAL levels of mTREM-1 and nTREM-1 in PS45. BAL / blood ratios of mTREM-1 and nTREM-1. (c) WCC and CRP levels. Each bronchoscopy was 48 hours apart; VAP was present at the time of the first bronchoscopy.
Overall, both mTREM-1 in the BAL and its BAL / blood ratio show a reduction in the resolution of VAP in the majority of patients.

5.8.3 Changes in mTREM-1 and nTREM-1 with worsening VAP

For PS80, 2 bronchoscopies were performed following (figure 5.16). VAP developed at the time of the first bronchoscopy and clinically worsened. The CRP and WCC are concordant, rising slowly with development of infection. The BAL mTREM-1 rises (as does the BAL/blood ratio). The BAL nTREM-1 falls however. The blood mTREM-1 and nTREM-1 fall and the latter is at a faster rate than the BAL nTREM-1. Therefore the BAL/blood nTREM-1 ratio rises but with a shallower gradient than for mTREM-1. Overall, BAL mTREM-1 and the BAL/blood ratio highlight changes with progressive infection.
**Figure 5.16**

(a) BAL and blood mTREM-1 and nTREM-1 for PS80. (b) BAL/blood ratios for mTREM-1 and nTREM-1. (c) CRP and WCC levels. Each bronchoscopy was 48 hours apart; VAP was diagnosed at the time of the first bronchoscopy.
5.8.4 Changes in mTREM-1 and nTREM-1 during VAP development and resolution

The patient PS87 had four bronchoscopies. Clinically, he developed staphylococcal VAP but then clinically deteriorated, requiring antibiotic escalation (from Vancomycin to Linezolid). The VAP subsequently settled. The CRP and to a lesser extent the WCC rose, as previously described (figure 5.17). The BAL mTREM-1 mirrors the deterioration before resolution. The BAL/blood ratio of mTREM-1 highlights this signal better. The BAL nTREM-1 shows the same signal but with a lower magnitude.

**Figure 5.17**

![TREM-1 kinetic data for PS87](image)

(a) Blood and BAL levels of mTREM-1 and nTREM-1 in PS87. (b) BAL / blood ratios of mTREM-1 and nTREM-1. (c) WCC and CRP levels. Each bronchoscopy was 48 hours apart; VAP was present at day 2 and worsened by day 4 before resolving.
5.9. **TREM-2**

TREM-2 levels were studied in 6 patients. One patient (PS87) had VAP that developed, worsened and then resolved over the course of 4 bronchoscopies. The other five patients had mTREM-2 and nTREM-2 measured prior to the onset of VAP. The data for PS72 will be shown as an example, but the data for 4 other patients (PS80, PS81, PS84 and PS86) is also shown in table 5.3.

Figure 5.18 shows the changes in TREM-2 during development and resolution of VAP in PS87. Figure 5.19 shows the changes during development of VAP for patient PS72.

During the development of VAP, blood mTREM-2 remains static (PS87) or drops marginally (PS72). BAL mTREM-2 rises (PS72) or falls slightly before rising (PS87). The BAL / blood ratio rises gradually (PS72) or falls slightly then rises higher than baseline (PS87). In the other patients, the BAL/blood ratio rises steadily.

Just as for TREM-1, the neutrophilic changes in TREM-2 are harder to understand. In the blood (PS87), nTREM-2 rises then falls; in PS72 the level falls then rises. In the BAL, nTREM-2 falls then rises (PS87) or remains reasonably static (PS72). The BAL/blood ratio falls then rises as infection develops and resolves (PS87) or rises then falls (PS72) when infection develops. Overall, there was no clear pattern for the neutrophilic changes.
Figure 5.18

(a) TREM-2 changes in the blood and BAL in neutrophils and monocytes for PS87 during development and resolution of infection.  (b) Changes in the BAL/blood ratio of mTREM-2 and nTREM-2.  (c) CRP and WCC levels. Bronchoscopies were performed 48 hours apart. VAP was diagnosed at the time of the second bronchoscopy, worsened at the time of the third and was resolving at the time of the fourth bronchoscopy.
Figure 5.19. (a) TREM-2 changes for monocytes and neutrophils in the blood and BAL (PS72) during development of infection. (b) Changes in the BAL/blood ratio for mTREM-2 and nTREM-2. (c) CRP and WCC levels. Bronchoscopies were performed 48 hours apart. VAP was diagnosed at the time of the third bronchoscopy.
Table 5.3 mTREM-2 and nTREM-2 changes with development of VAP

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Table 5.3. mTREM-2 and nTREM-2 changes with development of VAP. For PS72, VAP was diagnosed at the time of the 3\textsuperscript{rd} bronchoscopy. For PS80, VAP was present at the time of the 1\textsuperscript{st} bronchoscopy. For PS81, PS84 and PS86, VAP developed at the time of the 2\textsuperscript{nd} bronchoscopy. Serial changes in monocytic and neutrophilic TREM-2 in blood, BAL and the calculated BAL/blood ratio are shown together with CRP (mg/L) and peripheral blood WCC (x10\textsuperscript{9}/L). For PS80 and PS81, only two bronchoscopies were performed. Overall, bronchoscopies were performed 48 hours apart. In general, the BAL mTREM-2 rose with the development of VAP.
5.10. Discussion

The data for the standalone markers will be discussed first, followed by the temporal changes in mTREM-1, nTREM-1 and mTREM-2.

5.10.1. Standalone markers

mTREM-1 (blood) was raised in CAP compared with healthy volunteers This is in keeping with that measured by Porfyridis et al (2010) in a larger patient cohort [133]. The levels of mTREM-1 in the ventilated groups did not significantly differ from each other, indicating that perhaps that the changes in VAP (see later) are compartmentalised. Figure 5.20 demonstrates the levels of mTREM-1 in patients with VAP and CAP compared with healthy volunteers. In comparison with the study by Porfyridis et al (2010), our study did not include patients with non-CAP acute medical illness (non-ventilated) and therefore the utility of blood mTREM-1 as a diagnostic biomarker cannot be assessed.

**Figure 5.20. Blood mTREM-1 levels in pneumonia**

![Blood monocytic TREM-1 in pneumonia](image)

**Figure 5.20.** The mTREM-1 levels for the pneumonic groups have been highlighted with medians and inter-quartile ranges (IQR). The levels in CAP are significantly higher than the healthy volunteers. CAP > HV (p=0.001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
In the CAP study (Porfyridis et al, 2010), there was a significantly higher level of nTREM-1 in blood in patients with CAP, compared with acute medical patients without pneumonia. Our study did not see any difference from healthy volunteers. It is possible that this difference may be due to a lower number of patients in our study or that we did not study acutely unwell non-CAP patients.

Considering BAL, the elevated level of mTREM-1 in VAP is in agreement with the nine patients with VAP/CAP (ventilated) in the study by Richeldi et al (2004) but with a far greater number of patients and different study groups [137]. In addition, some of the patients with sarcoidosis in the NVC group had elevated BAL levels of mTREM-1. This has been confirmed in a study conducted and published after acquisition of our results which showed elevated BAL mTREM-1 in patients with sarcoidosis [138]. The BAL mCD11b levels appear dimorphic and warrant further investigation to delineate any subsets of the VAP population.

The BAL/blood ratios give rise to a variety of discussion points. The BAL/blood mTREM-1 ratio increased discrimination between the groups as compared with the BAL level alone. The blood mTREM-1 levels in health varied, suggesting inter-patient variation. The ratio therefore may be a means of reducing this variability and potentially improving its diagnostic value. Secondly, the ratio differentiated pulmonary and non-pulmonary infection (VAP and VSE). In a complex clinical situation that is common in ICU patients, where infections at multiple sites are common and difficult to discriminate, this may be an advantageous aspect of a biomarker.

Thirdly, the ratios in each patient group were below 1.0. This means that the blood level of mTREM-1 is higher than the BAL level. This appears to be contradictory to the predicted biology of TREM-1, where elevated levels of surface TREM-1 are seen in infective conditions. However, this pattern is still apparent in the data – the levels of mTREM-1 are higher in VAP than the other patient groups. A likely explanation is that the surface receptor TREM-1 exists in conjunction with its soluble counterpart, sTREM-1. Cleavage of the surface receptor by matrix metalloproteinases is thought to be the main reason for sTREM-1 release from the cell (although direct secretion may also
be a source). If surface receptor was cleaved then it would result in a lower level of receptor. In our patient groups, the level of BAL mTREM-1 is higher in VAP than other groups, consistent with increased expression with infection. The BAL / blood ratios lower than 1.0 are suggestive of cleavage occurring in the pulmonary compartment. There may be similar levels of cleavage amongst the groups, or perhaps a reduced amount in the VAP group to explain the higher mTREM-1 level. In the previous chapter, we illustrated the levels of the matrix metalloproteinases and their inhibitors, MMP-8, 9 and TIMP. The levels did not differ between the groups. This result was at variance with data by Wilkinson et al (2012) who showed elevated levels of MMP-8 and MMP-9 in VAP [136]. Our data was acquired from only a proportion of the patients in our study and it may be that a larger cohort would have highlighted a difference. However, regardless of the levels of MMP-8 and MMP-9, it is likely that cleavage of the mTREM-1 surface receptor in part explains the results seen.

In comparison with mTREM-1, the nTREM-1 BAL/blood ratio did not differ between the groups. The majority of the patients showed a ratio below 1, indicating levels higher in the blood than BAL, consistent with receptor shedding. Considering the mCD11b BAL/blood ratio had ratios above and below 1.0. The CD11b molecule is not cleaved from the surface like mTREM-1 and nTREM-1. Examination of the ratio in the VSE group (figure 5.11) suggests that here there is no pulmonary activation of this receptor and it is therefore low in comparison to the blood. Extrapolating this to the remainder of the patients suggest a dimorphic picture, some in whom mCD11b is activated and others where it is not. Perhaps mCD11b is not elevated in all cases of VAP. In this regard, Hoogerwerf et al (2008) assessed the levels of CD11b on neutrophils in volunteers who had lipopolysaccharide and lipoteichoic acid instilled into their lung. They saw a difference in that nCD11b was elevated in patients with LPS instilled but not LTA. Perhaps some of the differences in the ratios of our patients are due to differences in immune responses to gram-positive and gram-negative infection [51]. The mCD62L and nCD62L BAL/blood ratios were below 1.0. CD62L is a receptor that facilitates migration of macrophages and neutrophils across the alveolar
epithelium. In doing so, it is shed from the surface to create soluble CD62L (not measured by us). Therefore our data is in accordance with the known biology of the molecule and is confirmation of the validity of the assay.

Overall, there are a number of positive aspects to this study that warrant further mention. The first is that measurement of surface markers in the blood and BAL in VAP and other disease states is feasible and yields diagnostic information. The assays allow simultaneous measurement of blood and BAL levels as well as calculation of a BAL / blood ratio. We believe that the BAL / blood ratio allows the opportunity to correct for variable levels of expression that occur naturally between patients and result in a reduction of inter-patient variation. This is particularly demonstrated by mTREM-1, where the BAL / blood ratio increased the discrimination between the groups compared with the BAL level alone.

The second point to note is that often the blood level alone did not differentiate the groups; measurement of the pulmonary compartment was required. This has implications for diagnostic biomarkers because it is likely that blood biomarkers would be unable to diagnose VAP and that pulmonary sampling would be required, which is potentially not as straightforward. An advantage of tissue-site sampling though is that it can allow differentiation of infective states, by highlighting where the infection lies. In this regard, the BAL / blood ratio of mTREM-1 and the BAL level of mTREM-1 were elevated in VAP, but not in the VSE group – a cohort of patients who had non-pulmonary infection. Given that the blood compartment is unable to differentiate pulmonary from non-pulmonary sepsis, measurement of biomarkers in multiple compartments could offer the opportunity to rule-in and rule-out tissue sites as areas of potential infection.

The third issue is the differences seen between neutrophils and monocytes in the study. Data has shown significant differences between patient groups for the monocytic receptors, but not for the neutrophils. There may be several explanations for this. The first is that the number of patients in the study is too low to detect a difference. However, visual inspection of the levels for the
neutrophils does not show a pattern / trend that is failing to achieve significance. Therefore this is unlikely to be a major factor. The second is that the temporal receptor level changes are different for neutrophils than monocytes and that we are missing the changes in neutrophils by sampling at a comparatively late time. This is indeed possible and was assessed by sampling at multiple time points (see later). A final reason is that there is a difference in the biology of the receptors on neutrophils as opposed to monocytes. Gibot et al (2005) assessed mTREM-1 and nTREM-1 levels in the blood of mice with septic shock. They showed that the level of mTREM-1 rose but that it did not with nTREM-1. Such a pattern could explain the discrepant results we have seen in our study [139].

Do the results seen with the surface and soluble receptors fit with the pathogenesis of VAP? If we consider the pulmonary compartment (fig 5.21) a putative model can be constructed. The monocyte migrates from the blood to the lung and in doing so sheds mCD62L. The cell possesses mCD11b, mTREM-1 and Toll-like receptors. Activation of the cell by TLR and mTREM-1 ligation allows increased mTREM-1 expression, increased mTREM-1 cleavage resulting in sTREM-1 secretion and potentially increased mCD11b expression. mTREM-1 activation results in IL-1β and IL-8 release and therefore these would be increased in the BAL of patients with VAP. Differences may occur depending on whether the infection was gram-positive, gram-negative or fungal (or viral).
Figure 5.21. Putative model for pulmonary compartment in VAP

The monocyte possesses CD11b and TREM-1 surface receptors. In some cases, activation occurs, in conjunction with ligation of a toll-like receptor. This may lead to increased CD11b expression and increased surface TREM-1 levels. Cleavage of the latter releases sTREM-1. Activation of the surface receptors, in particular mTREM-1 releases IL-1β and IL-8.

5.10.2. Temporal changes in mTREM-1 and nTREM-1

Patients were sampled prior to the development of VAP, during resolution of VAP, during worsening of VAP and a combination of these periods. A number of themes emerge. Firstly, in the majority of cases, BAL mTREM-1 accurately follows the clinical progress of infection development and resolution, including changes in CRP. This also tends to be true for the BAL/blood mTREM-1 ratio. Secondly, BAL nTREM-1 often demonstrates one of two patterns – either rising / falling in a similar pattern to mTREM-1 but with a lower magnitude than mTREM-1, or it falls as mTREM-1 rises. The latter pattern may be akin to the published data showing falling nTREM-1 with rising mTREM-1 [42]. Perhaps in each patient both processes occur – nTREM-1 and mTREM-1 rise and also monocytes/neutrophils shed their receptors, in a metalloproteinase
dependent manner. The degree to which each one develops determines the pattern of either a shallow rise or a fall. WCC levels do not seem to correlate well with development and resolution of infection on their own.

Overall, there is compartmentalisation of effect, with the blood patterns in part explaining differences between the BAL levels and the BAL/blood ratios. BAL mTREM-1 appears to rise with infection and fall with resolution. nTREM-1 can either rise or may also fall with infection development. This may explain why the results for nTREM-1 are less significant than for mTREM-1.

Further analysis of the results from these patients can include comparisons with the previously derived diagnostic threshold levels. These were derived by analysis of ROC curves for each marker. The threshold was chosen as the point on the ROC curve with the optimum sensitivity and specificity (Youden index [63]). The BAL/blood nTREM-1 ratio has poor sensitivity and specificity. This can be seen with the temporal analyses where levels did not correlate with VAP. There is broad agreement for the BAL levels of mTREM-1 and nTREM-1 to diagnose VAP, as is the case with the BAL/blood mTREM-1 ratio.

Of interest, patients had evidence of raised BAL mTREM-1 and nTREM-1 prior to clinical infection. This suggests activation of these receptors prior to VAP diagnosis. There is controversy over VAP diagnosis in that it is diagnosed 48 hours or more after intubation. This is to differentiate it from infection that is already present/developing prior to intubation. The temporal analysis would be consistent with pulmonary immune system activation in the development of VAP (subclinical VAP) and prior to clinical manifestations.

5.10.3. Temporal changes in mTREM-2 and nTREM-2

Overall, the data was strongest for the BAL/blood ratio of mTREM-2 which demonstrated that the levels generally rose and fell with infection development and resolution. This is akin to the data from TREM-1 and was initially surprising to us given that TREM-2 was initially predicted to be a negative regulator of inflammation. The data is however in keeping with rises in infection seen by Chen et al (2013), albeit in a murine peritonitis model.
To our knowledge, this is the first time TREM-2 and the BAL/blood ratio has been measured in VAP. This data warrants further exploration in future studies to determine whether it too may perform as a suitable biomarker and also to better understand the nature of its interaction with TREM-1 in VAP.

5.10.4. Temporal changes in CRP and WCC

Although not diagnostic for VAP, CRP and WCC levels broadly rose with VAP development and fell with resolution. Povoa et al [67] measured serial CRP levels in 47 patients with microbiologically proven VAP in a mixed medical/surgical ICU. Temporal measurement of CRP over 4 days differentiated four groups, with varying mortalities. Patient survival occurred where CRP fell (a fast or slow responder). Patients who died frequently had either a persistent CRP level despite antibiotic therapy (indicating perhaps the antibiotic choice to be incorrect) or exhibited a biphasic profile of a fall and secondary rise. Overall, if the ratio of CRP on day 4 to day 0 (VAP diagnosed) was above 0.6, this correlated with non-resolving VAP. A study by Lisboa et al was also in agreement with this [141]. A CRP ratio of 0.8 (d4 to d0) rather than 0.6, 96 hours after VAP treatment, indicated adequate antibiotic effect (area under ROC curve 0.86, sensitivity 77%, specificity 87%). In generalised sepsis rather than pure VAP, Yentis et al found that in ICU patients a fall of serum CRP by 25% from the previous day was associated with sepsis resolution. The sensitivity was 97%, specificity 95% and positive predictive value was 97% [142].
5.11. Conclusions

BAL mTREM-1 and its BAL/blood ratio rise with the development of VAP and resolve with its clearance.

BAL nTREM-1 does correlate with BAL mTREM-1 in many cases but sometimes the level drops in comparison to mTREM-1. The latter data fits with basic science data. The net result of some patients having a rise in nTREM-1 and some having a fall in nTREM-1 is that the population levels provide lower diagnostic utility than mTREM-1.

The absolute levels of mTREM-1 and nTREM-1 in the BAL would be consistent a VAP process developing over time (ie that VAP can be developing prior to 48 hours) even though it is not formally diagnosed until after 48 hours following intubation.

Finally, mTREM-2 may be a biomarker worthy of further study to assess its utility in diagnosing VAP in combination with mTREM-1.
Chapter 6

Construction of a biomarker panel to classify patients with and without VAP

6.1. Introduction

In previous chapters, the utility of standalone biomarkers to differentiate VAP and non-VAP has been discussed. In a complex clinical condition such as VAP, where patients are ventilated in ICU and infection are caused by a variety of micro-organisms, single markers may have limited clinical utility. We therefore investigated a combination of the biomarkers to determine the ability to differentiate our patient populations. In constructing a panel, markers may be added which on their own have limited clinical utility but which identify cases correctly that other markers do not. Such markers would not be readily identified by examining the diagnostic accuracy of that single analyte. Similarly, if two markers differentiate cases strongly, both may not be required together in the panel as they associate with each other; measurement of the second marker may not add further new diagnostic information compared with measurement of just the first. In the chronic lung disease setting, a biomarker panel has been constructed by our Department and validated for the differentiation of sarcoid from other interstitial lung disease and systemic sclerosis, using Fisher discriminant analysis [125]. Overall, a biomarker panel should include sufficient markers to allocate patients to the correct disease category, whilst minimising the number of analytes to reduce the potential cost and complexity associated with measurement of multiple markers.

6.2. Aims

1. Construction of a biomarker panel to discriminate patients with VAP from those with non-VAP

2. Validation of the biomarker panel to determine its utility in predicting patients with VAP.
6.3. Methodology and statistical analysis

6.3.1. Rationale

Cases were classified into VAP, ventilated non-VAP (a combination of patients with no infection and those with non-pulmonary sepsis) and non-ventilated controls. The reason for combining the two patient groups into a non-VAP group was to create three patient groups of similar size; if the VC and VSE groups were not combined, the patient numbers would be insufficient for analysis. The rationale for including the NVC (non-ventilated controls) in the analysis was to determine whether the biomarker panel could distinguish ventilated from non-ventilated patients and also to include patients with chronic lung conditions (COPD) who comprise a significant proportion of the intensive care population.

In order to combine the VC and VSE groups, it is essential that the enlarged group of patients do not differ. We compared the data for VAP, VC, VSE and NVC with that from the three groups (VAP, ventilated non-VAP and NVC). There was no significant difference between the data. The BAL (figure 6.1) once again showed a similar pattern (VAP > non-VAP, p<0.0001 and VAP > NVC, p<0.0001).

The BAL/blood ratio (figure 6.2) showed a similar pattern to that of the four groups.
**Figure 6.1.** BAL mTREM-1 levels with combined ventilated control groups

(a) BAL monocytic TREM-1

![Box-whisker plots](image)

**Figure 6.1.** BAL mTREM-1 levels where the two ventilated control groups (non-infected and non-pulmonary sepsis) have been combined. Box-whisker plots (median and IQR with range) highlighted and outliers marked with dots. VAP > non-VAP and NVC (p<0.001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.
Figure 6.2. BAL/blood mTREM-1 ratios (ventilated controls combined)

Figure 6.2. BAL/blood mTREM-1 ratios with the two ventilated control groups combined (ventilated non-infected and ventilated with sepsis-elsewhere i.e. both groups do not have pulmonary sepsis). VAP > non-VAP and NVC (p<0.001). Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction.

The pooled data for the surface markers in the 3 groups has been summarised in table 6.1. Again, this shows no significant changes from the four groups. Table 6.2 shows the patient characteristics of the three groups. Combining these two groups into a ventilated control group without VAP, there were 33 patients. Their median age was 62 years. 52% were male. The median APACHE II score was 15 and the median CPIS 3. Microbiology was positive in 12% of patients. 70% were receiving antibiotics at the time of sampling. None of the patients had VAP. CXR shadowing was present in 55% and 30% of patients were receiving steroids (low-dose). 39% were post-op and 15% were burns patients.
Table 6.1. Expression of cell surface proteins in patients with VAP, non-VAP and NVC

<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>Non-VAP</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTREM-1</td>
<td>5.1 (3.2-8.6)</td>
<td>4.6 (3.1-6.1)</td>
<td>6.5 (4.3-10.9)</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>4.7 (2.6-7.3)</td>
<td>3.8 (2.3-6.1)</td>
<td>4.5 (3.1-7.4)</td>
</tr>
<tr>
<td>mCD11b</td>
<td>47.2 (30.0-70.0)</td>
<td>43.3 (27.6-52.3)</td>
<td>39.2 (21.7-51.8)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>44.0 (33.4-91.9)</td>
<td>59.8 (43.4-82.9)</td>
<td>49.0 (38.0-81.0)</td>
</tr>
<tr>
<td>mCD62L</td>
<td>9.4 (7.3-15.1)</td>
<td>9.5 (7.4-13.2)</td>
<td>5.4 (3.9-9.4)</td>
</tr>
<tr>
<td>nCD62L</td>
<td>9.6 (6.0-17.0)</td>
<td>8.3 (6.0-10.5)</td>
<td>8.6 (6.8-10.5)</td>
</tr>
<tr>
<td><strong>BALF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTREM-1</td>
<td>3.9 (2.5-5.4)</td>
<td>1.6 (1.1-2.3)</td>
<td>1.8 (1.2-2.9)</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>2.0 (1.7-3.3)</td>
<td>1.5 (1.2-2.2)</td>
<td>1.7 (1.3-3.0)</td>
</tr>
<tr>
<td>mCD11b</td>
<td>25.2 (9.0-81.2)</td>
<td>18.6 (13.7-31.2)</td>
<td>21.0 (6.9-47.3)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>47.0 (15.1-86.0)</td>
<td>32.9 (20.3-62.5)</td>
<td>24.0 (6.0-73.5)</td>
</tr>
<tr>
<td>mCD62L</td>
<td>1.2 (1.0-1.5)</td>
<td>1.1 (1.0-1.3)</td>
<td>1.2 (1.0-1.4)</td>
</tr>
<tr>
<td>nCD62L</td>
<td>1.4 (1.0-2.1)</td>
<td>1.1 (1.0-1.4)</td>
<td>1.2 (1.0-1.7)</td>
</tr>
<tr>
<td><strong>BALF/blood ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTREM-1</td>
<td>0.8 (0.5-1.0)</td>
<td>0.4 (0.2-0.5)</td>
<td>0.3 (0.2-0.4)</td>
</tr>
<tr>
<td>nTREM-1</td>
<td>0.6 (0.2-0.8)</td>
<td>0.4 (0.3-0.8)</td>
<td>0.4 (0.2-1.1)</td>
</tr>
<tr>
<td>mCD11b</td>
<td>0.53 (0.4-2.3)</td>
<td>0.4 (0.2-0.7)</td>
<td>0.5 (0.2-1.3)</td>
</tr>
<tr>
<td>nCD11b</td>
<td>0.7 (0.5-2.0)</td>
<td>0.5 (0.2-0.9)</td>
<td>0.5 (0.1-1.4)</td>
</tr>
<tr>
<td>mCD62L</td>
<td>0.2 (0.1-0.5)</td>
<td>0.1 (0.1-0.2)</td>
<td>0.2 (0.1-0.3)</td>
</tr>
<tr>
<td>nCD62L</td>
<td>0.2 (0.1-0.3)</td>
<td>0.2 (0.1-0.2)</td>
<td>0.2 (0.1-0.2)</td>
</tr>
</tbody>
</table>
Table 6.1.
The median and interquartile range for each patient group is reported. Statistically significant differences between groups were determined using the Mann-Whitney U and post hoc Dunn correction as follows: VAP and non-VAP versus NVC (p<0.001)*, VAP versus NVC (p<0.001)† and non-VAP versus NVC (p<0.05)‡, VAP versus non-VAP and NVC (p<0.001)§, VAP versus non-VAP (p<0.01)¶, VAP versus non-VAP (p<0.001)††, VAP versus NVC (p<0.01)‡‡, VAP versus non-VAP (p<0.001)§§ and NVC > non-VAP (p<0.01)‖. Thus there were no significant differences when the VSE and VC groups were combined into the single ventilated non-VAP group. Analysis was performed using Mann-Whitney U-tests and the Kruskal-Wallis tests using Dunn’s post-hoc correction. The BAL levels represent ELF levels following dilutional correction.
Table 6.2 Characteristics of patients recruited to the study

<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>Non-VAP</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>27</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>Age</td>
<td>68 (23-84)</td>
<td>62 (18-89)</td>
<td>59 (18-84)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>70</td>
<td>52</td>
<td>61</td>
</tr>
<tr>
<td>APACHE II</td>
<td>18 (5-45)</td>
<td>15 (2-24)</td>
<td>N/A</td>
</tr>
<tr>
<td>CPIS</td>
<td>7 (6-9)</td>
<td>3 (0-5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Microbiology (% +ve)</td>
<td>100</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Smoking (% current / ex)</td>
<td>44/15</td>
<td>30/21</td>
<td>35/13</td>
</tr>
<tr>
<td>Antibiotics (% receiving)</td>
<td>89</td>
<td>70</td>
<td>32</td>
</tr>
<tr>
<td>CXR (% with shadowing)</td>
<td>96</td>
<td>55</td>
<td>81</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>30</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>28-day mortality (%)</td>
<td>11</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Surgery (%)</td>
<td>37</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>Burns (% of cases)</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>WCC (x10⁹/L)</td>
<td>15 (4-24)</td>
<td>9 (3-27)</td>
<td>7 (3-18)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>84 (7-320)</td>
<td>102(2-341)†</td>
<td>6 (1-296)†</td>
</tr>
</tbody>
</table>

Table 6.2. The median and range (lowest-highest) is shown for each group, with the VC and VSE groups combined into a single non-VAP group. APACHE II and CPIS are only applicable to the ventilated patients. Statistically significant differences between the groups were determined using the Mann-Whitney U test and Kruskal-Wallis tests with post-hoc Dunn correction and are indicated as follows: VAP versus NVC (p < 0.001)* and non-VAP versus NVC (p < 0.001)†.
6.3.2. Methodology

A database was constructed with each case and the following biomarkers:

**Blood**

(i) CRP

(ii) WCC

(iii) sTREM-1

(iv) IL-6

(v) PCT

(vi) mTREM-1

(vii) nTREM-1

(viii) mCD11b

(ix) nCD11b

(x) mCD62L (L-selectin)

(xi) nCD62L (L-selectin)

**BAL**

(xii) sTREM-1

(xiii) IL-1β

(xiv) IL-6

(xv) IL-8

(xvi) PCT

(xvii) mTREM-1

(xviii) nTREM-1
For the biomarkers IL-1β and IL-8, blood levels were below the limit of detection. Therefore neither the blood nor the BAL/blood ratios were available. Similarly, WCC and CRP were measured in the blood only and therefore BAL and BAL/blood ratios are unavailable. The matrix metalloproteases and the 16S RNA levels were measured in separate assays after the biomarker panel had been constructed. Their analysis was only performed on a subset of patients. They are therefore not included in the biomarker panel.
6.3.3. Statistical analysis

6.3.3a. Construction of biomarker panel

We constructed the panel using Fisher discriminant analysis (FDA). This statistical technique allows construction of a linear set of functions derived from the markers that accurately classifies patients into a **categorical label**, in this case patient group. This differentiates it from regression analysis where the classification dependent variable is numerical. Discriminant analysis classifies patients into two or more groups using quantitative variables. Fisher analysis is utilised where classification into more than 2 patient groups is required. It was first described nearly 80 years ago [143]. It is also normally conducted where the markers are normally distributed. We believed that combining the 31 analytes would result in a population resembling a normal distribution and our department has the greatest familiarity with this technique. Fisher analysis attempts to maximize the discrimination between the groups, whilst keeping differences in variance to a minimum. If samples are described by two variables on x and y axes, FDA aims to create a vector that the variables are mapped to. The vector should increase the scatter between the groups (class separation), but minimize scatter inside each group (variance). This is then repeated for multiple variables to determine the optimal combination of ‘vectors’ between them [144].

Furthermore, stepwise forward linear regression analysis was conducted by Dr. Pantelidis (Imperial College Healthcare NHS Trust) in conjunction with Professor Athol Wells (Royal Brompton Hospital). In this technique, linear functions are created that describe the probability of being in the VAP group, as opposed to Fisher analysis which aims to separate three or more groups groups maximally. A number of functions are combined to create a panel that can optimally predict VAP. In stepwise forward analysis, the panel is built up by choosing the marker that best predicts VAP, then adding further markers sequentially until there is no improvement in diagnostic accuracy. Rapid computational methods are used to test each new function in combination with the prior sequence of functions. The analysis completes when either there is no improvement in accuracy or there are no further markers to add.
'Forward' stepwise analysis starts with no markers whereas 'backwards' stepwise analysis starts with all the markers and removes one or more to determine the optimal combination. We used 'forward' analysis as we wished to create a panel with the minimal combination of markers. The results agreed with the data from Fisher analysis.

6.3.3b. Validation

Once a panel has been constructed, it ought to be applied to data acquired from new patients to determine whether the patient group can be accurately predicted from the model. However, given the finite time and resources associated with the project, recruitment of new patients was not feasible and remains a limitation of the study. However, we conducted two further types of internal validity analysis.

The first analysis included the 'leave one out' classification (cross-validation). In this, the effect of outliers are determined and excluded. Each one of the 91 patients was excluded from the analysis in turn and the Fisher analysis re-conducted. If there was a strong effect from outliers, then the data would demonstrate high variability.

The second analysis involved cross-validation by splitting the data into a training and validation cohort with a 60:40 split. This technique has previously been used to validate a biomarker panel [125]. Randomly, 60% of patients are chosen to construct the panel. In effect, for the biomarker panel, new classification function coefficients were generated for each analyte [145-147].

The data is then applied to the 40% of patients not previously used, as a test cohort (as if the patients were new ones that were recruited). This analysis is repeated ten times and the summary utility of the panel is determined. This has the advantage of testing the panel on 'new data' but the disadvantage is that the panel is constructed with a smaller subset of patients and therefore may be exposed to greater variability.
6.3.3c. Fisher discriminant analysis

Fisher's analysis was used with the SPSS v19 software package (SPSS, Chicago, IL, USA) to determine the optimal biomarker combination to discriminate VAP and non-VAP groups. The biomarker variable was entered into the "model" if the significance level of its F-value was <0.05 (95% confidence level) and removed on subsequent analysis if it rose above 0.1. The constructed model was then used to classify the 91 patients into their predicted group.

6.4. Results

A seven marker bioscore comprising the BAL/blood ratio of mTREM-1 and mCD11b, BAL levels of sTREM-1, IL-8 and IL-1β, together with blood levels of CRP and IL-6 was generated by the analysis as being optimal in discriminating VAP and non-VAP patients (table 6.3). It correctly classified 100% of the non-VAP patients and 88.9% of the VAP patients (figure 6.3). The non-ventilated control patients were defined as non-VAP in 90.3% of cases. The 'leave one out' validity analysis produced the same results for the VAP and non-VAP groups. In the cross-validation model, the average predictive accuracies for patients in the test cohort were 98.5% for non-VAP and 71% for VAP.

Table 6.3 Analyte panel identified by Fisher discriminant analysis
Table 6.3. A seven marker panel was generated using Fisher Discriminant analysis, with mTREM-1 and mCD11b (BAL/blood ratio), CRP and IL-6 blood and sTREM-1, IL-1β and IL-8 in BAL. The analyte level is multiplied by the function and then summated to determine patient group classification.
**Figure 6.3 Utility of a seven-marker panel to discriminate patient groups**

![Table showing classification results]

**Figure 6.3.** Patients in group 1 are VAP, group 2 are ventilated non-VAP and ‘ungrouped’ patients are non-ventilated control. 24/27 patients (88.9%) with VAP are correctly predicted to be in the VAP group. 100% of ventilated non-VAP patients are predicted to be in the non-VAP group. 28/31 (90.3%) of non-ventilated controls are predicted to have non-VAP. Fisher Discriminant Analysis was used.
We compared the utility of the panel by removing one marker and re-analysing the accuracy. The marker adding the least ‘extra information’ was IL-8 in BAL. Removal of this reduced the accuracy of classifying ventilated non-VAP patients from 100% to 97% with cross-validation (figure 6.4).

**Figure 6.4 Utility of a six-analyte panel to discriminate patient groups**

![Table](image)

---

**Figure 6.4.** BAL IL-8 was excluded from the 7-marker panel. Group 1 is VAP, Group 2 is ventilated non-VAP and ungrouped cases are the non-ventilated controls. Although there is a slight increase in the correct classification of the non-ventilated controls, there is a drop in the utility of the panel to correctly classify the ventilated non-VAP from 100% to 97% following cross-validation. Fisher Discriminant Analysis was used.
Removal of a further marker reduced accuracy further. The least disruptive removal was IL-1β. This reduced the VAP diagnostic accuracy to 85.2% (figure 6.5).

**Figure 6.5 Utility of a five-marker panel to discriminate patient groups**

![Classification Results Table]

<table>
<thead>
<tr>
<th>Original Count</th>
<th>VAP</th>
<th>nonVAP</th>
<th>Predicted Group Membership</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>23</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>32</td>
<td>33</td>
</tr>
<tr>
<td>Ungrouped cases</td>
<td>2</td>
<td>2</td>
<td>29</td>
<td>31</td>
</tr>
<tr>
<td>% 1</td>
<td></td>
<td>85.2</td>
<td>14.8</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.0</td>
<td>97.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Ungrouped cases</td>
<td>6.5</td>
<td>93.5</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Count</td>
<td>1</td>
<td>23</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>32</td>
<td>97.0</td>
<td>100.0</td>
</tr>
<tr>
<td>% 1</td>
<td></td>
<td>85.2</td>
<td>14.8</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.0</td>
<td>97.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

a. Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
b. 91.7% of original grouped cases correctly classified.
c. 91.7% of cross-validated grouped cases correctly classified.

**Figure 6.5.** IL-8 (BAL) and IL-1β (BAL) were omitted. Group 1 is VAP, Group 2 is ventilated non-VAP and the ungrouped cases are the non-ventilated control patients. The accuracy of the panel to identify VAP cases drops to 85.2% and the ventilated non-VAP to 97.0%. The non-ventilated controls are correctly classified 93.5% of times. Fisher Discriminant Analysis was used.
ROC curves were constructed to demonstrate the utility of the biomarker panel to classify patients into VAP, ventilated non-VAP and non-ventilated control (Figure 6.6). The AUROCs were high, 0.98 (0.96-1.00) for VAP, 0.95 (0.91-0.99) for ventilated non-VAP and 0.94 (0.89-0.99) for non-ventilated control. Table 6.4 shows the other data for the diagnostic performance of the panel. The sensitivity and specificity of the panel to classify patients with VAP was 95% and 93%, with a positive likelihood ratio of 12.9. This demonstrates the enhanced utility of a panel compared with standalone markers.

Figure 6.6 ROC curves for the biomarker panel

Figure 6.6. The AUROCs for VAP were 0.98, ventilated non-VAP 0.95 and non-ventilated control (NVC) 0.94.
<table>
<thead>
<tr>
<th></th>
<th>VAP</th>
<th>Non-VAP</th>
<th>NVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area under ROC</td>
<td>0.98 (0.96-1.00)</td>
<td>0.95 (0.91-0.99)</td>
<td>0.94 (0.89-0.99)</td>
</tr>
<tr>
<td>curve (AUC) and 95% CI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>95</td>
<td>97</td>
<td>84</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>93</td>
<td>84</td>
<td>97</td>
</tr>
<tr>
<td>Likelihood ratio</td>
<td>12.9</td>
<td>6.3</td>
<td>25.2</td>
</tr>
</tbody>
</table>

**Table 6.4**. The AUROCs are high for the biomarker panel, as are the sensitivities, specificities and the positive likelihood ratios. The data was analysed using Fisher Discriminant Analysis.
The analysis was redone using stepwise forward linear regression. The functions identified were based on the following markers: CRP (blood), IL-6 (blood), sTREM-1 (BAL), IL-1β (BAL), IL-8 (BAL), mTREM-1 (BAL/blood ratio) and mCD11b (BAL/blood ratio). This was an identical to the 7-marker panel generated using Fisher analysis, confirming its validity.

The data for this analysis is shown in Table 6.5. The model was built up one marker at a time. The optimal first marker was mTREM-1 (BAL/blood). Additions to this (in order) were IL-8 (BAL), mCD11b (BAL/blood), sTREM-1 (BAL), IL-6 (blood), IL-1β (BAL) and CRP (blood). After the seventh analyte was added, no further improvement in the model occurred (data not shown). Addition of each additional marker up to the seventh did improve the accuracy of the model however.
Table 6.5. Summary data for stepwise forward linear regression analysis

<table>
<thead>
<tr>
<th>Model</th>
<th>R</th>
<th>$R^2$</th>
<th>Adjusted $R^2$</th>
<th>SE of estimate</th>
<th>$R^2$ change</th>
<th>$F$ change</th>
<th>df1</th>
<th>df2</th>
<th>Sig $F$ change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.527&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.277</td>
<td>0.265</td>
<td>0.430</td>
<td>0.277</td>
<td>22.276</td>
<td>1</td>
<td>58</td>
<td>0.000</td>
</tr>
<tr>
<td>2</td>
<td>0.697&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.485</td>
<td>0.467</td>
<td>0.366</td>
<td>0.208</td>
<td>23.028</td>
<td>1</td>
<td>57</td>
<td>0.000</td>
</tr>
<tr>
<td>3</td>
<td>0.750&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.562</td>
<td>0.539</td>
<td>0.341</td>
<td>0.077</td>
<td>9.836</td>
<td>1</td>
<td>56</td>
<td>0.003</td>
</tr>
<tr>
<td>4</td>
<td>0.795&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.631</td>
<td>0.605</td>
<td>0.315</td>
<td>0.069</td>
<td>10.330</td>
<td>1</td>
<td>55</td>
<td>0.002</td>
</tr>
<tr>
<td>5</td>
<td>0.819&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.671</td>
<td>0.640</td>
<td>0.301</td>
<td>0.039</td>
<td>6.445</td>
<td>1</td>
<td>54</td>
<td>0.014</td>
</tr>
<tr>
<td>6</td>
<td>0.839&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.702</td>
<td>0.688</td>
<td>0.289</td>
<td>0.031</td>
<td>5.524</td>
<td>1</td>
<td>53</td>
<td>0.023</td>
</tr>
<tr>
<td>7</td>
<td>0.853&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.727</td>
<td>0.690</td>
<td>0.279</td>
<td>0.025</td>
<td>4.811</td>
<td>1</td>
<td>52</td>
<td>0.033</td>
</tr>
</tbody>
</table>

Table 6.5. Differentiation of VAP from non-VAP. The functions used in the analysis are: (a) mTREM-1 (BAL/blood); (b) As (a) plus IL-8 (BAL); (c) as (b) plus mCD11b (BAL/blood); (d) as (c) plus sTREM-1 (BAL); (e) as (d) plus IL-6 (blood); (f) as (e) plus IL-1β (BAL); (g) as (f) plus CRP (blood). $R^2$ denotes the coefficient of determination and determines how well the data fits a straight line. The adjusted $R^2$ has the same meaning but is independent of the number of data points. $R^2$ change shows the difference of $R^2$ from the preceding model to the next. $F$ change shows whether the addition of a function improved the accuracy of the model (the higher the better). The df1 and df2 represent degrees of freedom. ‘Sig $F$ change’ demonstrates whether the improvement in accuracy by addition of a function is significant or not.
By comparing the true values of the analyte functions with that predicted by the model for the patient groups, ‘residuals’ (the difference between observed and expected) are generated. These may be plotted and should approximate to a normal distribution if the model is correct. The data is presented in figure 6.7 and fits a bell-shaped curve.

**Figure 6.6 Frequency plot of regression standardised residual**

![Histogram](image)

**Figure 6.7.** ‘Residuals’ are the difference between the observed data and that predicted by the model generated by linear regression analysis. The plot should approximate to a normal distribution to confirm that the model correctly fits the data. As can be seen, the data fits the bell shaped curve.
Similarly, the P-P plot of cumulative predicted and observed probabilities based on the residuals should be linear (figure 6.8). The results shown for VAP and non-VAP groups conform to this. Overall, if a ROC curve was to be constructed for the ability of the panel to classify patient to VAP or non-VAP, the AUC would be 0.997.

**Figure 6.8 Probability-probability plot (linear regression analysis)**

![Normal P-P Plot of Regression Standardized Residual](image)

**Figure 6.8.** Probability-Probability (P-P) plot for observed versus expected cumulative probabilities as determined by linear regression analysis. ‘Residual’ refers to the difference between the observed data and that predicted by the model. The linearity confirms that the model has validity.
6.5. Discussion

A biomarker panel was constructed and its utility in classifying patients into VAP, ventilated non-VAP and non-ventilated controls determined. The panel does not take into account the clinical state of the patient. A panel used in reality would be used in ventilated patients with suspected VAP. Therefore it would not be required to classify patients into non-ventilated control. The utility of the panel can thus be seen to be high, correctly classifying all of the non-VAP (ventilated) patients.

Given the limited resources, the panel was not tested on new patients and therefore statistical methods were used to confirm the robustness of the panel. This could be considered to be a limitation of the study. The ‘leave one out’ and use of training/validation cohorts confirmed a minimal effect of outliers. However, the limitation was that the validation cohorts became small (40% of 91 patients) and therefore the results of the cross-validation showed some variability. A further method of assessing the panel was to remove analytes to leave a 6-marker and a 5-marker panel. It could be seen that the utility diminished as this was done and it was concluded that a 7-marker panel was optimal. Finally, we re-analysed the data using stepwise forward linear regression. This correlated with our original analysis. The linear regression model used stepwise forward analysis to add analytes in sequence. Each analyte was kept in the model if it significantly improved predictive accuracy. Adding the seven markers we identified with Fisher analysis in turn improved the model, but addition of an eighth marker onwards did not.

The panel includes the levels of IL-1β and IL-8 in BAL. This is in accordance with data from Conway-Morris et al (2009), who have seen increased levels of these analytes in BAL in VAP [14].

Clinically, there is a debate as to whether it is feasible to measure a large number of analytes simultaneously in all patients with suspected VAP. Larger studies may elucidate a biomarker panel with a smaller number of markers to rule-in VAP, or act as a rule-out test. Furthermore, once a panel of markers has been identified, simpler methods of analysis may allow testing to be performed more cheaply.
6.6. Conclusions

We have successfully developed a 7-marker bioscore that accurately classifies patients with VAP and non-VAP. Internal validity is strong.

Such a panel may offer the potential to diagnose or refute patients with VAP in a complex clinical environment. We wish to test this biomarker in the clinical setting with new patients with suspected VAP to determine its clinical utility.
Chapter 7

Discussion

7.1. Introduction

This study has examined a combination of soluble and surface biomarkers in the blood and pulmonary compartments, alone and in combination to discriminate patients with VAP and non-VAP. The results address a potential weakness of previous studies which have measured only soluble mediators, often in one compartment, and therefore may not appreciate the dynamic interaction between cell surface receptors and their soluble counterparts (e.g. mTREM-1 and sTREM-1 respectively), and site-specific flux between the alveoli and blood [48]. There is a need for reliable biomarkers in VAP in order to speed up diagnosis and allow increased antibiotic stewardship. In this regard, biomarkers such as those identified by us could provide a same-day result, a significant advantage to the treating clinician [148, 149].

7.2. Standalone markers

The standalone markers have been investigated for their utility in discriminating VAP from the other ventilated and non-ventilated groups. The conventional markers CRP and WCC have limited utility in this regard as does PCT. The blood markers did not differentiate VAP from the other groups, suggesting VAP to involve compartmentalization. In this regard, sTREM-1, IL-1β and IL-8 are elevated in the BALF. The sTREM-1 data agrees with Gibot et al (2004) and Determann et al (2005) but differs from Song et al (2007), Oudhuis et al (2009), Anand et al (2008) and Conway-Morris et al (2009) in being elevated in VAP. Reasons for this include assay differences, patient selection, prior thawing/re-freezing of samples in other studies, VAP definitions and method of sample acquisition [14, 100-102, 104, 105]. Gibot et al (2004) used immunoblotting to detect sTREM-1 and BAL levels were not corrected for BALF dilution. Samples were acquired via mini-BAL rather than BAL [100]. Their cutoff level was 5 pg/ml in comparison to ours (13.6 µg/ml with dilutional correction). Determann et al (2005) used non-directed BAL but
used an ELISA technique to measure sTREM-1 as with our study. No correction for BALF dilution was made and microbiological culture was quantitative [101]. Their cutoff level was 200 pg/ml. Song et al (2007) used blind BAL to assess sTREM-1 (by ELISA) in patients with Pseudomonas Aeruginosa VAP only [102]. The studies by Horonenko et al (2007) and Anand et al (2008) were performed in medical ICUs only, though used quantitative culture of BAL [103, 104]. Huh et al (2008) examined sTREM-1 levels in patients with bilateral lung infiltrates only, by means of non-directed BAL [107]. Finally, Oudhuis et al (2009) used quantitative culture of BALF, but corrected for urea dilution [105].

To our knowledge, the expression of surface TREM-1 has not been assessed before as a diagnostic tool for VAP. Measurement of surface markers in the blood and BAL in VAP and other disease states is feasible and yields diagnostic information. The monocytic surface receptor mTREM-1 and its neutrophilic counterpart nTREM-1 are compartmentalized within the lung, with increased expression in VAP. The results are in agreement with increased mTREM-1 levels in a small cohort of patients with CAP [137].

The surface receptor mTREM-1 levels in the lung were surprisingly lower than in the blood, with BALF/blood ratios less than 1. This is likely due to increased shedding of up-regulated BALF mTREM-1 within the lung, as evidenced by the significantly greater increase in soluble sTREM-1 compared with blood. A potential mechanism is likely to involve the balance between bacterial induced metalloproteinase (MMP) mediated cleavage of cleavage of TREM-1 from surface of monocytes / neutrophils and action of specific MMP inhibitors [45]. Moreover, neutrophil derived MMP production is seen to increase markedly in BALF as compared to plasma from patients with hospital acquired pneumonia, whereas the specific tissue inhibitors of MMP (TIMP) were increased in plasma compared to BALF [126]. In our study, the MMP levels did not differ between the groups. However, the assays were only performed on a subset of patients from the cohort. Samples had also been stored for 2-3 years (though had not previously been thawed), but this should not have affected the results [150]. A further caveat is that MMP activity levels may vary rendering interpretation of absolute levels difficult [126]. We did not
perform functional assays of MMP-8 and MMP-9 activity, but this could be considered in a future study. mTREM-1 and nTREM-1 levels may be affected by recruitment of cells from the bone marrow and by differential apoptotic effects between groups, neither of which have been examined in this study. In particular mTREM-1 activation leads to a reduction in cell apoptosis [151].

7.3. Monocytes versus neutrophils

The BALF/blood ratios of neutrophil-based nTREM-1 and nCD11b were not raised in VAP, in contrast to their monocytic counterparts. Reasons for this were explored in the chapter on temporal changes. It appears that there are rises in nTREM-1, but in many patients these are of a lower magnitude than for mTREM-1, thus failing to show a significant difference when the data is pooled. The timings of the rise appeared different too. This difference is consistent with data from patients in septic shock, in whom blood mTREM-1 but not nTREM-1 levels increased compared with controls [139]. Expression of TREM-1 on neutrophils initially falls over minutes and then increases following in vitro LPS stimulation. In contrast TREM-1 levels on monocytes steadily increase over hours. It is therefore possible that the recruitment timescale for the main study missed early neutrophilic changes [46]. One way of assessing further the dynamic interaction between surface receptor expression and receptor shedding may be to assay the receptors in the presence of protease inhibitors. Absolute sTREM-1 release from receptor shedding is likely to be skewed by the large differential in cell numbers between neutrophils and macrophages, with far greater numbers of the former [152].

7.4. BAL / blood ratio

Our assays simultaneously measured blood and BAL levels of biomarkers to calculate a BAL / blood ratio. We believe that the BAL / blood ratio allows the opportunity to correct for variable levels of expression that occur naturally between patients and would otherwise increase inter-patient variation. This is
particularly demonstrated by mTREM-1, where the BAL / blood ratio resulted in a further improvement in diagnostic classification. Indeed, the BALF/blood ratio of mTREM-1, mCD11b and sTREM-1 were significantly higher in patients with VAP compared with those without VAP suggesting that these biomarkers may be useful indicators of site-specific infections. Others have found elevated BALF IL-1β and IL-6 levels in VAP, when using a lung to blood ratio like in this study [92]. The BALF/blood ratios of soluble cytokines were non-discriminatory in this study, perhaps due to significant compartmentalization by the time of sampling, producing very low blood levels.

7.5. Site-specific diagnosis of infection

The blood levels of the biomarkers were insufficient to discriminate between the patient groups; pulmonary sampling was required. The implication of this is that a simple blood test to diagnose VAP may be unlikely and that alveolar sampling is required, which is potentially not as straightforward. Our study identified markers such as the BALF/blood ratio of mTREM-1 which were able to distinguish VAP from non-pulmonary sepsis. In a complex ICU patient, the ability to differentiate these conditions may have significant clinical value. In previous studies, it has been shown that pulmonary infection may be distinguished from abdominal infection by combining BALF sTREM-1 and blood Procalcitonin measurement, although with lesser discrimination than our use of combined cell surface/soluble markers [82]. More recently, Ramirez et al reported the discriminative ability of site-sampled sTREM-1 for identifying pulmonary from abdominal infection as a cause for respiratory failure in a critically ill cohort [153]. Analysis of site-specific inflammatory markers may be useful in distinguishing pulmonary from non-pulmonary infection, although unlike cellular markers of infection, there may be greater variability in measurement of soluble proteins due to the need to correct for dilution following BALF sampling. We wish to address this aspect of the study further in future studies, with a larger number of patients with non-pulmonary sepsis.
7.6. Biomarker panel

Given that VAP may be caused by a variety of micro-organisms, with the potential for variable activation of pulmonary immune markers, we sought to construct a panel of analytes that could be used to identify VAP. The study demonstrates that a combination of cell surface and soluble markers of inflammation, in particular TREM-1, sampled in blood and BALF simultaneously, can discriminate VAP from ventilated patients without evidence of pulmonary sepsis accurately. The panel was used to classify patients into VAP, ventilated non-VAP or non-ventilated control without prior knowledge of the clinical state of the patient. It correctly classified all of the ventilated non-VAP patients.

Given the limited timescale, the panel was not tested on new patients. Statistical methods confirmed that the internal validity of the panel was strong.

The 'leave one out' analysis demonstrated a minimal effect of outliers. The training/validation cohorts demonstrated the panel to be robust. However, the limitation was that the validation cohorts became small (40% of 91 patients) and therefore the results of the cross-validation showed some variability.

A further method of assessing the panel was to remove analytes to leave a 6-marker and a 5-marker panel. It could be seen that the utility diminished as this was done and it was concluded that a 7-marker panel was optimal.

Finally, we re-analysed the data using stepwise forward linear regression. This correlated with our original analysis. Significantly, the panel included the levels of IL-1β and IL-8 in BAL. This is in accordance with data from Conway-Morris et al (2009), who have seen increased levels of these analytes in BAL in VAP [14].

Clinically, there is debate as to whether it is feasible and worthwhile to measure a large number of analytes simultaneously in all patients with suspected VAP. This would depend on how accurate the panel was in terms of positive and negative predictive values. Larger studies may elucidate a biomarker panel with a smaller number of markers to rule-in VAP, or act as a
rule-out test. Furthermore, once a panel of markers has been identified, simpler methods of analysis may allow testing to be performed more cheaply.

In summary, we have identified a panel that can differentiate VAP from non-VAP and non-ventilated controls. We wish to investigate this further in an unselected cohort of patients with suspected VAP.

**7.7. Temporal changes in mTREM-1 and nTREM-1**

A putative biomarker ought to rise early with infection and fall with resolution of the illness. We identified BAL mTREM-1 and the BAL/blood ratio of mTREM-1 as potential diagnostic biomarkers for VAP and therefore sought to understand changes in their levels over time. It would also allow comparison of the monocyte and neutrophil receptors to understand their relevance for sampling at a single point of time after 48 hours of ventilation, which would be when clinical suspicion of VAP would occur. We sampled a cohort of patients prior to VAP development, during resolution of VAP, during worsening of VAP and a combination of all three of these in one patient. Peripheral blood WCC levels did not correlate well with development and resolution of infection.

BAL mTREM-1 levels and the BAL/blood ratio generally correlated with the clinical status of the patient. We would like to investigate this further in future studies with larger numbers of patients as temporal changes have implications for sampling at one time point and comparing with a diagnostic threshold level.

There was a difference between the data for mTREM-1 and nTREM-1. nTREM-1 levels may peak at an earlier timeframe than mTREM-1 following cell activation [42]. Both receptors levels are also a balance between expression and cleavage to form the soluble receptor.

Finally, some patients (PS72, PS81, PS86 and PS87) had evidence of raised BAL mTREM-1 and nTREM-1 even though they had not yet got clinical infection. This suggests activation of these receptors prior to VAP diagnosis. There is controversy over VAP diagnosis in that it is diagnosed 48 hours or
more after intubation. This is to differentiate it from infection that is already present or is developing prior to intubation. The temporal analysis would be consistent with pulmonary immune system activation in the development of VAP (subclinical VAP) and prior to clinical manifestations. If the VAP process started before 48 hours, nTREM-1 may have risen and then started to fall prior to clinical suspicion and therefore biomarker sampling. In this regard, mTREM-1 may therefore be a more suitable biomarker, as we have seen with the study.

7.8. The ideal biomarker

Sackett and Haynes (2002) highlighted that a diagnostic biomarker ought to differentiate cases from healthy controls [62]; Increased levels should be associated with a higher probability of disease; levels should distinguish positive cases from negative ones in a cohort of suspected cases; finally, measurement and implementation of the biomarker should improve outcomes. Morrow and de Lemos (2007) suggested that an ideal biomarker should be easy to measure, add new information not obtained clinically or by other tests and aid patient management [58]. We assessed the success of our studies against these benchmarks. Firstly, sTREM-1 and mTREM-1 in the blood of patients with CAP was significantly higher than healthy controls. Considering VAP, several markers were raised compared with non-ventilated controls. The BAL mTREM-1 and BAL/blood mTREM-1 were raised in VAP. However, the level of expression of mTREM-1 or the ratio did not correlate with the severity of VAP per se. We wish to investigate the markers in a cohort of patients with suspected VAP.

In terms of ease of measurement, each assay was straightforward to conduct. As previously described, there is debate as to the expense and ease of measuring multiple simultaneous markers in patients. However, our study suggested blood markers on their own to lack utility. It is likely that in a complex clinical condition such as VAP, measurement of multiple markers will be required. This will be justified if their clinical utility is high (for which our study provides encouragement). Once our biomarker panel has been tested in
further patients, if the markers still retain diagnostic utility, they can be assessed to determine their ability to improve patient management and antibiotic stewardship.

7.9. The cellular immune response in VAP

Our study has focused on the changes in surface expression of receptors on monocytes and neutrophils. However, other changes occur in VAP that we did not address and could be examined in future studies. Pelekanou and colleagues (2009) found that in VAP, there was a reduction in peripheral blood CD4 positive T-cells in VAP, an increase in monocyte apoptosis and a reduction in monocyte responsiveness to stimulation, which may be associated with endotoxin tolerance [154].

7.10. Pathogenesis of VAP

A number of the biomarkers were significantly raised in VAP. Perera et al (2001) discussed that CD11b interacted with TLR and CD14 (monocyte receptor) to activate the cell in response to LPS stimulation [56]. Anas et al (2010) highlighted the crucial role of CD14 and macrophages in the development of pneumonia [155]. We constructed a putative model (figure 7.1) to link together several of these cytokines and receptors. A monocyte may migrate from the blood to the lung and in doing so sheds mCD62L. It possesses mCD11b, mTREM-1 and Toll-like receptors. Activation of the cell by TLR and mTREM-1 ligation allows increased mTREM-1 expression, increased mTREM-1 cleavage resulting in sTREM-1 secretion (which also appears from nTREM-1) and potentially increased mCD11b expression. Surface TREM-1 may act as a link in the pathway from infective organism, to upregulation of the inflammatory cytokines. Experimentally, mTREM-1 activation in conjunction with lipopolysaccharide (LPS) increases IL-8 and IL-1β release, with amplification seen in septic shock, as in our study [42, 156]. Differences may occur depending on whether the infection was gram-positive, gram-negative or fungal (or viral). mTREM-2 may modify the immune
response as it has effects in polymicrobial sepsis to promote bacterial clearance [140]. Many of these effects may occur with neutrophils too. Klesney-Tait et al (2013) used TREM-1 knockout mice to demonstrate that TREM-1 has a crucial role in allowing transepithelial migration of neutrophils from the blood to the lung. Mice lacking the TREM-1 gene had a significantly lower level of polymorph migration in response to bacterial challenge [157].

The crucial role of TREM-1 in modulation of sepsis has been tested experimentally by its blockade. In studies by Gibot et al (2006) and Wang et al (2012) animal models of pseudomonal infection (pneumonia and sepsis respectively) were used to demonstrate that TREM-1 abrogation by means of blocking peptides or antibodies reduced the severity of infection and protected animals from lethality [158]. However, blockade may also increase the severity of pneumonia. Lagler et al (2009) blocked TREM-1 activation in mice with pneumococcal pneumonia. Sepsis was worsened by TREM-1 inhibition and the researchers cautioned against the use of TREM-1 blockade to modulate sepsis [159]. Two reasons may explain the discrepant results. The first is that there are differences between the pathogenesis of gram-positive and gram-negative infection, with TREM-1 blockade favouring gram-negative (pseudomonal) infection but worsening gram-positive (pneumococcal) sepsis. The second relies on the strength of the immune response. TREM-1 amplifies the immune response to infection. If, in the pseudomonal models, the sepsis and SIRS response was overwhelming then TREM-1 abrogation may have attenuated the inflammatory response, increasing survival. On the other hand, if the immune response to pneumococcus was merely adequate but not overwhelming, blockade of TREM-1 may have made the response insufficient to treat the infection and increased lethality. Overall, modulation of TREM-1 may provide a therapeutic avenue for septic patients, such as those with VAP but a better understanding of its role is required.
Figure 7.1. Putative model for pulmonary compartment in VAP

The monocyte possesses CD11b and TREM-1 surface receptors. In some cases, activation occurs, in conjunction with ligation of a toll-like receptor. This may lead to increased CD11b expression and increased surface TREM-1 levels. Cleavage of the latter by Matrix metalloproteinases releases sTREM-1. Activation of the surface receptors, in particular mTREM-1 releases IL-1β and IL-8. TREM-2 may have a role in modification of the immune response.
7.11. Monocyte subsets

mTREM-1 predominates on CD14 an CD16 positive macrophages [160, 161]. For the study, mTREM-1 levels were assessed on the global monocyte population of CD14 positive cells. However, it is possible that the effects seen were restricted to one or more subset populations of cells. Future studies may utilise further monocyte markers to differentiate these populations and allow measurement of mTREM-1 on each type of cell.

7.12. Problems encountered during the study

The initial phase of the study sought to develop assays to measure sTREM-1 and surface TREM-1 (mTREM-1 and nTREM-1). Predecessors had attempted to use in-house ELISA kits developed with manufacturer antibodies. However, this was unsuccessful with failure to create reproducible standard curves. One reason for this may have been the quality of the purchased antibodies, which were recalled from supply approximately three months after the commencement of my study [106]. We decided against using in-house kits and purchased ready to use ELISA plates and reagents prior to the recall of the other antibodies; our kits were not affected. The flow cytometry assays were developed ourselves over the first 3–4 months of the study.

A major problem encountered was an outbreak of the multi-resistant bacterium Acinetobacter Baumannii on the ICU at Chelsea and Westminster. This led to a curtailment of admissions to ICU for approximately 6 months and a resultant decline in the ability to recruit ICU patients into the study. The study was thus extended by a further period of 9 months – 1 year to allow sufficient recruitment.

The final issue to discuss was that during the period of the study, only semi-quantitative microbiological analysis was available. Quantitative analysis only became available after completion of the study. We believe that our results would have been similar if quantitative analysis had been used, as we found elevated levels of IL-1β and IL-8 in the BAL of patients with VAP. This was in accordance with a study from a group using quantitative analysis [14].
7.13. Limitations

We included patients, who based on the CPIS scoring system, plus semi quantitative microbiological testing were highly likely to have the presence or absence of VAP in order to test putative biomarkers. The new CDC guidelines for identifying patients with VAP for surveillance control accept semi-quantitative and quantitative cultures [28]. The diagnostic definitions used were also concordant when the European HELICS criteria for pneumonia were used [32]. Yet, in spite of utilizing standard criteria, the limits of these definitions for accurate diagnosis of VAP are recognised, because of such factors as sampling site variation, and prior antibiotic usage [162]. Reassuringly, from a biological perspective, the raised BAL IL-1β and IL-8 levels in VAP from our study concur with a group utilizing different diagnostic methodology, implying the validity to such approaches [14].

Second, our study did not encompass the whole range of infective aetiologies. For instance, no patients had Legionella pneumonia and few had bilateral lung infiltrates. The latter group present great diagnostic challenge and would benefit most from a suitable biomarker. An increased number of patients with acute lung injury could have influenced the results. Another practical consideration is that flow cytometry is a specialized technique. It requires samples with sufficient numbers of cells, which mandates invasive bronchoscopy and makes serial biomarker analysis challenging. However, BALF samples are the current standard of care a microbiological diagnosis of VAP and to our knowledge there were no adverse events associated with bronchoscopy [27, 163, 164].

Another consideration is the number of patients receiving antibiotics and steroids, with potential immunomodulatory activity. Given the prevalence of these key standard interventions in critically ill patients, we believe this pragmatic approach enhances the applicability of the findings. Finally, the immune response to infecting pathogens in VAP, as in sepsis, is likely to involve neutrophils, monocytes and lymphocytes [165]. As such we have not necessarily looked at all potentially relevant phagocytic or T cell markers. That said, the value of biomarker panels that include sTREM-1, PCT and CD64 on
neutrophils has recently demonstrated the ability to predict sepsis in the setting of unselected critical illness, confirming the need to pursue such discriminatory panels in VAP, as in other disease states [165, 166].

7.14. Future studies

(i) Use quantitative microbiology for sample analysis

(ii) Measure mTREM-1, nTREM-1 and sTREM-1 in the presence of metalloproteinase inhibitors to determine the role of receptor shedding.

(iii) Determine the role of TREM-2 in a larger number of patients with and without VAP.

(iii) Study the role of T-cells in the pathogenesis of VAP.

(iv) Measure mTREM-1, nTREM-1 and sTREM-1 in other body fluids to calculate a tissue/blood ratio and determine if this is increased in infection.

(v) Determine the role of TREM-1 on different macrophage subsets in VAP.

(vi) Assess the biomarker panel in a new cohort of patients with suspected VAP.

(vii) Measure changes in mTREM-1, nTREM-1 and sTREM-1 in cells stimulated ex vivo with fungal (candidal) antigens to better understand the role of TREM-1 in fungal VAP.

(viii) Stimulate BAL macrophages and neutrophils ex vivo with LPS and LTA and determine the profile of mTREM-1, nTREM-1 and sTREM-1 expression over time to simulate VAP.

(ix) Use luminex analysis to measure analyte levels.

7.15. Summary

In conclusion, a 7 biomarker panel compromising of soluble and cell-surface inflammatory markers including TREM-1 in combination with BALF/blood ratio differentiates VAP from non-pulmonary infection with high diagnostic
accuracy. Further prospective multi-center studies are needed, that incorporate these practically relevant and easily measurable biomarkers, to confirm the value of such a diagnostic bioscore in suspected VAP.
Appendix A

Approval letter from NRES
09 September 2008

Dr Suveer Singh
Consultant Intensivist & Pulmonary physician
Chelsea and Westminster Hospital NHS Trust
Intensive Care Unit
Chelsea & Westminster Hospital
369, Fulham Road, LONDON
SW10 9NH

Dear Dr Singh,

Full title of study: Evaluation of the expression of surface TREM-1 (Triggered Receptor Expressed on Myeloid cells and soluble TREM-1 in blood and bronchoalveolar lavage samples in patients admitted to intensive care, patients with chronic lung disease and patients with inhalational burn injuries to determine its value as a marker of infection

REC reference number: 08/H0702/61

The Research Ethics Committee reviewed the above application at the meeting held on 03 September 2008. Thank you for attending to discuss the study.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

[Other conditions specified by the REC – optional]
Approved documents

The documents reviewed and approved at the meeting were:

<table>
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<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<td>02 July 2008</td>
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<td>Protocol</td>
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<td>14 August 2008</td>
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<td>Covering Letter</td>
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<td>Peer Review</td>
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<td>23 August 2008</td>
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<td>03 July 2008</td>
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<tr>
<td>Participant Information Sheet</td>
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<td>03 July 2008</td>
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<tr>
<td>Participant Consent Form</td>
<td></td>
<td>03 July 2008</td>
</tr>
<tr>
<td>Patient Information Sheet for adult volunteers (non ventilated)</td>
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<td>03 July 2008</td>
</tr>
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Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

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.

After ethical review

Now that you have completed the application process, please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0702/61 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project
Yours sincerely

[Signature]

Mrs JR IrwinHunt
Chair

Email: janet.carter@redbridge-pct.nhs.uk

Enclosures:

List of names and professions of members who were present at the meeting
"After ethical review - guidance for researchers"
Site approval form (SF1)
Barking and Havering Local Research Ethics Committee

Attendance at Committee meeting on 03 September 2008

<table>
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<tr>
<td>Mrs. J. IrwinHunt</td>
<td>Chair</td>
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<tr>
<td>Mrs. T. Bland</td>
<td>Lay Member</td>
</tr>
<tr>
<td>Mr. C. Chowdhury</td>
<td>ENT Consultant</td>
</tr>
<tr>
<td>Dr. D. Hollanders</td>
<td>Consultant Physician</td>
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<tr>
<td>Mr. A. Jabbari</td>
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<tr>
<td>Dr. A. Mohan</td>
<td>GP</td>
</tr>
<tr>
<td>Miss E. Visentin</td>
<td>Research Nurse *</td>
</tr>
<tr>
<td>Janett Carter</td>
<td>Administrator</td>
</tr>
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</table>
Barking and Havering Local Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, including the new sites approved.

<table>
<thead>
<tr>
<th>REC reference number:</th>
<th>08/H0702/61</th>
<th>Issue number:</th>
<th>0</th>
<th>Date of issue:</th>
<th>09 September 2008</th>
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**Chief Investigator:**
Dr Suvir Singh

**Full title of study:**
Evaluation of the expression of surface TREM-1 (Triggered Receptor Expressed on Myeloid cells and soluble TREM-1 in blood and bronchoalveolar lavage samples in patients admitted to intensive care, patients with chronic lung disease and patients with inhalational burn injuries to determine its value as a marker of infection.

This study was given a favourable ethical opinion by Barking and Havering Local Research Ethics Committee on 03 September 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

<table>
<thead>
<tr>
<th>Principal Investigator</th>
<th>Post</th>
<th>Research site</th>
<th>Site assessor</th>
<th>Date of favourable opinion for this site</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>Dr. Suvir Singh</td>
<td>Post Consultant Innsitve &amp; Pulmonary Physician</td>
<td>Research site Chelsea &amp; Westminster Hospital NHS Trust</td>
<td>Site assessor Barking &amp; Havering REC</td>
<td>Date of favourable opinion for this site 3rd September 2008</td>
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Approved by the Chair on behalf of the REC:

[Signature of Chair/Co-ordinator]

(delete as applicable)

[Name]
19 November 2008

Dr Suveer Singh
Consultant Intensivist & Pulmonary physician
Intensive Care Unit
Chelsea & Westminster Hospital
369, Fulham Road, LONDON
SW10 9NH

Dear Dr Singh

Study title: Evaluation of the expression of surface TREM-1 (Triggered Receptor Expressed on Myeloid cells and soluble TREM-1 in blood and bronchoalveolar lavage samples in patients admitted to intensive care, patients with chronic lung disease and patients with inhalational burn injuries to determine its value as a marker of infection

REC reference: 08/H0702/61
Protocol number: 1
Amendment number: 1
Amendment date: 11 November 2008

Thank you for your letter of 12 November 2008, notifying the Committee of the above amendment.

The amendment has been considered by the Chair on the 18th November 2008 and has and approved by Chair's Action on the 18th November 2008.

The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<td>CV Dr Vimal Grover</td>
<td>1</td>
<td>11 November 2008</td>
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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.

Yours sincerely

Janett Carter  
Committee Co-ordinator

E-mail: janet.carter@redbridge-oct.nhs.uk
Appendix B

Research and Development letter of approval from Chelsea and Westminster Hospital
Contract of Research and Development Approval (Non-IMP)

Between

Dr Suveer Singh
Chelsea and Westminster NHS Foundation Trust
369 Fulham Road
LONDON
SW10 9NH

and

Chelsea and Westminster NHS Foundation Trust
369 Fulham Road
LONDON
SW10 9NH

The Principal Investigator

The Trust

in respect to the study:

**Protocol Reference (where applicable):** NA

**Study Title:**

Evaluation of the expression of surface TREM-1 (Triggered Receptor Expressed on Myeloid cells and soluble TREM-1 in blood and bronchoalveolar lavage samples in patients admitted to intensive care, patients with chronic lung disease and patients with inhalational burn injuries to determine its value as a marker of infection

**R&D Reference Number:** ANA09001CN

**Research Ethics Number:** 08/H0702/61

**Date of Issue:** August 19, 2009

Please quote the above reference numbers in any communications relating to this project.

In Acceptance of this Research and Development Approval, The Principal Investigator agrees to the Following:

<table>
<thead>
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<th>Principal Investigator – Please Initial</th>
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<tbody>
<tr>
<td>Although not a CTIMP trial I agree to uphold the principles of Good Clinical Practice throughout all conduct and documentation relating to this trial, including ensuring all the Study Team are trained to perform the tasks they are delegated, and that any delegation of PI responsibility is recorded appropriately</td>
</tr>
<tr>
<td>Report all Serious Protocol Deviations to the Research and Development Support Office, in accordance with the Research and Development Support Office Standard Operating Procedure OP10</td>
</tr>
<tr>
<td>Undertake project monitoring, in accordance with the monitoring plan for your research study</td>
</tr>
<tr>
<td>To keep the Research and Development Support Office informed of all:</td>
</tr>
<tr>
<td>• Amendments to study protocols</td>
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<tr>
<td>• Amendments to financial arrangements</td>
</tr>
<tr>
<td>• Changes to Study Team</td>
</tr>
<tr>
<td>In accordance with the Human Tissue Act 2004 – I agree to destroy all tissue samples, as described in the Act, upon completion of the research project for which they were specifically approved by the Research Ethics Committee</td>
</tr>
<tr>
<td>Where this study is on the UKCRN Portfolio, I agree to enter accrual data on the portfolio within the timeframes set by the UKCRN</td>
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</table>

Christopher Braime – Interim Research Governance Manager
Version 0.1 – Thursday March 26th 2009
E: RESEARCH APPROVAL LETTERS\Anaesthetics\2009\20090819- ANA09001CN.doc
To undertake R&D quarterly centralised project monitoring within the timeframes set by the Research and Development Support Office

I agree to notify the Research and Development Support Office when the study closes

I agree to the following responsibilities, as set out in the Research Governance Framework for Health and Social Care 2005:

- A senior individual must be designated as PI for any research undertaken within the Trust. This individual will take responsibility for the conduct of the Research and is accountable for this to the Trust, and if appropriate, to the Sponsor
- The PI must have suitable experience and expertise in the design and conduct of research
- Ensure the dignity, rights, safety and well-being of participants are given priority at all times
- Ensure that research is carried out in accordance with the Research Governance Framework
- Ensure that controlled trials are registered
- Ensure that the Chief Executive, or their designate, is informed of all research and where appropriate indemnity is given
- Ensure that studies comply with all legal and ethical requirements
- Ensure that other professionals involved in the care of the research subject are made aware of their participation in the study
- Ensure each member of the research team is adequately qualified
- Ensure all students and new researchers have adequate supervision, support, and training
- Ensure any protocol amendments are approved by the Research Ethics Committee
- Ensure procedures are in place to guarantee the collection and confidentiality of high quality data
- Ensure appropriate archiving of research data
- Make the findings from the research open to critical review after which these are disseminated promptly and fed back to the participants
- Accept a key role in the detection and prevention of scientific misconduct by adopting the role of guarantor on published outputs
- Ensure all data and documentation associated with the study are available for audits
- Ensure arrangements are in place for the management of financial and other resources provided for the study, including the managements of any intellectual property arising

Training Requirements:

<table>
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<th>Member of Study Team Requiring Training</th>
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Trust SOPs / Procedures required for the conduct of this trial:

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<th>Location</th>
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Christopher Braime – Interim Research Governance Manager
Version 0.1 – Thursday March 26th 2009
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<td>[Signature]</td>
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<tr>
<td></td>
<td>DR SUVEER SINGH</td>
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This approval is not valid until both parties have signed, and a copy has been returned by hand to the Research and Development Support Office, who will then activate your project on the ReDA database – at this point, your project is active.
Appendix C

Consent form (ventilated patient)
PATIENT INFORMATION SHEET

Adult volunteer

INVESTIGATORS NAMES: Dr Vimal Grover, Dr. Stefan Gurney, Dr Neil Soni, Dr Suveer Singh
INVESTIGATIONAL SITE: Chelsea and Westminster Hospital

STUDY TITLE

Measurement of surface TREM [Triggered Receptor Expressed on Myeloid cells] levels in specimen collected from lung fluid and blood to detect infection.

INVITATION

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled ‘Medical Research and You’. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, LONDON N16 OBW.

Thank you for taking the time to read this form.

EXPLANATION OF PROJECT:

Ventilator associated Pneumonia (lung infection after being on the lung support machine) is a common cause of prolonged stay on the intensive care unit. It is difficult to diagnose this ventilator associated pneumonia and therefore a laboratory based test to help doctors detect this illness may lead to improvement in the management of this condition.

We are studying patients admitted to the intensive care unit. Measurement of an immune protein called TREM will allow doctors to detect ventilator related pneumonia more accurately and faster than standard tests. We will be using the samples derived from blind bronchial sampling, bronchoscopic alveolar lavage (BAL) and blood taken from you. Some of these procedures are done routinely on patients admitted to the intensive care unit. Blind bronchial sampling and taking blood are done on a daily basis whereas bronchoscopic alveolar lavage (BAL) is undertaken post intubation, when there is a clinical indication.

TREM [Triggered Receptor Expressed on Myeloid cells] is a protein produced in patients who are very ill (i.e. sepsis). It would not be raised in individuals without any infection.

We will be evaluating the significance of TREM secretion in body fluids (BAL & serum) to detect bacterial or fungal infection.

PERIOD OF STUDY

We plan to undertake the study until January 2012. We plan to recruit 100 patients in our study.

Why have I been chosen?

You have been chosen as you are being / were admitted to the intensive care unit during which your breathing is supported by the ventilator (breathing machine)
Procedures undertaken

1. Fibreoptic bronchoscopy test

   This is done after intubation (after you have been put onto the lung support machine) on the intensive care unit depending on clinical requirements. Samples will also be taken for research at those times.

   Bronchoscopy is a routine investigation that allows the doctor to examine directly the large air passage in the lung. You will already be under sedation, as part of your treatment on the Intensive care unit (ICU) and therefore will not notice the procedure. The bronchoscope, which is a narrow flexible instrument, is then passed through the endotracheal tube (ETT), the breathing tube that has already been placed on your admission to the ICU, and into the airways of your lungs. The airways are inspected for any signs of inflammation. Bronchial washings (flushing small quantities of salty water into and out of the lung) are taken. In addition, we will use a thin soft probe with a cotton wool-like tip to obtain a lung fluid sample. This is the end of diagnostic bronchoscopy.

Side effects and possible risks of the procedure

There are small risks associated with having a bronchoscopy and also small risks from having additional samples taken for research studies.

Risks of Bronchoscopy

We monitor your oxygen level throughout the procedure so you are not at risk from low oxygen levels. If there is a fall in oxygen levels, then the procedure is stopped, until oxygen levels recover. If biopsies were taken (which is not the case for this study, although may be part of the routine bronchoscopic investigation), then a rare complication (1:2000) of bronchoscopy is pneumothorax. This is a collapse of the lung, which is treated by inserting a tube through the chest wall, and connecting to a negative pressure bag system, that allows the lungs to expand.

Risk of Taking Research Samples

Bronchoscopic microsampling may cause only minor additional risk. Washing the air passages is also safe since the salty water is sucked out of the lungs as soon as it is put in. A BAL of 20ml to 120ml usually causes no side effect. However in 5% of cases the washing causes a fall in blood oxygen levels. Since your oxygen levels are monitored we would know if you are getting short of oxygen and we would give you more oxygen to breath through the ventilator. We would stop the procedure if this did not improve oxygen levels, until improvement. Taking additional samples will make the bronchoscopy last about 3-5 minutes longer than it would otherwise be. However it is not necessary to give you more sedative or a different sedative from that we ordinarily use and the slightly longer procedure does not mean extra risk.

2. Blind Bronchial Sampling (BBS)

   This is done daily for research purposes.

   This is done by passing a catheter through the ventilation tube and 20ml of normal saline is instilled. The secretions dissolve in the normal saline instilled which is suctioned out. This also helps in clearing the secretions. As this procedure is done not under direct vision i.e. not using a fibre-optic scope, it is termed blind.

   This is quicker to perform than the one assisted by the fibre-optic bronchoscope. Hence, there is decreased risk of complications mentioned earlier.

3. Venesection:

   This is done daily for research purposes. It is done from a fresh site to avoid any bacterial contamination from pre-inserted lines.

   We will be collecting a 6-10mls sample of blood from a peripheral vein whenever a bronchoscopy procedure is undertaken. This is done under strict aseptic precautions. To minimize any discomfort
while performing this procedure, local anaesthetic is instilled before inserting a needle into the vein to draw blood. There is a slight risk of bruising at the site of venepuncture which will subside soon.

4. Ventilator condensate:
The breathing machine (ventilator) has a container attached to the tubing to collect water that condenses from the patient’s exhaled breath. This is normally thrown away. We will obtain 20-40ml of this fluid (maximally on a daily basis) to measure the proteins in it. This will not affect your care in any way and does not require any invasive procedure.

Confidentiality

After obtaining the samples from you, they will be anonymised by giving them encoded reference number. All names and personal data which can identify you with the sample will be discarded.

Use of samples obtained

We will take samples to laboratory and we will measure levels of TREM. The results obtained will be without significance for you personally; i.e. they will not guide your clinical management. The results of the study will be submitted for publication in scientific journals, and they can be made available to you, if you wish to know at the end of the study. Any remaining bronchoscopy specimens will be analyzed for routine measurements of infection, and you will not be identifiable in any reports of the study.

All samples will be destroyed at the end of the study.

If you decide to withdraw from the study after the procedure, and before submission of data for publication in scientific journals, we will discard those samples and data derived from them.

Participation in the study

Finally, we would like to thank you for your interest in the study, even if you have decided not to take part. There is no pressure on you to participate in the study and having agreed to participate you are free to withdraw at any stage without having to provide any reason. Your decision to participate or not will not affect in any way the treatment you may be receiving at this hospital.

There will be no direct benefits to you for taking part in this study. It may allow us to establish the role of TREM in the early diagnosis of the source of infection, in the future.

If you do decide to take part in the study all information relating to the study will be kept confidentially and you will not be identifiable in any reports of the study.

If you need any help or advice at any time following the fibreoptic bronchoscopy procedure, you can contact us at any time. Please call: Dr Suveer Singh (Tel: 020 8746 8472), Consultant Physician in Respiratory and Intensive Care Medicine, Chelsea & Westminster Hospital 369 Fulham Road, London SW10 9NH.

Thank you very much for your kindness in considering this project.
Research Subject Consent Form

Title of Project: Use of surface TREM-1 as a marker in detecting infection

Patient Hospital Number: Patient Study Identification:

Patient Initials:

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Patient/ Volunteer Declaration (please INITIAL all boxes which apply)

I have been given the chance to read and understand the information sheet relating to the above study

I have been given the opportunity to ask questions and discuss the study

I have been made aware of the risks/ benefits

I understand that authorized individuals may look at my medical notes and give permission for these individuals to have access

I understand that I am free to withdraw from this study at any time without prejudice to my future care/treatment

---

Patient Hospital Number: Patient Study Identification

I agree to take part in the above study

Signature ........................................ Name ................................................

Date ........................................................................................................

Person responsible for obtaining Informed Consent:
'To the best of my knowledge I have provided the above individual with sufficient information to enable them to give informed consent'.

Signature ........................................ Name ................................................

Date ........................................................................................................

Position .............................................................................................

Witnessed by:

Signature ........................................ Name ................................................

Date ........................................................................................................

Position .............................................................................................
Appendix D
Consent form (non-ventilated patient)
PATIENT INFORMATION SHEET

Adult volunteer (non ventilated)

INVESTIGATORS NAMES: Dr Vimal Grover, Dr. Stefan Gurney, Dr Neil Soni, Dr Suveer Singh
INVESTIGATIONAL SITE: Chelsea and Westminster Hospital

STUDY TITLE
Measurement of surface TREM [Triggered Receptor Expressed on Myeloid cells] levels in specimen collected from lung fluid and blood to detect infection.

INVITATION
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) publish a leaflet entitled ‘Medical Research and You’. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, LONDON N16 OBW.

Thank you for taking the time to read this form.

EXPLANATION OF PROJECT:
Ventilator associated Pneumonia (lung infection after being on the lung support machine) is a common cause of prolonged stay on the intensive care unit.
We are studying patients admitted to the intensive care unit to evaluate the correlation of TREM secretion in infection. We will be using the samples derived from blind bronchial sampling, bronchoscopic alveolar lavage (BAL) and blood taken from you. Some of these procedures are done routinely on patients admitted to the intensive care unit. Blind bronchial sampling and taking blood are done on a daily basis whereas bronchoscopic alveolar lavage (BAL) is undertaken post intubation, when there is a clinical indication.

You are being invited to participate, because you do NOT have a severe infection, and are undergoing routine bronchoscopy. Thus, we think this will be an important comparison with those patients with severe infections on lung support machines.

TREM [Triggered Receptor Expressed on Myeloid cells] is a substance produced in patients who are very ill (i.e. sepsis). It is produced by the blood cells to enhance the inflammatory response to bacterial or fungal infection. It may not be raised as much in individuals without any infection, but may be increased in other lung conditions.
We will be evaluating the significance of TREM secretion in body fluids (BAL & serum) to detect bacterial or fungal infection.

PERIOD OF STUDY
We plan to undertake the study until January 2012. We plan to recruit 100 patients in our study.

Why have I been chosen?
You have been chosen as you are undergoing routine bronchoscopy to investigate your lungs.
**Procedures undertaken**

1. **Fibreoptic bronchoscopy test**

   *This is done as clinical requirement as part of management of your lung investigations.*

   Bronchoscopy is a routine diagnostic examination that allows the doctor to examine directly the large air passage in the lung. You will be asked not to eat or drink anything from midnight prior to the bronchoscopy. You will be given oxygen to breathe throughout the procedure. A local anaesthetic called lidocaine is then sprayed on to the back of the mouth and into the nose. This anaesthetic numbs the nerves so that the bronchoscope can be easily inserted into the wind passage without discomfort. Lidocaine is the same as the local anaesthetic used by dentists. If you are allergic to any local anaesthetic agents you should let us know and you will be withdrawn from the study. A sedative such as midazolam and/or fentanyl is injected through the vein to make you more relaxed and drowsy. You may be given Ventolin, which is a medicine that will prevent any narrowing of the airway tube, to inhale from a nebuliser, which produces a fine mist for inhalation, after the bronchoscopy.

   The bronchoscope, which is a flexible instrument, is then passed usually through the mouth and down the back of the throat. More local anaesthetic is then placed on the vocal cords and the bronchoscope passed through the voice box and into the lungs. Then airways are inspected for any signs of disease. Bronchial washings (flushing small quantities of salty water into and out of the lung) are taken. In addition, we will use a thin soft probe with a cotton wool-like tip to obtain a lung fluid sample. This is the end of diagnostic bronchoscopy.

   Bronchoscopies will only be performed by a C&WH approved bronchoscopist (Dr Suveer Singh, Dr Pallav Shah and colleagues under their direct supervision).

   This is done under sedation and hence, you would not be subject to any discomfort.

2. **Side effects and possible risks of the procedure**

   There are small risks associated with having a bronchoscopy and also small risks from having additional samples taken for research studies.

   **Risks of Bronchoscopy**

   Passing a tube into the airways sometimes causes a minor nosebleed (although we usually use the oral route, thus avoiding this complication). After your examination, the lining of your mouth and throat will remain numb just in the same way as your mouth would after a dental procedure. You will experience a sore throat and will be coughing because of irritation in the chest. There may also be the possibility of coughing bloody sputum. These symptoms will wear off within the next 2 hours or so. You should not eat or drink for at least 2 - 3 hours. This precaution is necessary to keep food or liquids from accidentally entering the windpipe or lungs. Bronchoscopy is a standard diagnostic procedure. Fewer than one in ten people get a ‘flu’ like reaction about 6 hours after the test sometime with a fever. This only lasts a few hours and can be helped by taking paracetamol. You will have a sore throat for approximately 4 hours after the procedure. There may also be the possibility of coughing up a little blood. Using a mild sedative can cause your breathing to slow down as you become drowsy.

   We monitor your oxygen level throughout the procedure so you are not at risk from low oxygen levels. If there is a fall in oxygen levels, then the procedure is stopped, until oxygen levels recover. If biopsies were taken (which is not the case for this study, although may be part of the routine bronchoscopic investigation), then a rare complication (1:2000) of bronchoscopy is pneumothorax. This is a collapse of the lung, which is treated by inserting a tube through the chest wall, and connecting to a negative pressure bag system, that allows the lungs to expand.

   **Risk of Taking Research Samples**

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There will be no direct benefits to you for taking part in this study. It may allow us to establish the role of TREM in the early diagnosis of the source of infection, in the future.
If you do decide to take part in the study all information relating to the study will be kept confidentially and you will not be identifiable in any reports of the study.
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Date ………………………………………………………………………

Person responsible for obtaining Informed Consent:
'To the best of my knowledge I have provided the above individual with sufficient information to enable them to give informed consent'.

Signature………………………………………………Name……………………………………

Date…………………………………………………………Position…………………………

Witnessed by:

Signature ………………………………………Name ………………………………………

Date ……………………………………………………………Position…………………………
References


40. Ramanathan, B., Minton, J.E., Ross, C.R. & Blecha, F., Cloning of porcine triggering receptor expressed on myeloid cells-1 (TREM-1) and its induction by lipopolysaccharide, peptidoglycan, and Salmonella


