A biomarker-based exclusion of ventilator-associated pneumonia: towards improved antibiotic stewardship.

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Abstract

Ventilator-associated pneumonia (VAP) is a common complicating condition amongst patients mechanically ventilated in the Intensive Care Unit (ICU). It is a common reason for antibiotics to be administered. The diagnosis of VAP is challenging and amongst patients in whom VAP is suspected, approximately a third will have infection confirmed. Therefore many patients receive antibiotics for VAP despite the condition not being present. Antimicrobial resistance (AMR) is a growing global concern and the overuse of antibiotics is an important factor in increasing AMR. The ICU is an environment with high antibiotic use and improving antibiotic stewardship is a priority. Rapid biomarker-based diagnostics could achieve this by expediting the diagnostic process.

In this thesis I present the findings of a multi-centre validation study of a novel bronchoalveolar lavage-based biomarker test. The diagnostic value of the measured biomarkers is discussed and the optimum biomarker-based diagnostic test for use in VAP is presented. I subsequently present a multi-centre randomised controlled trial in which the biomarker-based test is assessed in the clinical environment to determine whether it does indeed result in improved antibiotic stewardship. Trial outcomes are reported and implications are discussed.

Dedicated to Liz, Jack and Edward, my loving family.

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Abbreviation list

AFD Antibiotic-free days

AGNB Aerobic Gram-negative bacilli

AM Alveolar macrophages

AMR Antimicrobial resistance

APACHE Acute physiology and chronic health evaluation

ARDS Acute respiratory distress syndrome

ATS American Thoracic Society

AUROC Area under receiver operator characteristic curve

BAL Bronchoalveolar lavage

BD Becton Dickinson

CBA Cytometric bead array

CDC Centers for Disease Control and Prevention

cfu Colony forming unit

CLABSI Central line associated bloodstream infection

CONSORT Consolidated Standards of Reporting Trials

CPIS Clinical pulmonary infection score

cpm Counts per minute

CRP C-reactive protein

CSF Cerebrospinal fluid

CT computerised tomography

CXR Chest x-ray

DDD Defined daily dose

DID DDD per 1000 inhabitants per day

DMEC Data monitoring and ethics committee.

DNA Deoxyribonucleic acid

DSI Daily sedation interruption

DVT Deep vein thrombosis

EARS-Net European Antimicrobial Resistance Surveillance Network

ECDC European Centre for Disease Control

ECM Extracellular matrix

ELF Epithelial lining fluid

ELISA Enzyme-linked immunosorbent assay

ES Enhanced sensitivity

ESAC European Surveillance of Antimicrobial Consumption

ESBL Extended-spectrum beta-lactamases

ESI-MS Electrospray ionisation-mass spectrometry

ETA Endotracheal aspirate

ETT Endotracheal tube

G-CSF Granulocyte colony-stimulating factor

GC-MS Gas chromatography and mass spectrometry

GI Gastrointestinal

GNB Gram-negative bacilli

GPCR G-protein coupled receptors

H2RB Histamine H_2-receptor blockers

HAP Hospital-acquired pneumonia

HCAI Healthcare-associated infection

HELICS Hospitals in Europe Link for Infection Control through Surveillance

HH Heat humidification

HME Heat and moisture exchanger

HNE Human neutrophil elastase

HPE Health Protection England

ICU Intensive care unit

IL-1Ra Interleukin-1 receptor anatagonist

IL-1α Interleukin-1 alpha

IL-1β Interleukin-1 beta

IL-8 Interleukin-8

IL-R1 Interleukin-receptor 1

IL-R2 Interleukin-receptor 2

INR International normalised ratio

IQR Interquartile range

ITT Intention to treat

IVAC Infection-related ventilator-associated complication

LOS Length of stay

LR Likelihood ratio

LRTI Lower respiratory tract infection

MALDI-TOF Matrix-assisted laser desorption ionisation-time of flight spectrometry

MAP Mean arterial pressure

MAS Macrophage activation syndrome

MCP-1 Monocyte chemo-attractant peptide-1

MDR Multi-drug resistant

MIP-2 Macrophage inflammatory protein-2

MMP Matrix metalloproteinase

MPO Myeloperoxidase

MRSA Methicillin-resistant *Staphylococcus aureus*

N-acetyl PGP N-acetyl Pro-Gly-Pro

NADPH Nicotinamide adenine dinucleotide phostate

NCTU Newcastle clinical trials unit

NETS Neutrophil extracelluar traps

NHS National Health Service

NHSN National Healthcare Safety Network

NLR NOD-like receptors

NLRC4 NLR family, CARD Domain Containing 4

NLRP3 NLR family, Pyrin Domain Containing 3

NNT Number needed to treat

NOD Nucleotide-binding oligomerisation domain-like receptors

NPV Negative predictive value

OD Optical density

OR Odds ratio

PCR Polymerase chain reaction

PCR Polymerase chain reaction

PCT procalcitonin

PE Phycoerythrin

PEEP Positive end expiratory pressure

PNA FISH peptide nuclei acid-based fluorescence *in situ* hybridisation

PPI Proton pump inhibtors

PPM Potentially pathogenic microorganisms

PPV Positive predictive value

PSB Protected specimen brush

PTP Post-test probability

QC Quality control

RCT Randomised controlled trial

ROC Receiver operator characteristic curve

RR Relative risk

rRNA ribosomal ribonucleic acid

RRR Relative risk reduction

RRT Renal replacement therapy

SBT Spontaneous breathing trials

SDD Selective decontamination of the digestive tract

SIRS Systemic inflammatory response syndrome

SLPI Secretory leucocyte protease inhibitor

SOD Selective oropharyngeal decontaminaton

SOFA Sequential organ failure assessment

SOP Standard operating procedure

SSD Subglottic secretion drainage

STARD Standards for Reporting of Diagnostic Accuracy

sTREM-1 Soluble triggering receptor expressed on myeloid cells-1

SUP Stress ulcer prophylaxis

t-PA Tissue-type plasminogen activator

TIMP Tissue inhibitors of metalloproteinases

TLR Toll-like receptor

TNFα Tumour necrosis factor alpha

u-PA Urinary-type plasminogen activator

VAC Ventilator-associated condition

VAP Ventilator-associated pneumonia

VAT ventilator-associated tracheobronchitis

VFD Ventilator-free days

VOC Volatile organic compounds

VRE Vancomycin-resistant *Enterococcus*

WCC White cell count

WHO World Health Organization

α1-PI Alpha-1 protease inhibitors

Chapter 1. Introduction

1.1 Definition of ventilator-associated pneumonia (VAP)

Ventilator-associated pneumonia is hospital-acquired pneumonia (HAP) occurring in patients who are undergoing intubation and mechanical ventilation. Conceptually this is inflammation of the gas exchange areas of the lung, the parenchyma, caused by an infectious agent. Definitions of VAP are difficult since obtaining histological evidence of alveolar inflammation (pneumonia) is impractical in critical care patients and there is little consensus on histological and clinical definitions. It is for these reasons that working definitions and surrogate 'gold standards' of VAP diagnosis have been surrounded by considerable controversy for decades.

The histological features of VAP, although considered to be the true 'gold standard' of diagnosis, lack consensus. Histological evidence demonstrates that VAP has a predominance for dependent regions of the lung, is patchy and can be distributed within large areas of non-specific alveolar damage resulting in histological evidence of pneumonia only being apparent on one or two slices of lung segment, which could easily be missed(Rouby *et al.*, 1992).

In a prospective study of 39 patients who died while receiving mechanical ventilation, lung histology was evaluated by four pathologists(Corley *et al.*, 1997). The pathologist assessed specimens for pneumonia based on consensus of pathologist opinion and according to previously described criteria by Johanson *et al*(Johanson *et al.*, 1988). There was consensus between all four pathologists on diagnosis for 77% of patients (7 patients with pneumonia and 23 without pneumonia). Six patients judged not to have pneumonia by consensus pathologist opinion were reclassified as having mild to moderate pneumonia based on the predefined criteria. Interestingly one patient judged to have pneumonia due to invasive fungi by consensus of opinion, was not classed as pneumonia by criteria due to an absence of inflammation. Fungi are generally considered to not be pathogenic organisms in VAP(American Thoracic Society, 2005), yet in this study a total of four patients had invasive fungi, one with typical neutrophilic infiltration of bronchopneumonia and another with lymphocyte and plasma cell inflammation.

The consensus reached in this study describes the earliest stages of pneumonia as beginning in small bronchi with accumulation of neutrophils. Adjacent alveoli become

oedematous before complete infiltration and obliteration by neutrophils. This advances to macroscopic consolidation or formation of abscess in some cases. In the later stages, the infiltrate can be replaced by a myxomatous connective tissue resulting in an organising pneumonitis. Macrophages can be identified in the alveolar space scavenging debris, with neutrophils remaining in fibroinflammatory tissue.

Since histological evidence is not routinely available, clinical definitions based on collections of signs and clinical investigations make up working definitions of VAP. There are numerous criteria, which vary greatly in their complexity and can be divided into clinical definitions (have diagnostic utility at the bedside) and surveillance definitions (used on a population scale to monitor VAP epidemiology). All definitions require the onset of symptoms to be 48 hours after the initiation of mechanical ventilation. VAP is often divided into early- and late-onset VAP, although the cut-off varies in the literature between 4-7 days(Trouillet *et al.*, 1998; Chastre and Fagon, 2002; American Thoracic Society, 2005; Forel *et al.*, 2012)

The predominant clinical definitions used are those put forward by the American Thoracic Society and Infectious Diseases Society of America(American Thoracic Society, 2005),the British Society of Antimicrobial Chemotherapy (BSAC) criteria(Masterton *et al.*, 2008) and the Association of Medical Microbiology and Infectious Diseases Canada(Rotstein *et al.*, 2008). These criteria are based in clinical signs and chest x-ray (CXR) findings and are summarised in **Table 1**.

Definition	Radiological	Clinical	Clinical	Use of CPIS
American Thoracic Society & Infectious Diseases Society of America	New or progressive CXR infiltrate	And at least 2 of: Temperature >38°C Leucocytosis or leucopaenia Purulent tracheal secretions		Recommend: If CPIS ≤6 for 3 days, early discontinuation antibiotics
British Society of Antimicrobial Chemotherapy	New or persistent CXR infiltrate	And: Purulent tracheal secretions	And: Increased oxygen requirement Core temperature >38.3°C Leucocytosis (>10,000/mm^3) or leucopaenia (<4000/mm^3)	Recommend: CPIS to guide short-course therapy
Association of Medical Microbiology and Infectious Disease Canada	New infiltrates, progressive changes or air bronchogram on CXR	And at least 2 of: Temperature >38°C or <36°C Leucocytosis or leucopaenia Purulent tracheal secretions Decrease PaO_2		Recommended: Calculate CPIS. If <6 then infection unlikely and decision for antibiotics should be carefully considered

Table 1: Clinical definitions of VAP. CPIS, clinical pulmonary infection score(Pugin *et al.*, 1991; Singh *et al.*, 2000)

All three of the above guidelines recommend the use of the clinical pulmonary infection score (CPIS) to guide antibiotic use. The CPIS was proposed by Pugin *et al* as an early clinical scoring system for VAP(Pugin *et al.*, 1991). The CPIS was derived in a small cohort of 28 patients of whom 13 had VAP. The CPIS criteria are presented in **Table 2**. In the original derivation study, a CPIS >6 identified patients with VAP. The diagnostic accuracy of this score will be discussed in a later section. It should be noted that the CPIS includes the results of semi-quantitative culture from endotracheal aspirate (ETA). Therefore the full score can only be calculated 2-3 days after the day of suspicion. The greatest value of a clinical infection score would be to correctly identify patients at the moment of suspicion. At this point the results of semi-quantitative cultures would not be known and so a modified CPIS is used which excludes the final microbiology criteria.

Diagnostic feature	Score = 0	1	2
Tracheal secretions	Rare	Abundant	Abundant and purulent
CXR infiltrate	None	Diffuse	Localised
Temperature (^{o}C)	≥36.5 and ≤38.4	≥38.5 and ≤38.9	≥39 or ≤36
White blood cell count ($x10^9$/L)	≥4.0 and ≤11.0	<4.0 or >11.0	<4.0 or >11.0 plus band forms ≥50%
PaO_2/FiO_2 (mmHg)	>240 or ARDS		≤240 and no ARDS
Culture of tracheal aspirate (semi-quantitative 0-+++)	No growth or ≤+	Growth >+	Culture >+ plus same bacteria seen on Gram stain

Table 2: The Clinical Pulmonary Infection Score (CPIS)(Pugin *et al.*, 1991).

The clinical criteria described above are relatively simple criteria that aim to guide clinical decision-making on an individual patient basis. In contrast, case-definitions for surveillance purposes are more cumbersome. These are generally used for retrospective

analysis of population level data. The two main definitions used are the Hospitals in Europe Link for Infection Control through Surveillance (HELICS) criteria which is now coordinated by the European Centre for Disease Prevention and Control (ECDC)(European Centre for Disease Control, 2010) and the American Centers for Disease Control and Prevention (CDC) definition(Horan, Andrus and Dudeck, 2008) **(Table 3)**. The CDC criteria have in recent years been superseded by a new diagnostic paradigm which will be discussed in a later section. The new criteria no longer define VAP, rather a collection of ventilator-associated complications.

HELICS	Radiological Two or more serial CXR or computerised tomography (CT) scans suggestive of pneumonia in patients with underlying cardiac or pulmonary disease. In patients without pulmonary or cardiac disease one definitive CXR or CT is sufficient.
	Clinical And at least one of: Fever >38°C Leucopaenia (<4000 WBC/mm^3) or leucocytosis ($\geq$12,000 WBC/mm^3)
	Clinical And at least one of the following (or at least two if clinical pneumonia only (PN4 and PN5 below)). New onset of purulent sputum or change in character of sputum. Cough, dyspnoea or tachypnoea. Suggestive auscultation (rales, bronchical breathing, ronchi or wheeze). Worsening gas exchange (eg. O_2 desaturation, increased O_2 requirements or increased ventilation demand).
	Microbiological PN1: Positive quantitative culture for minimally contaminated lower respiratory tract specimen (eg. bronchoalveolar lavage (BAL) or protected specimen brush (PSB)). PN2: Positive quantitative culture for possibly contaminated lower respiratory tract specimen (eg. endotracheal aspirate (ETA)). PN3: Alternative microbiology methods including blood culture, pleural fluid culture, needle aspiration from pulmonary or pleural abscess, histological examination or positive examination for viruses or particular pathogens (eg. *Legionella*, *Mycoplasma*). PN4:Others including sputum culture or non-quantitative lower respiratory tract culture. PN5: No positive microbiology.

CDC	Radiological X-ray findings must have at least one of: new or progressive and persistent infiltrate; consolidation; cavitation or pneumatocoeles in infants ≤1 year. These findings need to be present in two serial x-rays in patients with underlying disease or in one x-ray in patients without underlying disease.
	Clinical At least one of: Fever >38°C. Leucopaenia (<4000 WBC/mm^3) or leucocytosis (≥12,000 WBC/mm^3). Altered mental status with no other cause in ≥70 years old.
	Clinical And at least one of (two if clinical diagnosis only PNU1 (below)): New onset of purulent sputum, change in sputum character, increased respiratory secretions or increased suction requirements. New onset or worsening cough, dyspnoea or tachypnoea. Rales or bronchial breathing. Worsening gas exchange.
	Microbiology PNU1: 'Clinically defined pneumonia' in the absence of microbiology data. PNU2: 'Pneumonia with common bacterial or filamentous fungal pathogens and specific lab findings'. These specific lab findings include blood culture; pleural fluid culture; positive quantitative culture from minimally contaminated lower respiratory tract sample (BAL or PSB); ≥5% BAL-obtained cells containing intracellular bacteria on direct examination; or histological examination. PNU2: 'Pneumonia with viral, *Legionella*, *Chlamydia, Mycoplasma*, and other uncommon pathogens and specific lab findings'. PNU3: 'Pneumonia in immunocompromised patients'. Criteria for immunocompromised patients are less stringent than for immune competent patients.

Table 3: VAP criteria used for surveillance.

1.2 Epidemiology

1.2.1 VAP in the context of healthcare-associated infections

Healthcare-associated infections (HCAI) are a priority for the National Health Service (NHS). It is estimated that 300,000 HCAI occur annually and result in at least 5000 deaths a year(National Institute for Health and Care Excellence, 2014). The estimated cost of HCAI is £1 billion per year(National Audit Office and Office, 2000; National Audit Office (NAO), 2009) and it has been estimated that a high proportion of HCAI could be preventable (up to 70% catheter-related blood stream infections)(Umscheid *et al.*, 2011), allowing for a significant resource reallocation.

The overall prevalence of HCAI from UK and European point prevalence surveys has been very similar. The estimated prevalence of all HCAI in England is 6.4%(Health Protection Agency, 2011) and in Europe 5.7%(European Centre for Disease Prevention and Control, 2012b). In both of these surveys ICU wards have represented a minority of patients (2.6% in UK and 5% in Europe) yet have had the highest prevalence of HCAI compared to other specialties, with 23% in the UK and 19.5% in Europe. Recent data from the USA reports 9% of critical care patients surveyed have a HCAI(Magill *et al.*, 2014). A point prevalence survey by Health Protection Scotland specifically with regards to ICU HCAI reports a prevalence of 5.6% in Scottish ICUs(Health Protection Scotland, 2011).

Respiratory infection accounts for a significant proportion of all HCAI. HAP accounts for 23% of HCAI in the UK and 26% of HCAI across Europe. Pneumonia represents approximately 50% of HCAI on the ICU(Health Protection Agency, 2011; Health Protection Scotland, 2011). 80% of HAP in Scottish ICUs were classed as VAP(Health Protection Scotland, 2011).

1.2.2 Epidemiology of VAP

The accuracy of defining the epidemiology of VAP is determined by the surveillance definition used. As previously described, surveillance case-definitions vary considerably to clinical definitions. This has led to a discrepancy between clinically observed rates of VAP and those that are reported by national or international surveillance systems(Michetti *et al.*, 2012).

The reported incidence of VAP in clinical studies generally ranges from 9-28%(Chastre and Fagon, 2002). Randomised controlled trial (RCT) data from 1998, which measured VAP as an outcome measure, reported VAP occurring in 18% of patients undergoing

mechanical ventilation(D. J. Cook *et al.*, 1998). Two contemporary epidemiological studies, one a global study of 1,873 patients found that VAP occurred in 15.6% of mechanically ventilated patients(Kollef *et al.*, 2014) and a European study of 1,735 patients found that VAP occurred in 14-19% of mechanically ventilated patients (depending on age grouping)(Blot *et al.*, 2014).

Different methods of respiratory sampling can significantly alter the observed VAP rate. In a single-centre before-and-after study, diagnosis based on clinical criteria and endotracheal aspirate (ETA) was changed to VAP diagnosis based on culture of bronchoalveolar lavage (BAL) above a threshold of $>10^4$ colony forming units per ml (cfu/ml)(Conway Morris *et al.*, 2009). The reported incidence of VAP fell from 18 per 1000 ventilator days in the ETA period to 9 per 1000 ventilator days in the BAL period.

The incidence of VAP varies amongst patient groups. VAP is more common in trauma patients than other patient groups. In a retrospective analysis using the CDC/National Healthcare Safety Network (NHSN) criteria for VAP the incidence in trauma patients was 18% and 3% for non-trauma patients(Cook, Norwood and Berne, 2010). This study also found that the mortality amongst patients with VAP in the trauma and non-trauma groups was 11% and 34% respectively, from which the authors conclude that although VAP is more common in trauma patients it may well be of less consequence in this patient group. Patients with acute respiratory distress syndrome (ARDS) are a particularly challenging group in which to diagnose VAP. In a prospective study to determine the incidence of VAP in patients with ARDS, patients meeting inclusion criteria for ARDS underwent bronchoscopically guided respiratory sampling(Markowicz *et al.*, 2000). VAP was diagnosed on the basis of culture growth at specific thresholds for the sampling methods used. VAP occurred in 36.5% of patients with ARDS in comparison to 23% of non-ARDS patients. In a further trial evaluating neuromuscular blockade in ARDS, VAP occurred in 28.9% of patients but the occurrence of VAP was not associated with an increase in mortality(Forel *et al.*, 2012).

1.2.3 VAP surveillance

The surveillance of VAP and its relevance to the epidemiology of VAP requires specific discussion. A considerable number of interventions have been studied for the prevention of VAP and these have shown that VAP rates could be reduced(Lorente, Blot and Rello, 2007; O'Grady, Murray and Ames, 2012). This has led to a perception that VAP is entirely preventable and that VAP rates can be used as an indicator of quality of care(Klompas, 2010; Walsh, Morris and Simpson, 2013). However, as described in the

previous section, the case definitions used can greatly influence the number of cases. It should be noted that infection control teams that are separate to the ICU teams, using case definitions that differ from the clinical diagnosis, commonly perform surveillance. In the main, case definitions used are the HELICS criteria in Europe and the CDC criteria in the USA.

The CDC/NHSN have reported a steady decline in VAP rates. For comparison, the incidence of VAP in trauma patients (expressed as number of VAP cases per 1000 ventilator-days) was 15.2 in 2004(Center for Disease Control, 2004); 10.2 in 2007(Edwards *et al.*, 2007); 9.3 in 2008(Edwards *et al.*, 2008); 8.1 in 2009(Edwards *et al.*, 2009) and 6.0 in 2010(Dudeck *et al.*, 2011). These observations are in contrast to clinically observed VAP rates which in the trauma patient population remains close to 20%(Michetti *et al.*, 2012). These falling rates raise concerns about the objectivity of reporting and the vulnerability of case definitions to pressure to reduce VAP rates(Halpern *et al.*, 2012). Surveillance criteria results in a much lower incidence of VAP in comparison to clinical case definitions(Skrupky *et al.*, 2012).

In an effort to improve consistency of surveillance reporting of VAP in the USA, the CDC have developed entirely new case definitions, the ventilator-associated condition (VAC) which encompassed the new terms of infection-related ventilator-associated complication (IVAC), possible pneumonia and probable pneumonia(Klompas, 2013). A recent study comparing these criteria to prospective surveillance (with CPIS as reference standard), found that VAC identified 33% of prospectively identified VAP, IVAC 18% and 'possible' or 'probable VAP' 17%(Klein Klouwenberg *et al.*, 2014). The most common conditions contributing to VAC were pneumonia (including pre-existing and new onset pneumonia) (28%); volume overload (28%); atelectasis/sputum plug (15%); pleural effusion (12%); acute neurological event (12%); new onset systemic inflammatory response syndrome (SIRS)/sepsis (extra-pulmonary) (11%); and abdominal distension (11%). This study highlights that the new CDC surveillance paradigm does not represent surveillance of VAP and is a collection of respiratory complications in the ICU. This paradigm shift brings together multiple pathologies, not to aid diagnosis or treatment but to improve objectivity of surveillance within a single healthcare system (USA). Furthermore there is limited evidence as to whether VAP prevention strategies (that led to the idea of using VAP as a quality indicator), can have an impact on VAC. A recent study of spontaneous breathing trials (SBT) and daily sedation interruption (DSI) found that VAC were reduced per mechanical ventilation

episode (not ventilator-days) but pneumonias were not reduced(Klompas *et al.*, 2015). This study demonstrated that reducing time on mechanical ventilation reduced the time at risk of its complications rather than the prevention of VAP.

1.3 Microbiology of VAP

The pathogens implicated in VAP characteristically depend on the time until onset (early vs late VAP), the patient group and previous antibiotic exposure. The method of respiratory sampling influences the anatomical region tested and confidence that the microbiology represents the pulmonary infection rather than respiratory tract colonisation (this will be discussed further in *Diagnosis*). A recent global study of 1,873 patients enrolled from the USA, Europe, Latin America and Asia Pacific identified *Pseudomonas aeruginosa* as the most common pathogen causing VAP followed by *Staphylococcus aureus*, *Acinetobacter* species, *Klebsiella* species, *Enterobacter* and *Escherichia coli*(Kollef *et al.*, 2014). The predominance of Gram-negative bacilli in VAP has been recognised for many years. In a prominent review, the microbiology of 24 studies demonstrated that Gram negative bacilli comprised approximately 60% of pathogens and the predominant Gram-positive pathogen being *S. aureus* accounting for 20% of cases(Chastre and Fagon, 2002). *P. aeruginosa* accounted for 24% of cases, *S. aureus* 20%, *Acinetobacter* spp. 8%, Enterobacteriacease 14%, *Haemophilus* spp. 10% and *Streptococcus* spp. 8%. The German Nosocomial Infection Surveillance System reported on the 5811 cases of VAP between 2005-2007(Kohlenberg *et al.*, 2010). Gram-negative bacteria accounted for 59%, Gram-positive 26% and Fungi 12%. The most common pathgens were *S. aureus* (18%), *P. aeruginosa* (15.7%), *Klebsiella* spp. (10.2%), *E. coli* (9.8%) and *Candida* spp. (9.3%). VAP is often polymicrobial occurring in 25% of cases in a large scale surveillance study in Asian countries(Chung *et al.*, 2011), although the rate of polymicrobial positivity has been shown to vary considerably between patients who underwent BAL (12.3%) and those who had ETA (45.5%)(Fagon *et al.*, 2000). VAP that occurs within 7 days of mechanical ventilation is more likely to be due to Enterobacteriaceae, *Haemophilus* spp, *S. aureus* and *Streptococcus* spp.(Trouillet *et al.*, 1998). Beyond 7 days of mechanical ventilation *P. aeruginosa, Acinetobacter baumannii* and *Stenotrophomonas maltophilia* become prominent. Furthermore previous exposure to antibiotics and greater level of comorbidities are associated with antibiotic-resistance(Trouillet *et al.*, 1998; Depuydt *et al.*, 2008).

Aetiological patterns may vary between patient populations too. In a comparison of trauma and non-trauma patients, there were significantly more VAP cases caused by *Haemophilus influenzae*, *Enterobacter* spp. and *Acinetoacter baumannii* in trauma patients(Cook, Norwood and Berne, 2010). In the context of ARDS, Gram-negative bacilli are more common(Markowicz *et al.*, 2000).

Newer microbial detection methods are shedding new light on the aetiology of VAP and the pattern of transcolonisation to the lungs. Bacterial DNA polymerase chain reaction (PCR) detection of bacteria from BAL samples in patients with suspected VAP revealed bacteria not previously detected in BAL including *Haemophilus* spp., *Terrahaemophilus aromaticivorans, Shigella* spp., *Pseudomonas fluorescens, Janthinobacterium lividum, Streptococcus* spp., *Gemella* spp., *Dialister* spp., *Megasphaera* spp., *Firmincutes* spp., *Lachnospiraceae* spp., *Porphyromonas* spp. and *Prevotella* spp.(Bahrani-Mougeot *et al.*, 2007). Interestingly for some individuals in that study, known VAP-associated pathogens were detected only by PCR and not standard culture, including *H. influenzae, S. pneumonia* and *E. coli*.

The role of *Candida* spp. in the respiratory tract is uncertain. As noted above, it is frequently isolated from cultures and expert opinion and clinical guidelines advise that it is a colonising pathogen and not implicated in infection unless the patient is immunosuppressed(Chastre and Fagon, 2002; American Thoracic Society, 2005). In a retrospective analysis of patients with *Candida* spp. in the respiratory tract from a large RCT(Delisle *et al.*, 2008), the presence of *Candida* spp. on respiratory culture was independently associated with hospital-mortality. Furthermore limited evidence does suggest that the presence of *Candida* spp. in comparison to control patients (no suspicion of VAP), is associated with increase inflammatory markers including pro-calcitonin, C-reactive protein and Interleukin-6(Williamson *et al.*, 2011). However this issue remains unresolved and as to whether patients should undergo treatment for *Candida* spp. is uncertain(Albert *et al.*, 2014).

1.4 Pathogenesis

The critically ill patient on a mechanical ventilator is vulnerable to acquiring nosocomial infections. In health the upper airway provides protection to the lower airway predominantly by the epiglottis and cough reflex. These barriers are bypassed by endotracheal intubation. Furthermore inspired gases are humidified by the upper airway, and this process is also circumvented by mechanical ventilation. If gases were not humidified, normal respiratory tract function would be severely impaired(Fonkalsrud *et*

al., 1975). It is not only these mechanical changes but also the state of immunity that renders the patient vulnerable. Critically ill patients have been shown to have immune impairment with dysfunctions in regulatory T cells and neutrophils associated with 2.4 and 6.9 fold increased risk of nosocomial infection respectively. When these two dysfunctions where present in conjunction with monocyte dysfunction, 75% of these patients acquired a nosocomial infection (Conway Morris *et al.*, 2013). Patients who have suffered a traumatic brain injury are recognised to have a high prevalence of VAP and an immune paralysis has been described in association with this type of injury(Dziedzic, Slowik and Szczudlik, 2004)

The predominant mechanism of infection of the lung is by micro-aspiration although haematological spread or local infiltrations (from the pleural space) are also relevant routes of infection(Estes and Meduri, 1995). In health micro-aspiration can occur while sleeping(Gleeson, Maxwell and Eggli, 1997) yet the balance between inoculation and host defence is such that pneumonia does not develop. There are changes in the critically ill patient in terms of organisms/pathogens and also alterations in host immunity.

Central to this mechanism are the organisms that colonise sites of potential trans-colonisation, the upper gastrointestinal tract and the oropharynx. Culture-independent methods of bacterial detection, using detection of bacterial 16s rRNA by microarray, has shown that in healthy subjects the characteristic family of bacteria colonising the oropharynx are *Streptococcaceae, Lachnospiraceae* and *Clostridia*(Lemon, 2010).Gastric contents, thought previously to be inhospitable to bacteria, shows a diverse range of normal flora, when detected by bacterial 16s rDNA, with 5 predominant genera of *Streptococcus, Prevotella, Rothia, Fusobacterium* and *Veillonella*(Bik *et al.*, 2006; Nardone and Compare, 2015). In comparison to healthy subjects, hospitalised patients have a predominance of aerobic Gram-negative bacilli (AGNB) colonising the oropharynx(Filius *et al.*, 2005). Bacteria demonstrate tissue tropism, where tissues support growth of specific bacteria, which is dependent on adhesion molecules and tissue factors. For example fibronectin is a factor present in salivary fluid that favours *Streptococcus* spp. binding in the oropharynx(Avila, Ojcius and Yilmaz, 2009) and *P. aeruginosa* favours binding on tracheal epithelium and may well be able to colonise the trachea without prior oropharyngeal colonisation(de Latorre *et al.*, 1995). A clear relationship between bacteria identified by molecular analysis in the oral cavity and the BAL of VAP patients supports the oropharyngeal route of

translocation(Bahrani-Mougeot *et al.*, 2007). In relation to colonisation of the stomach contents, bacterial growth is influenced by stomach pH. Acid suppression therapy is common in critically ill patients as a means of prophylaxis against stress ulceration. Raising the stomach pH promotes bacterial growth and allows growth of potentially pathogenic AGNB(Bonten *et al.*, 1995). Although clinical studies have demonstrated an association between the use of acid suppression and VAP(Messori *et al.*, 2000), other studies have not demonstrated a clear gastro-pulmonary route of infection for VAP(Bonten *et al.*, 1995). In addition to the physiological changes that promote colonisation of these sites by AGNB, the endotracheal tube (ETT) becomes an important source of bacteria. *Staphylococcus aureus* has a particular tendency to adhere to the surfaces of invasive devices(Jones *et al.*, 1997). It therefore follows, as previously described, that the predominant pathogens implicated in VAP are *P. aeruginosa*, *S. aureus*, *Acinetotbacter* species, *Klebsiella* species, *Enterobacter* species and *Escherichia coli*(Kollef *et al.*, 2014).

Infectious inoculate accesses the lower respiratory tract past the endotracheal tube. This is possible because the endotracheal tube does not represent a complete barrier between the upper and lower respiratory tract. The endotracheal tube balloon that forms the tracheal seal is high-volume, low-pressure, designed to reduce the risk of tracheal ischaemia and stenosis. When the cuff is inflated multiple folds occur in the cuff that allows supraglottic secretions to pass around the cuff(Seegobin and van Hasselt, 1986).

Once the pathogen has gained access to the respiratory tract, the innate immune response to pulmonary parenchymal infection is characterised by a neutrophilic infiltration of the alveolar spaces. Lung macrophages, including alveolar and interstitial macrophages, are the first line of defence with phagocytic, cytotoxic and cytokine release functions(Franke-Ullmann *et al.*, 1996). Mouse studies demonstrate that alveolar macrophages (AM) play a central role in the initiation of the early pro-inflammatory response releasing cytokines such as interleukin (IL)–1β, IL-1α, tumour necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)–2 (mouse analogue of IL-8) and chemokine CXCL1(Pittet *et al.*, 2011). AM therefore are implicated in neutrophil recruitment and AM depletion is associated with reduced neutrophil recruitment(Kooguchi *et al.*, 1998; Pittet *et al.*, 2011). In addition alveolar epithelial cells are involved in neutrophil recruitment through the release of potent chemo-attractants such as IL-8(Harada *et al.*, 1994; Thorley *et al.*, 2007; Craig *et al.*, 2009).

IL-1β belongs to the IL-1 cytokine family, which includes 11 different cytokines. IL-1β is a highly inflammatory cytokine and the margin for benefit and toxicity in humans is narrow(Dinarello, 1996, 2011). Binding of IL-1 to its receptor initiates recruitment of the adaptor protein MyD88 to the Toll-IL-1 receptor domain, causes phosphorylation of kinases, and NK-κB translocates to the nucleus, resulting in the expression of a range of inflammatory cytokines(Dinarello, 2011). IL-1β is therefore tightly regulated by two mechanisms. Firstly its action is antagonised by interleukin-1 receptor antagonist (IL-1Ra), which binds avidly to interleukin-receptor-1 (IL-R1) to block the binding of IL-1β. There is a second IL-1 receptor, IL-1R2. This does not transduce any action and therefore acts as a 'sink' for IL-1β and the second mechanism for its regulation(Boraschi and Tagliabue, 2013). IL-1β is produced by a wide range of cells including dendritic cells, monocytes, macrophages, mast cells, neutrophils, B and T cells, endothelial cells, epithelial cells and dying cells(Sims and Smith, 2010). It has actions on: dendritic cells, increasing cytokine production; macrophages, increasing cytokine production and phagocytosis; and on neutrophils, increasing survival, adhesion (and therefore migration), oxidative burst and protease release(Borregaard, 2010; Sims and Smith, 2010). IL-1β is produced in an inactive form and requires cleavage by caspase-1, which itself is activated by protein complexes termed the 'inflammasome'(Netea *et al.*, 2010). Several inflammasomes have been described all of which include members of the NOD-like receptor (NLR) family of proteins. Stimuli that activate the NLRP3 inflammsome include bacterial RNA, muramyl dipeptide (bacterial origin) and danger-associated molecular patterns (eg. uric acid crystals and amyloid-β)(Jo *et al.*, 2016). The NLRC4 inflammasome complex is activated by intracellular flagellin that is independent of toll-like receptor (TLR)-5 binding allowing direct activation of the inflammasome by bacteria(Miao *et al.*, 2006). The classical model of IL-1β production is that two triggers are needed for its production, the first to trigger transcription and the second to induce inflammasome activation(Netea *et al.*, 2010). In addition to the importance of inflammasome production of IL-1β, pro-IL-1β can be cleaved in a caspase-1-independent manner by neutrophil- and macrophage-derived serine proteases including proteinase-3, human neutrophil elastase (HNE) and cathepsin-G(Netea *et al.*, 2010; Karmakar *et al.*, 2012). This could be an important mechanism of IL-1β production in environments where the neutrophil is the predominant cell population.

Considering the central role that IL-1β has in inflammation, blocking its action using a recombinant IL-1Ra (Anakinra), has been used to treat a wide range of inflammatory

diseases(Dinarello, Simon and van der Meer, 2012). One of the earliest uses of Anakinra was in trials as a treatment for sepsis, but no mortality benefit was demonstrated(Fisher *et al.*, 1994; Opal *et al.*, 1997). Interestingly, a recent retrospective analysis of previous trial data(Opal *et al.*, 1997), examined patients with sepsis and features of macrophage activation syndrome(Shakoory *et al.*, 2016). Macrophage activation syndrome (MAS) presents with a cytokine storm, with clinical features similar to severe sepsis. MAS can lead to multi-organ dysfunction and has been treated with anakinra(Dinarello, Simon and van der Meer, 2012). In this retrospective analysis, the presence of MAS was defined as the presence of hepatic and coagulation dysfunction. Patients with sepsis and these features of MAS did appear to have a survival benefit with Anakinra, with a 28-day mortality of 34.6% in Anakinra group versus 64.7% in placebo group(Shakoory *et al.*, 2016). This interesting re-analysis calls for the reappraisal of the role of IL-1β blockade in sepsis.

The ability of the neutrophil to arrive at a site of infection is central to the host defence. Neutrophils respond to chemotactic cytokines, chemokines, and IL-8 (or CXCL8) is a potent neutrophil chemokine(Mukaida, 2003; Nathan, 2006; de Oliveira *et al.*, 2013). IL-8 is produced by a range of cells including monocytes, T cells, neutrophils, natural killer cells, endothelial cells, fibroblasts and epithelial cells(Mukaida, 2003). IL-8 binds to two seven trans-membrane G-protein coupled (GPCR) receptors, CXCR1 and CXCR2, and in addition IL-8 can bind to glycosaminoglycans(Jin, Xu and Hereld, 2008; Kufareva, Salanga and Handel, 2015). Binding to the GPCR primarily results in chemotaxis through the dynamic formation of the cellular actin cytoskeleton resulting in cell polarity and cell motility in response to chemotactic gradients. Neutrophils express both CXCR1 and 2, and IL-8 is one of only three known chemokines that bind to both receptors in the neutrophil (these are IL-8, CXCL-6 and N-acetyl PGP). Many ligands bind to CXCR2 but none bind solely to CXCR1(Stillie *et al.*, 2009). CXCR1 and 2 result in different actions in the neutrophil, which may be concentration dependent. CXCR2 activation causes the release of matrix metalloproteinase (MMP)-9 and HNE and at low chemokine concentration, is purely chemotactic. At high chemokine concentration, CXCR1 activation results in respiratory burst. Both receptors mediate chemotaxis, intracellular calcium influx and phagocytosis. IL-8 is released in response to a range of insults causing inflammation. IL-8 is released in response to several *P.aeruginosa* derived factors including flagellin, procyanin and nucleoside diphosphate kinase(DiMango *et al.*, 1995; Chai *et al.*, 2014; Kim *et al.*, 2014); bacterial products such as lipopolysaccharide(Maniar-Hew *et al.*, 2013); environmental factors including

hypoxia(Keglowich *et al.*, 2014) and over-stretch of the lung, relevant in the mechanically ventilated patient(Kotani *et al.*, 2004). High levels of IL-8 have been detected in various pulmonary pathological states including pneumonia(Boutten *et al.*, 1996; Abul *et al.*, 2001), asthma(Ordonez *et al.*, 2000), ARDS(Aggarwal *et al.*, 2000; Mukaida, 2003; Agrawal *et al.*, 2012) and VAP(Conway Morris *et al.*, 2010). Interestingly, the IL-8 response to inflammation appears to vary with age(Dalboni *et al.*, 2013; Maniar-Hew *et al.*, 2013), which is of significance given that this questions the relevance of experimental models of inflammation to an increasingly elderly critical care population.

Neutrophils that migrate to the alveolar space are the predominant cell involved in the killing of bacteria. They are phagocytes with a armamentarium of bactericidal factors which are housed within granules(Faurschou and Borregaard, 2003; Segal, 2005; Nathan, 2006). There are five groups of granule: the specific, gelatinase, azurophilic, lysosomes and secretory granules(Segal, 2005; Nathan, 2006). Granule contents is determined by the stage of cell and granule development and so granules have overlapping contents(Faurschou and Borregaard, 2003). The specific and gelatinase granules contain lactoferrin, lipocalin, lysozyme, MMP-8, MMP-9 and MMP-25(Nathan, 2006). The azurophilic granule contains myeloperoxidase (MPO) which converts hydrogen peroxide to hypochlorous acid, defensins and the serine proteases cathepsin G, HNE and protease 3(Nathan, 2006). Lysosomes contain acid hydrolases and secretory granules act as a reservoir of membrane components that may serve to replenish the membrane used during phagocytosis(Segal, 2005). The components of these granules are used to kill bacteria but also serve other functions. Killing of bacteria is primarily within the phagosome, a vacuole within the cell containing the phagocytosed particle (eg. bacterium)(Reeves *et al.*, 2002; Segal, 2005). Activation of the neutrophil, by IL-8 for example, triggers degranulation whereby the granule contents are released into either phagosome or extracellularly(Lacy, 2006; Khokha, Murthy and Weiss, 2013). Animal models have demonstrated that mice deficient in cathepsin G are susceptible to infection by *S. aureus*, mice deficient in elastase were susceptible to *C. albicans*(Reeves *et al.*, 2002) and both proteases are necessary for resistance against *A. fumigatus*(Tkalcevic *et al.*, 2000). Reeves *et al* demonstrated that despite the acidic contents of the granules being deposited into the vacuole, the pH ranged from 6 to 8(Reeves *et al.*, 2002). This rise in pH is partly due to the movement of potassium into the vacuole via the NADPH oxidase, a membrane-bound enzyme complex. The hypertonic potassium within the vacuole causes the solubilisation of

cathepsin G and elastase previously bound (and inactive) to a sulphated proteoglycan matrix(Reeves *et al.*, 2002). This mechanism has called into question the previously held paradigm that reactive oxygen species produced by NADPH oxidase are the predominant mechanism of bacterial killing(Segal, 2005).

Granule contents are released into the extracellular matrix (ECM) of which many components of the ECM are substrates for the granule contents, in particular the proteases(Lacy, 2006). Although breakdown of the ECM is necessary to allow cell motility to the infected site, proteases are responsible for wider tissue damage and inflammation and so an array of antiproteases are released to neutralise the proteases(Williams *et al.*, 2006). However extracellular proteases serve other purposes such as cleavage and activation of cytokines(Karmakar *et al.*, 2012). Neutrophils may also be able to kill bacteria by production of neutrophil extracellular traps (NETS)(Brinkmann *et al.*, 2004). Brinkmann *et al* demonstrated that activated neutrophils produced fragile NETS, which contained HNE, cathepsin G, MPO, lactoferrin, MMP-9 and DNA. Neutrophils in which phagocytosis was blocked but NETS were still present were able to killing 30% of *S. flexneri* and *S. aureus*. NET production or “NETosis” is a form of programmed cell death with this particular bactericidal purpose(Brinkmann and Zychlinsky, 2012)..

Considering this neutrophilic infiltration it is unsurprising that nosocomial pneumonia has been shown to be associated with high levels of neutrophilic proteases including HNE, MMP-8 and MMP-9 (Hartog *et al.*, 2003; Wilkinson *et al.*, 2012).

1.4.1 Measurement of cytokines

There are a number of methods with which to quantify soluble cytokines in fluids. These methods are generally divided into enzyme-linked immunosorbent assay (ELISA) and multiplex assays. ELISA is based on a double antibody sandwich in which the base of a well is coated with an analyte-specific capture antibody that binds the cytokine of interest when incubated. Another analyte specific antibody complexed with an enzyme then binds to the cytokine and the colour change caused by the enzyme when a suitable substrate is added allows detection of the cytokine. ELISA allows quantifiable measurement and is highly reproducible and considering the experience that has built up since its introduction in the 1970s, it is considered the standard for measuring cytokines(Leng *et al.*, 2008). There are, however, a number of limitations to ELISA that have driven development of alternative assays. The main limitation is that only one cytokine can be measured per assay. Since the inflammatory process involves a large

number of mediators, measuring many cytokines is desirable but the repeated aliquots required for multiple ELISA can be prohibitive with some low volume clinical samples. Furthermore there are cost and time considerations when using multiple ELISA kits.

To overcome the limitations of ELISA, assays have been developed that allow measurement of multiple cytokines in a single assay, by multiplex array. These assays use flow cytometry, chemiluminescence or electrochemiluminescence(Zhou *et al.*, 2010). Chemiluminescence is used in multiplex ELISA assays in which multiple specific antibiodies are coated at specific locations on a 96 well plate. A similar set up is used in electrochemiluminescent assays where the antibodies are coated on an electric wired microplate. The use of flow cytometry is the most common format for multiplex arrays and these use proprietary antibody-coated beads that are detected by flow cytometry. Cytometric Bead Array (CBA) (BD Biosciences) is one such method.

Flow cytometry is a laser-based technology that measures ('-metry') cells ('cyto') in a stream of fluid ('flow'). The basic elements of flow cytometry are the fluidics, optics and electronics. The fluidics system is the flow of fluid that transports the sample core to interact with lasers. The optics system comprises lasers, lenses and prisms that focus the laser on the sample core and detectors that capture emitted and scattered light. The electronics systems convert the light signals into an electronic output. The principle of flow cytometry is that when a cell or particle interacts with the laser beam in the sample core it deflects the laser from its path. Therefore measurement of light in line with the laser source (forward scatter) reflects the particle size. Measurement of light at right angles to the laser source (side scatter) represents the internal complexity of the cell or particle. Markers of interest that are to be measured within or on the cell/particle surface can be conjugated, via monoclonal antibodies, with fluorescent dyes, flourochromes(Morgan *et al.*, 2004). Flourochromes are molecules that when excited by lasers, are raised to a higher energy state. When they return to their resting state they emit a characteristic wavelength of electromagnetic energy. These are also detected in the side scatter optics elements.

CBA uses these principles of flow cytometry to measure soluble factors. The proprietary CBA beads are 7.5μm in diameter and therefore appear as a single group on the forward scatter(Morgan *et al.*, 2004). Beads are dyed and so fluoresce at a specific intensity at 650nm when excited by an argon laser and detected in the FL3 channel. This allows different beads (eg. beads detecting IL-1β and IL-8) to be distinguished despite being the same size. The analyte of interest binds to the antibody-coated bead

and is then further complexed with another antibody that is bound to the detector reagent. This detector is a flourochrome such as phycoerythrin (PE) (which emits a fluorescent signal that is detected in the FL2 channel). The intensity of the FL2 signal is therefore proportional to the amount of analyte bound to the bead and so the concentration of analyte in solution.

The main benefit of CBA is the ability to detect a range of analytes in a single assay with less biological sample. This has benefits in terms of time and efficiency. Furthermore the detection range for the fluorescent signal is wide. There are however some limitations of CBA that must be considered. Firstly, unlike ELISA, which immobilises the analyte being measured, the reactions in CBA occur in suspension and cross-reactivity is a concern. Not only is there potential interaction with different reagents but also factors that can cause antibody-antigen interactions causing false negative or false positive measurements(Bartels and Ribel-Madsen, 2013). Secondly, the limit of detection for IL-1β and IL-8 (as with other flex sets) is reported by the manufacturer to be 1.2pg/ml. This is estimated from the median fluorescent intensity of the negative control plus 2 standard deviations. Caution should be taken when interpreting any values that fall below the lowest point on a standard curve which is generally 5-10pg/ml for flex sets. Lastly, good correlations of CBA with ELISA have been demonstrated but there is poor concurrence of quantitative values(Elshal and McCoy, 2006).

1.4.2 Sampling epithelial lining fluid by BAL

The cytokines described above are largely present in the milieu surrounding the cells. In the context of VAP, the cytokines are present in the epithelial lining fluid (ELF), which is sampled by BAL.

Performing a BAL will invariably sample both the bronchial area, bronchiolar area and the alveolar area. Using digital subtraction imaging techniques, it has been demonstrated that the first aliquot of saline remains close to the bronchoscope and subsequent aliquots were required to fill the alveolar space(Kelly *et al.*, 1987). For this reason it has been advocated that the aspirate of the first aliquot is discarded(Meduri and Chastre, 1992). Squamous epithelial cells are used as a marker of bronchial contamination and considerably more squamous epithelial cells have been demonstrated in the first 20ml BAL aliquot (45.0%) in comparison to the second 50ml BAL aliquot (7.6%)(Marquette *et al.*, 1995). Uncertainty over exact anatomical position of BAL sampling is inherent in BAL and previous expert opinion has stated that discarding the

bronchial wash is unreliable and that ensuring a sufficient BAL volume is used to dilute the effects of bronchial sampling and lowering the airways:alveolar material ratio is required(Haslam and Baughman, 1999). In fact current national guidelines for bronchoalveolar lavage in clinical practice, do not recommend discarding a bronchial sample(Meyer *et al.*, 2012; Du Rand *et al.*, 2013).

A minimum volume of 120mls has been recommended(Kelly *et al.*, 1987) and there is variation in recommendations in national guidelines with the British Thoracic Society recommending 60-180mls(Du Rand *et al.*, 2013) and the American Thoracic Society recommending 100-300mls(Meyer *et al.*, 2012). It should be noted that these volumes are not specific to BAL in the ICU (ATS recommendations are in relation to investigation of interstitial lung disease).

In cases of bilateral CXR changes, there is inevitably some uncertainty as to whether the BAL has been performed in the correct area. The main concern is the risk of a false negative result if the infected area is not sampled. VAP has been shown to be widely distributed histologically(Rouby *et al.*, 1992) which may account for the finding that the bacterial index of directed BAL and non-directed BAL, even if the non-directed BAL was in a different lobe or even the contralateral lung, were not significantly different(Pugin *et al.*, 1991). In current UK practice, generally more than one lobe is lavaged, volumes >100mls are also only used by approximately 10% of clinicians and the median volume being 20mls(Browne *et al.*, 2014). This would lead to the conclusion that most 'BAL' performed in the UK are in fact bronchial 'washes'. If an adequate BAL is being performed with the necessary volumes (at least 120mls), there are safety considerations for performing BAL in more than one lobe in ICU patients. Anecdotally the volume of 120mls used in these studies was considered to be high volume by many clinicians working within the study sites.

The ELF sampled by BAL is diluted by the instilled saline during the BAL. One of the main uncertainties with BAL is the degree to which the ELF is diluted by the saline and therefore to what degree the measured biomarker level truly reflects the biomarker level in the ELF(Haslam and Baughman, 1999). Urea is a small molecule (60 Daltons) that can freely diffuse across biological membranes. Measurement of BAL and serum urea, has been previously used to determine a correction factor for the amount of ELF dilution that has occurred and ELF has been shown to be diluted approximately 100 fold by BAL using the urea correction method(Rennard *et al.*, 1986). Since urea moves freely across membranes, down its concentration gradient, the amount of urea in BAL

does increase the longer the saline is allowed to remain in the lungs prior to aspiration ("dwell time")(Rennard *et al.*, 1986). The movement of urea during BAL is complex and the rationale for the use of urea may be too simplistic(Ward *et al.*, 1992). In a study of healthy volunteers BAL urea concentrations were lower in smokers than non-smokers, urea increased in BAL fluid between 3 sequential 60ml aliquots of instilled saline and significant amounts of intravenously injected radiotracer were found in BAL fluid demonstrating the dynamic movements of water and urea during BAL(Ward *et al.*, 2000).

1.5 Mortality and morbidity

1.5.1 Mortality

Estimating the mortality associated with VAP has proven to be complex. VAP complicates the condition that mandated the patient's admission to ICU. Therefore the admission condition and subsequent HCAI may both contribute to the risk of death. Separating the respective contributions has proven difficult. Furthermore the adequacy of treatment for VAP is inherent in its contribution to the risk of dying and in the context of estimating ICU mortality, an increased length of ICU stay results in a time-at-risk bias as these patients will have a prolonged observation period on ICU(Muscedere, Day and Heyland, 2010). Mortality rates are either reported as crude mortality rates or as attributable mortality. Attributable mortality is calculated by subtracting the baseline mortality for the cohort from the mortality in the VAP group to determine the contribution of VAP to the risk of death. Attributable mortality is generally expressed as relative risk (RR) or as an odds ratio (OR). Difficulties in determining attributable mortality in VAP have led to widely ranging estimates.

Crude mortality rates have been reported to be between 24-76%(Chastre and Fagon, 2002). This wide range of crude mortality rates is derived from relatively small studies. In a retrospective case-controlled study, 48 patients with VAP confirmed by semi-quantitative culture, the crude mortality was 54.2% and amongst controls 27.1%.(Fagon *et al.*, 1993). Similarly in a prospective cohort study in a community hospital VAP was associated with a 45.5% mortality compared to 32.2% amongst patients who did not acquire VAP, although this was not case-matched(Ibrahim *et al.*, 2001). High crude mortality rates have been reported in association with *Pseudomonas* spp. and *Acinetobacter* spp. with mortality rates of 73%(Fagon *et al.*, 1993, 1996). In contrast to these findings, a case-controlled study in a mixed medical and surgical ICU of 97 patients and did not find a significant difference in mortality between the VAP and case-

control group(Papazian *et al.*, 1996). Furthermore there was no significant increase in mortality when *Pseudomonas* spp. were the causative pathogens. Trauma patients are a group of patients in which VAP is a common complication but there does not appear to be an associated increase in mortality(Baker, Meredith and Haponik, 1996; Cook, Norwood and Berne, 2010).

Estimates of attributable mortality also vary widely. High attributable mortality rates were reported in a prospective, matched, risk-adjusted cohort(Bercault and Boulain, 2001). Crude mortality rates were 41% and 14% for the VAP and control groups respectively, which equates to an attributable mortality OR of 2.7 (95% confidence interval (CI) 1.8-3.1). Interestingly when sub-analysis was performed on VAP caused by antibiotic resistant or sensitive pathogens the OR was only significant for the resistant group (2.6, 95%CI 1.1-5.8 versus 1.8, 95% CI 0.9-3.9 for the sensitive group). In contrast to these high attributable mortality rates, a match controlled analysis performed on patients enrolled in a stress ulcer prophylaxis RCT did not find an increase in RR in patients with VAP (32.2% (95% CI -20.6-85.1))(Heyland *et al.*, 1999).

Pooling RR of mortality for VAP from observational studies in a meta-analysis revealed a RR of 1.27 (95% CI 1.15-1.39)(Melsen, Rovers and Bonten, 2009). This analysis did however include matched and non-matched observational studies and there was considerable heterogeneity between studies (I^2 69%). Interestingly there was very little heterogeneity in the sub-groups of trauma (I^2 1.3%) and ARDS (I^2 0%) and in these groups no additional attributable mortality was associated with VAP.

In an alternative approach, a meta-analysis of RCTs of VAP prevention interventions was performed(Melsen *et al.*, 2011), the rationale being that if there is an associated mortality with VAP then any reduction in VAP rates will result in a reduction in mortality. A ratio of relative risk reduction (RRR) of mortality to the RRR of acquiring VAP, was used to estimate the attributable mortality. The pooled RRR in mortality was 0.03 (95% CI -0.03-0.08) (no RCT reported a significant decrease in mortality) and the RRR in VAP was 0.33 (95% CI 0.23-0.41). The estimated attributable mortality was 9%.

Although the previously described reports attempted to control for confounding variables through matching, none of them considered the fluctuating risk of VAP over time. In an analysis by Bekaert *et al*, the authors attempted to overcome the following confounding factors: patients need to survive long enough to acquire VAP; patients with

VAP are more severely unwell throughout ICU admission, not only on admission; the interplay between disease severity and adequacy of treatment over time; and that discharge from ICU is a competing risk for ICU mortality(Bekaert *et al.*, 2011). This analysis was performed using data from a high quality database in which VAP was diagnosed not only by clinical criteria but also respiratory sampling by bronchoscopic methods and quantitative culture. This approach estimated a 2.3% increase in hazard of ICU death for each day following onset of VAP. The estimated attributable mortality fraction at 30 days was 4.4% (95% CI 1.6-7.0) and at 60 days was 5.9% (95% CI 2.5-9.1). Considering the crude mortality rates at 30 and 60 days were 23.3% and 25.6% respectively, this results in an attributable mortality at 30 days of 1% and 1.5% at 60 days. Although this is a detailed investigation, it alone should not lead the reader to conclude that there is no significant associated mortality with VAP. It highlights the complexity of elucidating the attributable mortality in complex patients in whom many factors influence the risk of death.

1.5.2 Length of stay

Unlike the attributable mortality of VAP there is much more consistent signal in regard to an associated increased length of ICU and hospital stay. The increased length of ICU stay amongst patients with VAP ranges from 4.7-8 days(Papazian *et al.*, 1996; Heyland *et al.*, 1999; Ibrahim *et al.*, 2001; Rello *et al.*, 2002; Safdar *et al.*, 2005)The increased length of hospital stay ranges from 11.5-23.4 days(Ibrahim *et al.*, 2001; Rello *et al.*, 2002; Cook, Norwood and Berne, 2010). The highest length of stay was reported in a prospective study of trauma patients in which the increased length of ICU stay was 17.8 days and hospital length of stay was 16.8 days although this was not a case-controlled study(Cook, Norwood and Berne, 2010). The prevention of VAP using subglottic secretion drainage (SSD) has not only prevented VAP but also reduced length of ICU stay(Muscedere *et al.*, 2011).

1.5.3 Healthcare resource utilistation

Estimating the cost attributable to VAP suffers the same difficulties of estimating mortality in patients with complex disease and the potential for multiple complicating diseases. The wide range of differences in healthcare systems further limits estimated costs. No detailed reports of the cost of VAP in the UK have been previously published. Data from the USA (as in studies described below) estimates cost from a patient, provider or insurance perspective. Case definitions of VAP can influence the estimated prevalence of VAP, which will also influence estimates of cost. In a retrospective, case-

controlled study in which patients with VAP were identified if ventilated for ≥2 days and met the criteria of the International Classification of Disease, Ninth Revision (ICD-9) code for VAP (997.31)(Kollef, Hamilton and Ernst, 2012), the estimated cost from a hospital perspective (indirect and direct costs) was $99,598 for patients with VAP and $59,770 for control patients. These costs are very similar to a previously reported provider perspective estimate(Rello *et al.*, 2002).

In a prospective analysis, Anderson *et al* estimated the cost of HCAIs from a systematic review of the literature from 1985-2005 and then applied these costs to the prospective HCAI rates for 32 participating community hospitals(Anderson *et al.*, 2007). With an estimated cost of $25,072 per VAP case and median of 2 (IQR 0-4) VAP cases per year per hospital, the median cost of VAP per year to the hospital was $50,144.

Another strategy was to estimated attributable cost in a meta-analysis based on the estimated attributable increased length of ICU stay and estimated specific treatment cost for VAP. The estimated cost was an additional $13,647 per VAP episode(Safdar *et al.*, 2005). Similar attributable costs were determined in two retrospective analyses with costs of $11,897(Warren *et al.*, 2003) and $15,986(Hugonnet *et al.*, 2004).

In contrast to these a further analysis from a hospital perspective did not find a significant cost associated with VAP(Restrepo *et al.*, 2010). The median costs were $76,730 for VAP cases and $41,250 for control cases. Following Medicare reimbursements to hospitals the median losses to hospitals were $32,140 for VAP cases and $19,360 for control cases, although this difference was not statistically significant.

1.6 Diagnostic test metrics

Diagnostic tests aim to correctly detect disease but tests rarely do this with 100% accuracy. There are two sides to diagnostic testing; the ability to correctly detect the disease in those that have the disease and the ability to correctly detect the absence of disease in those that do not have it. The ability of a test to correctly identify disease can be illustrated by the following 2x2 table:

	Disease present	Disease absent
Test positive	A (true positives)	B (false positives)
Test negative	C (false negatives)	D (true negatives)

The sensitivity is the proportion of true positives that are correctly identified by the test and is given by the equation: Sensitivity = A / (A+C) (Douglas G Altman and Bland, 1994).

The specificity is the proportion of true negatives that are correctly identified by the test and is given by the equation: Specificity = D / (B+D).

Sensitivity and specificity approach the diagnostic accuracy from the direction of the whether the disease is present or not. In clinical practice, however we generally approach from the position of the test result asking: what proportion of positive tests (or negative tests), correctly identify patients with the disease (or without the disease)? This is given by the predictive values (D G Altman and Bland, 1994).

The positive predictive value (PPV) is the proportion of patients with a positive result who are correctly diagnosed and is given by the equation: PPV = A / (A+B).

The negative predictive value (NPV) is the proportion of patients with a negative result who are correctly diagnosed and is given by the equation: NPV = D / (C+D).

1.7 Diagnosis of VAP

The lack of a gold standard and the need for surrogate definitions is a major limitation in clinical practice and in research. The clinical features that indicate *suspicion* of VAP are all limited in terms of specificity and none of the sampling techniques used to obtain respiratory samples are 100% accurate. The implication of this at the bedside is that antibiotics may well be used unnecessarily. In fact amongst patients with suspected VAP, pneumonia is confirmed in approximately 40%(Meduri *et al.*, 1994; Hellyer *et al.*, 2015), although diagnosis based on culture is heavily dependent on sampling technique used(Conway Morris *et al.*, 2009).

Diagnostic strategies are generally divided into two approaches. The first is a “clinical” strategy based on clinical findings and generally in conjunction with culture of ETA. The benefit of this approach is the ease of obtaining an ETA, which is not a skilled task. The main issue is the lack of specificity and therefore the risk of over diagnosis of VAP and the potential for overuse of antibiotics. An alternative is an “invasive” sampling approach. Respiratory samples for culture are obtained by bronchoscopic methods and the diagnosis of VAP is determined by quantitative cultures. The benefit of these methods is that the distal site of infection is sampled and so is less vulnerable to contamination from upper airway colonisation. The problems with this strategy is that bronchoscopy is a skilled task that is not necessarily available, sampling in one region

of the lung could miss infection in another region and there is some risk associated with performing the procedures.

In this section we will consider the evidence base for the clinical diagnosis and then the accuracy of respiratory sampling.

1.7.1 Clinical diagnosis

The most widely recognised features of suspected VAP are fever; leucocytosis or leucopenia; purulent tracheal secretions and new or worsening CXR infiltrates. These form the basis of the clinical and surveillance definitions outlined previously. Several studies have examined the accuracy of these clinical features against proven VAP at autopsy. A consideration from autopsy studies, particularly in relation to clinical data, is that there is a temporal dissociation between the clinical data recorded, the histological findings and the episode of suspected VAP. For example clinical data collected just before death may not represent the onset of clinical suspicion of VAP and alternatively, histological findings at death may not represent the histological findings at the moment of suspicion of VAP.

Torres *et al* carried out a prospective study of 30 patients who died while receiving mechanical ventilation(Torres *et al.*, 1994). Following death, patients underwent a protocol of several standardised respiratory sampling techniques followed by lung biopsies from the same area that BAL and PSB were performed in, guided by the light of the bronchoscope. The protocol was performed in the area that corresponded with the maximum CXR infiltrate and on the contralateral side. Histological pneumonia was defined as a focus of polymorphonuclear cell accumulation in the bronchioles and adjacent alveoli. Using histological evidence of pneumonia as evidence for VAP, fever had a sensitivity of 55% and a specificity of 58%; purulent tracheal secretions had a sensitivity of 83% and specificity of 33% and CXR infiltrates had a sensitivity of 78% and specificity of 42%. Alternative diagnoses for pulmonary infiltrates included alveolar haemorrhage, alveolar damage, atelectasis and pulmonary fibrosis. There were no significant differences in WCC between the VAP and non-VAP groups.

A further prospective study of 40 patients who died while on mechanical ventilation found poor agreement between clinical features and pneumonia on histology(Kirtland *et al.*, 1997). Kappa values describe the level of agreement ranging from 0.00-0.20 as slight, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial and 0.81-1.0 as almost perfect(Landis and Koch, 1977) Temperature had a kappa of 0.15, worsening

CXR 0.04, a reduction in PaO_2:FiO_2 ratio 0.11 and raised white cell count 0.03. A limitation of this study is that clinical data were obtained 48-72hrs before death.

Since case definitions of VAP are a composite of clinical observations and investigations, Tejerina *et al* determined the diagnostic utility of case definitions against histologically proven VAP(Tejerina *et al.*, 2010). In a retrospective analysis of patients who had undergone autopsy following death on mechanical ventilation, 253 patients were included of which 142 had histological evidence of pneumonia. The clinical definitions evaluated were:

1. 'Loose definition' of VAP: a new of worsening CXR infiltrate in the context of at least 2 of the following 3 findings: temperature >38°C or <35.5^{o}C; WCC >10,000/µl or <4000/µl; or purulent tracheal secretions.
2. 'Rigorous definition' of VAP: the above criteria but all 3 findings required in the context of a new or worsening CXR infiltrate.
3. CPIS >6.

Of patients who had proven pneumonia, 64% met the loose definitions, only 13% met the rigorous definition and 43% had a CPIS >6.The sensitivity and specificity for the loose definition was 65% and 36% respectively; for the rigorous definition, 15.5% and 91% respectively; and for a CPIS > 6, 46% and 60% respectively.

A further study defined 'microbiologically active pneumonia' in the presence of positive lung tissue culture and positive histology(Fabregas *et al.*, 1999). Twenty-five patients who died while mechanically ventilated were included. A high proportion of patients, 92%, had histological evidence of pneumonia in at least 1 lung biopsy but 52% of patients had 'microbiologically active pneumonia'. The sensitivity and specificity for a CXR infiltrate was 92% and 33% respectively; for leucocytosis the sensitivity was 77% and specificity was 58%; for purulent secretions the sensitivity was 69% and specificity was 42%. The ATS/IDSA criteria (see **Table** 1) had a sensitivity of 69% and specificity of 75% and a CPIS > 6 a sensitivity of 77% and specificity of 42%. In this study 68% of patients received antibiotics in the 48 hours before death. It is possible that a proportion of patients without 'microbiologically active' VAP could have had VAP but were culture negative due to the high levels of antibiotics used in these patients.

These studies demonstrated a low sensitivity and specificity of the CPIS. In the derivation study a CPIS > 6 correctly identified VAP with a sensitivity of 93% and a

specificity of 100%(Pugin *et al.*, 1991). This has failed to be validated in further studies. The CPIS correlates poorly with the bacterial index of pathogens cultured from BAL(Schurink *et al.*, 2004) and a score >6 detected significant pathogen growth from BAL with a sensitivity of 60% and a specificity of 59%(Fartoukh *et al.*, 2003). In a prospective study the diagnostic performance of the CPIS to identify VAP was poor with an area under the receiver operator characteristic curve (AUROC) of 0.47 (95% CI 0.42-0.53)(Lauzier *et al.*, 2008).

Chest x-ray forms part of all clinical definitions of VAP. In a retrospective analysis the last CXR before death was examined against histology proven pneumonia(Wunderink *et al.*, 1992). Alveolar infiltrates were present in 80% of patients and air bronchograms in 57%. Of patients with ARDS, 53% had asymmetric infiltrates. The presence of either a single or multiple air bronchograms identified pneumonia with a sensitivity of 83% and a specificity of 58%. A single air bronchogram had the highest specificity at 96% but a sensitivity of only 17%. Alveolar infiltrates identified pneumonia with a sensitivity of 88% and a specificity of 26%. In the regression analysis a single air bronchogram was the strongest predictor of VAP with a RR of 3.19 but this model predicted only 64% of VAP.

Sensitivities and specificities for these clinical parameters are summarised in **Table 4**

Reference	↑WCC	Fever	Purulent secretions	CXR changes	CPIS >6	CXR change AND 2 of: Fever ↑WCC Purulent secretions	CXR change AND ALL of: Fever ↑WCC Purulent secretions
Torres 1994	NS	Sn 55% Sp 58%	Sn 83% Sp 33%	Sn 78% Sp 42%			
Tejerina 2010					Sn 46% Sp 60%	Sn 65% Sp 36%	Sn 15% Sp 91%
Fabregas 1999	Sn 77% Sp 58%	Sn 46% Sp 42%	Sn 69% Sp 75%	Sn 92% Sp 33%	Sn 77% Sp 42%	Sn 69% Sp 75%	Sn 23% Sp 92%
Pugin 1991					Sn 93% Sp 100%		
Fartoukh 2003					Sn 60% Sp 59%		
Wunderink 1992*				Sn 88% Sp 26%			

Table 4: Summary of sensitivity (Sn) and specificity (Sp) of clinical parameters used in the diagnosis of VAP. *sensitivity and specificity for a range of radiological signs reported in reference, 'alveolar infiltrates' presented in this table. NS, no significant difference between VAP and non-VAP

1.7.2 Respiratory sampling

Samples from the respiratory tract can be obtained by bronchoscopic or non-bronchoscopic techniques. Bronchoscopic techniques include BAL and PSB. Although a standardised approach to BAL has been reported(Meduri and Chastre, 1992), there is considerable variation in practice in the UK(Browne *et al.*, 2014). The use of quantitative culture is used to distinguish between culture growth from colonising bacteria and from pneumonia. To consider the diagnostic accuracy of these techniques, data from human autopsy studies will be considered.

In a prospective study, 26 patients who died while receiving mechanical ventilation underwent PSB immediately post-mortem in the left lower lobe followed by thoracotomy and collection of peripheral lung specimens(Chastre *et al.*, 1984). Six patients had histological evidence of pneumonia and all of these patients had growth of a pathogen at $>10^4$ colony forming units per gram (cfu/g) of tissue. The 20 patients without pneumonia either had a sterile culture or growth $<4x10^3$ cfu/g. The correlation of PSB cfu/ml to tissue cfu/g was r=0.57. For patients on antibiotics this was lower (r=0.55) and for the 6 patients with pneumonia the correlation was higher (r=0.77). Operating characteristics of PSB fluid culture (cfu/ml) were determined to identify cases with a tissue culture of $>10^4$ cfu/g or histological pneumonia. Using a cut-off of $>10^3$ cfu/ml for PSB, the sensitivity to detect pneumonia (by histology) was 100% but specificity was 60% and to detect tissue culture of $>10^4$ cfu/g, the sensitivity was 100% and specificity was 61%. Excluding patients receiving antibiotics increased the specificity for detecting pneumonia to 87% and detecting lung tissue culture to 76%.

In a seminal paper, Chastre *et al* aimed to give a comprehensive description on the relationship of histological pneumonia to its microbiology(Chastre *et al.*, 1995). Twenty patients who died while undergoing mechanical ventilation underwent immediate BAL and PSB before thoracotomy and tissue specimens from corresponding segments were collected. In order to reduce confounding factors, patients were excluded if antibiotics had been commenced within 3 days of death or if the patient had previously developed bacterial pneumonia. Therefore patients only had pneumonia if it was acquired in the terminal phase of the patients' condition. The correlation between tissue cfu/g and histology was r=0.79. All patients with moderate to severe pneumonia had a tissue cfu > 10^4/g. Segments with mild pneumonia had tissue culture that ranged from sterile to $>10^4$

cfu/g. There were strong correlations between PSB and BAL culture and tissue culture (r=0.67 and r=0.75 respectively). Operating characteristics were determined for BAL or PSB (cfu/ml) to detect significant tissue microbiology (>10^4 cfu/g). For BAL at a threshold of >10^4 cfu/ml the sensitivity was 91% and specificity was 78%; for PSB at a threshold of >10^3 cfu/ml the sensitivity was 82% and specificity was 77%.

These papers represent the strongest evidence for the accuracy of invasive sampling and the relationship between the BAL/PSB sample and tissue culture and histology. However some important limitations must be considered. Firstly these studies have not studied a population of *suspected* VAP. Although pneumonia was evidenced on histology, how this corresponds to clinical suspicion is uncertain. By definition these were groups of severely ill and terminal patients. Secondly the BAL and PSB thresholds proposed by Chastre(Chastre *et al.*, 1995) have been widely accepted but it must be acknowledged that these were derived against a tissue culture threshold that does not encompass *all* pneumonia, failing to include mild pneumonia. How clinical features of pneumonia differ between patients with mild, moderate or severe pneumonia on histology is not known nor is it known whether all patients with some evidence of pneumonia at histology receive antibiotic treatment. Clinical outcomes have been shown to be different in patients above and below these thresholds(Fagon *et al.*, 1996) suggesting this is a clinically relevant distinction. Furthermore managing patients as non-VAP if culture growth is below these thresholds and discontinuing antibiotics early is not associated with worse outcome and may have benefits in terms of reducing antibiotic resistance and reducing antibiotic prescribing(Fagon *et al.*, 1988, 2000; Raman *et al.*, 2013).

Further cadaveric studies that aimed to validate the findings of Chastre *et al* have failed to report the same correlations of tissue culture to histology and the same diagnostic accuracy with BAL and PSB(Torres *et al.*, 1994; Marquette *et al.*, 1995; Kirtland *et al.*, 1997; Fabregas *et al.*, 1999). These studies have generally not controlled for antibiotic use or the presence of pre-existing pneumonia but are arguably a more clinically relevant population. The tissue culture threshold of >10^4 cfu/g was found to have a sensitivity of 27% and specificity of 66% in one study(Torres *et al.*, 1994) and a sensitivity of 11% and specificity of 93% in another (Kirtland *et al.*, 1997) against histological pneumonia. In a study by Rouby *et al,* 35% of patients with histological pneumonia were culture negative and 49% of culture positive patients had no histological evidence of pneumonia(Rouby *et al.*, 1992). However the use of antibiotics

in these studies could have resulted in falsely low or negative cultures(Fabregas *et al.*, 1996). The sensitivity and specificity of BAL ranged from 11-50% and 45-100% respectively; for PSB the sensitivity and specificity ranged from 33-83% and 50-100% respectively(Torres *et al.*, 1994; Marquette *et al.*, 1995; Kirtland *et al.*, 1997; Fabregas *et al.*, 1999). For blind mini-BAL the operating characteristics were report at a sensitivity of 70% and specificity of 69%(Rouby *et al.*, 1994). Endotracheal aspirate was evaluated in two studies which reported sensitivity from 69-87% and a specificity of 31-92%(Kirtland *et al.*, 1997; Fabregas *et al.*, 1999).

Table 5 summarises the diagnostic performance of respiratory sampling methods reported.

Reference	BAL	PSB	ETA	Blind-BAL/ Bronchial aspirate	Blind PSB
Fabregas 1999	Sn 39%* Sp 100%	Sn 62% Sp 75%	Sn 69% Sp 92%		
Kirtland 1997	Sn 11% Sp 80%	Sn 33% Sp 63%			Sn 22% Sp 77%
Chastre 1995	Sn 91% Sp 78%	Sn 82% Sp 77%			
Chastre 1984		Sn 100% Sp 61%			
Marquette 1995	Sn 47% Sp 100%	Sn 56% Sp 87%	Sn 56% Sp 86%		
Torres 1994	Sn 50% Sp 45%	Sn 36% Sp 50%		Sn 44% Sp 48%	
Rouby 1992				Sn 70% Sp 69%	

Table 5: Summary of diagnostic performance of difference respiratory sampling methods. Sensitivity (Sn) and Specificity (Sp). Reference criteria used by Chastre et al (1984, 1995) tissue culture of >10^4 cfu/g, Fabregas 1999, 'microbiologically active pneumonia'. Remaining studies use histology proven pneumonia.* Sensitivity and specificity for 'protected BAL'.

1.7.3 Clinical versus invasive diagnostic strategy: RCT evidence

Professional bodies have previously recommend the use of invasive respiratory sampling as best practice(American Thoracic Society, 2005) due to the higher specificity to exclude false positive cultures from proximal airway colonisation. More recently this recommendation has been challenged by a meta-analysis that did not support the use of invasive sampling(Berton, Kalil and Teixeira, 2012) and updated guidelines no longer recommend invasive sampling(Kalil *et al.*, 2016). It may not be surprising therefore, that there is considerable variation in practice around respiratory sampling(Koulenti *et al.*, 2009; Browne *et al.*, 2014). Clearly improvement in patient outcome is the predominant drive for adoption into clinical practice. There have been 5 RCTs that have compared bronchoscopic methods to clinical/non-bronchoscopic methods. The use of an invasive approach was invariably combined with quantitative culture; however these trials differ in whether the control arm included non-invasive sampling with quantitative culture or qualitative culture.

Of the 5 trials, two dominate the discussion, and have conflicting results(Fagon *et al.*, 2000; Canadian Critical Care Trials Group et, 2006). In a French multi-centre trial of 413 patients conducted over 31 ICUs, patients were randomised to a treatment algorithm of antibiotic initiation or de-escalation based on invasive sampling (BAL or PSB) or to a clinical management approach (ETA)(Fagon *et al.*, 2000). In both the invasive and the non-invasive groups the decision algorithm recommended antibiotics at two decision points, the immediate Gram stain result and later culture results. Cultures were positive from PSB in 37% of patients, for 34% of BAL and 86% of ETA. The mortality at 14 days was significantly lower in the invasive group over the non-invasive group (16.2% vs 25.8%, p=0.022). Furthermore there were significantly more antibiotic-free days (AFD) at 14 days in the invasive group over the non-invasive group with 5.0 +/- 5.1 and 2.2 +/- 3.5 respectively (p<0.001). Fourteen per cent of the invasive group did not receive any antibiotics up to 28 days in comparison to only 2 patients in the non-invasive group. There was no significant difference in ICU or hospital LOS or duration of mechanical ventilation.

In this trial, considering only those with negative cultures, 85% in the invasive group and 60% of the clinical group did not receive antibiotics. This degree of antibiotic stewardship in light of negative cultures is not reflected in other trials(Ruiz *et al.*, 2000; Solé Violán *et al.*, 2000; Canadian Critical Care Trials Group et, 2006). The algorithm used in the trial reflects best practice of de-escalation or discontinuation of antibiotics

following culture results. Furthermore the mortality difference could be explained by the fact that the invasive group had fewer patients receiving inappropriate antibiotics that failed to treat the cultured pathogens (inadequate treatment in 24 patients (11%) in the non-invasive group versus 1 patient in the invasive group).

The adequacy of treatment of VAP is inextricably linked to outcome and so any trial intervention must ensure that treatment adequacy is not a confounding factor. A large multi-centre trial conducted in Canada went some way to accommodate for this confounding factor ensuring that all patients got broad-spectrum empiric antibiotics(Canadian Critical Care Trials Group et, 2006). Seven hundred and forty patients from 28 ICUs were randomised in a 2 by 2 factorial design RCT. Factorial RCTs randomise on two levels to two interventions that are felt not to interact. In this trial the levels of randomisation were invasive sampling with BAL and quantitative culture versus clinical diagnosis and ETA; the second level was randomisation to monotherapy or combination therapy. This trial had important exclusions that limit its generalisation into routine practice. Patients were excluded if colonised with *S. aureus* or *Pseudomonas* spp., if allergic to the trial antibiotics (ciprofloxacin or meropenem) or if they had received the trial antibiotics prior to randomisation (within 24 hours for ciprofloxacin and within 7 days for meropenem). The protocolised antibiotics resulted in equally high degree of adequacy of treatment in both groups (89% in BAL group and 89.5% in clinical group).

In contrast to the French trial, which showed a considerable difference in positivity rate of microbiology culture, this trial found that BAL was positive in 60% of cases and ETA was positive in 52% of cases. There was no significant difference in mortality at 28 days with a relative risk reduction of 1.01 (95% CI 0.75-1.37). There was no difference in duration of mechanical ventilation or length of ICU or hospital stay. Furthermore there was no significant difference in number of days alive and without antibiotics between the BAL group and ETA group (10.4 +/- 7.5 and 10.6 +/- 7.9 respectively). However at day 6, 74.6% of the ETA group and 74.2% of the BAL group were receiving antibiotic therapy, which suggests that opportunities for discontinuation of antibiotics in the context of negative cultures were not taken. The authors do state that the decision to continue antibiotics in the context of a culture growth of $<10^4$ cfu/ml was, pragmatically, left to the treating physician. In addition the diagnosis of VAP was adjudicated retrospectively and the authors do state that any potentially pathogenic organism regardless of the number of cfu/ml was considered as a positive culture.

Three smaller single centre RCTs have also been carried out. Sanchez-Nieto *et al* reported a pilot study of 51 patients who were randomised to either invasive sampling by BAL and PSB with quantitative culture or ETA with quantitative culture(Sanchez-Nieto *et al.*, 1998). The use of quantitative culture in the control arm does not necessarily represent standard care. This study also reported high rates of positive culture, occurring in 67% of BAL, 58% of PSB and 67% of ETA. Although the trial was not powered at 51 patients to detect a mortality difference, there was no significant difference in mortality between the two groups. There was also no significant difference in LOS and duration of mechanical ventilation. The number of antibiotic days was not measured but there were more antibiotic modifications in the invasive group over the non-invasive group although whether this lead to fewer antibiotic days or more targeted therapy was not reported.

The trial by Fagon *et al* highlights the potential value different diagnostic methods could have on antibiotic prescribing. In a trial by Ruiz *et al*, patients were randomised to either invasive or non-invasive management(Ruiz *et al.*, 2000). In this trial antibiotics were continued if cultures were negative but clinical suspicion of VAP remained. The duration of antibiotic therapy was the same for both groups (12 +/- 4 days in the non-invasive group and 13 +/- 4 days in the invasive group). There was no difference in mortality, ICU LOS or duration of mechanical ventilation. Again the rates of positive culture were similar in both groups with 51% in the non-invasive group and 60% in the invasive group. Surprisingly all PSB performed had a positive culture and 50% of BAL had a positive culture.

In another single-centre RCT, 91 patients were randomised to either invasive or non-invasive sampling(Solé Violán *et al.*, 2000). The rate of culture positivity for the invasive group was 44% in comparison to 76% of the ETA group. There was no significant difference in mortality, ICU LOS or duration of mechanical ventilation. Although there were more antibiotic modifications in the invasive group over the non-invasive group (33% vs 12%), the number of antibiotic days was not reported. In fact the authors state that antibiotics were not altered following negative cultures.

The 5 RCTs are summarised in **Table 6**.

Reference	Sample size	Trial arms	Primary outcome(s)	Mortality	Antibiotic management
Fagon 2000	413	BAL/PSB vs clinical + ETA	Mortality at 14 days AFD at 14 days	Mortality difference -9.6 (-17.4 - -1.8) P=0.022	AFD difference 2.8 (1.9-3.6) P<0.001
CCCTG 2006	740	BAL vs clinical + ETA	28-day mortality	Relative risk of death in BAL vs ETA group: 1.01 (0.75-1.37) NS	Days alive without antibiotics: BAL 10.4+/-7.5 ETA 10.6+/-7.9
Ruiz 2000	76	BAL/PSB vs ETA	30-day mortality	Mortality ETA 46%, BAL 38% NS	Duration of antibiotics ETA 12+/-4 BAL/PSB 13+/-4
Sanchez-Nieto 1998	51	Quantitative ETA/BAL/PSB vs quantitative ETA (QEA)	Crude mortality	BAL/PSB mortality 46%, QEA mortality 26% NS	Antibiotic modifications: BAL/PSB group 42% QEA 16%.
Solé Violán 2000	91	Quantitative culture BAL or blind BAL vs clinical + ETA	Mortality Antibiotic change	Mortality: Quantitative 22.2% Qualitative 20.9% NS	Narrower spectrum change in 10 patients in quantitative group vs 3 in qualitative group

Table 6: Comparison of trials evaluating invasive sampling in comparison to non-invasive sampling. QEA, quantitative ETA

In a Cochrane review, the use of quantitative versus qualitative cultures was reviewed for clinical outcomes(Berton, Kalil and Teixeira, 2012). This review is as much a review of invasive versus non-invasive sampling. This meta-analysis concludes that there is insufficient evidence in terms of 28-day mortality (risk ratio 0.91, 95% CI 0.75-1.11), length of ICU stay (risk ratio 0.58, 95% CI -0.51-1.68) or antibiotic changes (risk ratio 1.53, 95% CI 0.54-4.39) to suggest that an invasive strategy is superior over a non-invasive strategy. However it is important to note that this review did not include the findings of Fagon *et al*(Fagon *et al.*, 2000) regarding antibiotic reductions because no other study measured AFD. The authors highlight that to confirm the risk ratio reduction in mortality suggested in this meta-analysis of 9% would require a trial of 7500 patients. It is therefore futile to make further attempts to demonstrate a mortality difference. There are however other relevant outcomes, and in the context of VAP antibiotic stewardship endpoints are important.

The CPIS was used in a RCT as a strategy to improve antibiotic stewardship in patients with new CXR infiltrates on a surgical ICU(Singh *et al.*, 2000). Patients with a CPIS > 6 were excluded from the study and they received treatment for pneumonia (despite the same group previously showing that only 50% of patients with CPIS > 6 had pneumonia(Singh *et al.*, 1998)). Patients with a CPIS ≤6 were randomised to receive either standard care (10-21 days of antibiotics) or ciprofloxacin for 3 days followed by a reassessment of CPIS at day 3 and discontinuation of antibiotics if the CPIS remained below 6. This study showed a reduction in antibiotic use in the CPIS-guided early discontinuation arm, a reduction in antibiotic-resistant pathogens, and a reduction in ICU length of stay, while maintaining equivalent outcomes in terms of mortality. There are several limitations to this study. Firstly only 58% of the patients were mechanically ventilated and it is important to highlight that the CPIS has not been derived or validated in a non-ventilated population. Secondly this study excluded patients with a CPIS of > 6 at screening and so does not really address the clinical problem of the overuse of antibiotics in patients with suspected VAP as it can be presumed that a significant proportion of those patients would not go on to have confirmed VAP. Although this study did result in a reduction in antibiotics, it highlights the extent of overuse of antibiotics for suspected VAP. A strategy of invasive BAL sampling and discontinuation of antibiotics following negative culture is likely to have a greater impact on reduction of antibiotics(Luyt, Chastre and Fagon, 2004).

1.8 Prevention of VAP

The pathogenesis of VAP has been previously discussed (section 1.4) in terms of the changes in bacterial colonisation of oropharynx, subglottic area, sinuses and gastrointestinal (GI) tract and then subsequent translocation to lower respiratory tract. Altering this process could prevent VAP. The ability to reduce VAP rates through preventative measures has had a significant effect on how this HCAI is perceived, with a growing sense that this is an entirely preventable disease(Klompas, 2010). Since VAP occurs in the context of critical illness which is associated with immune dysfunction(Conway Morris *et al.*, 2013), the ability to completely prevent this condition seems unlikely. Strategies can be considered as general approaches, strategies to prevent aspiration, strategies to prevent contamination of respiratory equipment and strategies to reduce colonisation.

1.8.1 General measures

Strategies to prevent VAP must be embedded in an environment of good hygiene practices to reduce the spread of HCAI by healthcare workers. The importance of hand hygiene in the reduction of HCAI is widely accepted(Pittet *et al.*, 2000). Similarly a general approach to minimise or avoid intubation and mechanical ventilation is important to avoid the specific intervention that is implicated in this HCAI. Patients are generally sedated while mechanically ventilated, for comfort. At a point when it is felt that the patient has recovered sufficiently to attempt to breathe on their own and ultimately be extubated, the sedation is either reduced or stopped and the patient undergoes a 'spontaneous breathing trial' (SBT). Protocolised daily sedation interruption (DSI) to the point that the patient wakes with an assessment made of whether sedation should be restarted, in comparison to clinician-guided management of sedation, resulted in significantly reduced duration of mechanical ventilation and ICU stay(Kress *et al.*, 2000). In a further RCT, SBT were undertaken in conjunction with DSI(Girard *et al.*, 2008). In the control group sedation practice was left to the discretion of the clinician, to maintain a level of arousal felt appropriate to the patient. An assessment was made on the safety of performing a SBT and if appropriate the SBT was undertaken. Decisions to extubate based on the success of the SBT were left to the discretion of the clinical team. In the intervention arm, sedation was stopped each day and if patients tolerated being awake, a SBT was undertaken. There were a greater number of ventilator-free days, shorter duration of ICU stay, shorter duration of hospital stay and reduced risk of death in the first year in the intervention arm.

In a meta-analysis of weaning protocol interventions, protocolised weaning was associated with a 25% (95% CI 9-39%) reduction in the mean duration of mechanical ventilation(Blackwood *et al.*, 2011). In a recent non-randomised study, SBT and DSI were implemented to determine the impact on VAC(Klompas *et al.*, 2015). The study found that VAC and IVAC were reduced per episode of mechanical ventilation (rather than in ventilator-days) with an OR 0.63 and 0.35 respectively. While this study did not prevent 'VAP', it does support the concept that time off the ventilator reduces the time at risk of the complications of mechanical ventilation.

With a similar purpose of minimising time on mechanical ventilation, non-invasive ventilation (NIV) has been used to assist in extubation from mechanical ventilation. NIV provides bi-level positive airway pressure via a tightly fitting facemask or hood. Two meta-analyses of randomised trials and quasi-randomised trials of NIV in comparison to invasive weaning strategies(Burns, Adhikari and Meade, 2006; Burns *et al.*, 2009) have demonstrated a benefit in patients who have undergone weaning via NIV with a reduction in mortality (RR 0.55, 95% CI 0.38-0.79), VAP (RR 0.29, 95% CI 0.19-0.45), ICU LOS (mean difference 6.27 days, 95% CI 3.78-8.77), hospital LOS (mean difference 7.19 days, 95% CI 3.58-10.80) and invasive mechanical ventilation (mean difference 7.81 days, 95% CI 4.31-11.31)(Burns *et al.*, 2009). The trials included in the meta-analyses were relatively small ranging from 21 to 90 participants and the majority of trials only included patients with chronic obstructive pulmonary disease (COPD). While it is unknown whether these findings can be generalised to a general ICU population, it does again support the concept that avoiding mechanical ventilation reduces its complications.

1.8.2 Prevention of aspiration

Aspiration of either oropharyngeal or gastric contents is implicated in the pathogenesis of VAP and strategies to prevent this are important(Estes and Meduri, 1995). In an observational study of patients nursed both in a supine position (0°) or at a 45-degree semi-recumbent position, a technetium (Tc)-99m sulphur colloid was instilled into the stomach via a nasogastric tube(Torres *et al.*, 1992). Bronchial secretions were sampled at intervals between 0 and 300 minutes. Radioactivity was measured in counts per minute (cpm) and there were higher cpm in the supine group over the semi-recumbent group, which rose steeply between 240 and 300 minutes in the supine group. In a RCT, patients were randomly assigned to being nursed in a supine position (0°) or in a semi-recumbent position (45°)(Drakulovic *et al.*, 1999). The primary outcome measure was

clinically suspected VAP and the trial was powered to show a 50% risk reduction by the semi-recumbent position requiring 182 patients. A secondary endpoint was microbiologically confirmed VAP which could be confirmed by a pathogen in either an ETA or by a bronchoscopic method (PSB or BAL). The trial was stopped at its interim analysis after randomisation of 90 patients due to a significant risk reduction in the semi-recumbent position group in clinically suspected VAP of 76% and microbiologically confirmed VAP at 78%. A significant limitation of this trial was the potential for bias, in that the primary endpoint of clinically suspected VAP is highly subjective and the trial had no means of blinding. One key issue with the external validity of this trial is that patients are rarely nursed completely supine but generally with some degree of elevation. In a further RCT patients were randomised to nursing at 10° elevation (supine) or 45° elevation (semi-recumbent) and the degree of bed elevation was monitored(van Nieuwenhoven *et al.*, 2006). The primary endpoint was confirmed VAP by criteria based on culture from BAL fluid. In this trial of 221 patients, one of the key outcomes was the feasibility of nursing patients at a 45° position. In the supine group bed elevation was $9.8^{o} \pm 3.9$ on day 1 and at day 7 was $14.8^{o} \pm 7.1^{o}$. In the semi-recumbent group the bed elevation was $29.2^{o} \pm 10.3^{o}$ on day 1 and $26.5^{o} \pm 8.2^{o}$ on day 7. In this trial there was no significant difference in confirmed VAP between the two trial arms. Although the exact degree of bed elevation is unclear, patients are almost universally nursed with some degree of elevation and an elevation of 30° is widely recommended(Lorente, Blot and Rello, 2007) with little controversy since this is a simple measure to implement.

Secretions above the ETT cuff are able to leak past the cuff, reaching the lower respiratory tract(Seegobin and van Hasselt, 1986). The ETT cuff is the predominant barrier and the pressure of this has to be sufficient for it to function in this regard. In an observational study of 83 patients primarily investigating the value of subglottic suction(Rello *et al.*, 1996), the investigators found that antibiotic use and subglottic suction was preventative against VAP. There was a trend towards patients with cuff pressures of $<20cmH_20$ to be at greater risk of VAP (RR 2.57, 95% CI 0.78-8.03) and in a multivariate analysis of patients who were not on antibiotics, a cuff pressure of $<20cmH_2O$ was independently associated with VAP (RR4.23, 95% CI 1.12-15.92).

Once the ETT cuff is inflated there is a space between the vocal cords and the top of the cuff and in this subglottic area secretions can accumulate and be aspirated into the airways. This area cannot be suctioned by conventional methods and so specialised ETT

are used that have a suction port to this space. The clinical benefits of subglottic secretion drainage (SSD) have now been evaluated in three meta-analyses(Dezfulian *et al.*, 2005; Muscedere *et al.*, 2011; Wang *et al.*, 2012). The first meta-analysis of 5 RCTs incorporating a total of 896 patients showed that SSD was associated with a relative risk reduction of VAP of 0.51 (95% CI 0.37-0.71)(Dezfulian *et al.*, 2005). In the sensitivity analysis, excluding a single trial that caused the heterogeneity in the group, SSD reduced duration of mechanical ventilation by 2 days (95% CI 1.7-2.3 days) and length of ICU stay by 3 days (95% CI 2.1-3.9). This meta-analysis was updated in two further meta-analyses. In the meta-analysis by Wang *et al* of 10 RCTs, SSD was associated with a RR reduction of 0.56 (95% CI 0.45-0.69), reduced duration of mechanical ventilation by 1.55 days (95% CI -2.40 - -0.71) and prolonged time until VAP by 3.9 days (95% CI 2.56-5.24)(Wang *et al.*, 2012). Similar to Dezfulian *et al*, the main effect appears to be on reducing early VAP rather than late VAP. In the meta-analysis by Muscadere *et al*, the risk ratio for VAP was similarly lower in the SSD group at 0.55 (95% CI 0.46-0.66) in a meta-analysis of 13 RCTs with no heterogeneity (I^2 0%)(Muscedere *et al.*, 2011). In this meta-analysis, not only was there an increased time until onset of VAP and shorter duration of mechanical ventilation, but also a reduced length of ICU stay (-1.52 days, 95% CI -2.94- -0.11). SSD represents a preventative strategy with one of the strongest evidence bases for its use. It is now recommended in VAP prevention guidelines(NICE, 2008; Institute for Healthcare Improvement, 2012) but despite this it is not widely used in UK ICUs(Baldwin, Gray and Chequers, 2014).

1.8.3 Prevention of contamination of equipment

Patients who are mechanically ventilated bypass the natural humidification process of the upper airways. Inspired gases must be humidified to allow normal respiratory tract function and prevent respiratory secretions becoming tenacious, which could have the potentially catastrophic consequence of ETT occlusion. Inspired gases can be humidified by two methods, the first of which is heat humidification (HH), which passes gases over a reservoir of heated water and in addition may have a heated coil in the inspiratory limb of the circuit to prevent condensation. The second method is a heat and moisture exchanger (HME). This is a filter placed at the ETT that has a hygroscopic salt (calcium or lithium chloride) that absorbs the patient's water vapour during expiration and then releases it on inspiration, thereby conserving the patient's own moisture and heat. While humidification is essential there is also concern that humidification can act as a source of contamination and result in VAP. In two early

trials of HME and HH, patients in the HME groups had occlusion or partial occlusion of the ETT, which led to the death of a patient in one trial(Martin *et al.*, 1990; Roustan *et al.*, 1992). A number of RCTs conducted since that have not found a signal for harm from HME use(Dreyfuss *et al.*, 1995; Boots *et al.*, 1997; Hurni *et al.*, 1997; Kirton *et al.*, 1997; Kollef *et al.*, 1998; Memish *et al.*, 2001; Thomachot *et al.*, 2002; Hess *et al.*, 2003; Lacherade *et al.*, 2005; Lorente *et al.*, 2006). As with many VAP trials, a number of RCTs are limited by diagnostic definitions, and outcomes based on clinical definitions are inherently at risk of bias. In a single-centre RCT of 164 patients in which VAP was defined based on BAL and PSB culture, no difference in VAP rates or in pharyngeal colonisation was found between HH and HME groups(Dreyfuss *et al.*, 1995). Furthermore in a multi-centre RCT of 369 patients, with a similar rigorous definition of VAP, no difference in VAP rates was found between HH and HME(Lacherade *et al.*, 2005). A meta-analysis of 8 RCTs found that VAP was lower in the HME group with an OR of 0.69 (0.51-0.94). When sub analyses were performed this was only significant in patients ventilated for greater than 7 days and in patients with a clinical diagnosis of VAP but not a microbiological diagnosis of VAP(Kola, Eckmanns and Gastmeier, 2005). In a further large meta-analysis of 33 trials, of which 27 were in adults, 25 were parallel design and 8 were a cross-over design, no difference was found in outcomes of pneumonia, airway occlusion or mortality between HH and HME(Kelly *et al.*, 2010). In subgroup analyses there was no difference in just adults or paediatric cases or between hydrophobic or hygroscopic HME filters.

Suctioning of respiratory secretions is critical in the care of ventilated patients. Open suction units require disconnecting the patient from the ventilator to introduce a single use suction catheter. This has a number of disadvantages including disruption to ventilation, loss of positive end expiratory pressure (PEEP) and exposure of healthcare workers to respiratory secretions. Closed suction units are placed in the circuit and allow suctioning without disconnection from the ventilator. Despite concerns that closed suction units could become contaminated and therefore be a risk factor for VAP, a meta-analysis did not find an increased risk of VAP between either method of suctioning(Subirana, Solà and Benito, 2007).

Since inspired gases are humidified, the condensate in the ventilator circuit runs the risk of becoming contaminated. For this reason it was practice in the 1980's for circuits to be changed every 24 hours to prevent contamination, however this frequency was found to be a risk factor for VAP in comparison to changes every 48 hours(Craven *et al.*,

1986). This may be due to increased manipulation of the circuit and therefore the risk of contaminated secretions entering the bronchial tree via the lumen of the ETT. Several studies have investigated the interval that the circuits should be changed at. In an observational study that used circuit changes at 2-day intervals, 7-day intervals and 30-day intervals, each for 1 year, the highest VAP rate was in the 2-day interval period at 11.88 cases per 1000 ventilator days and 3.34 and 6.28 per 1000 ventilator days in the 7- and 30-day interval periods respectively(Fink *et al.*, 1998). In two RCTs one using HH(Kollef *et al.*, 1995) and another using HME humidification(Lorente *et al.*, 2004), routine 7 day changes were compared to no routine changes. Ventilator circuits were changed at the discretion of clinicians if faulty or visibly soiled. In both trials there were no significant differences in rates of VAP between the two trial arms, suggesting that routine changes are unnecessary and costly. In the trial by Kollef *et al*, patients receiving circuit changes every 7 days had 247 circuit changes at the cost of $7410 in comparison to the group with no routine changes, which had a total of 11 circuit changes costing $330(Kollef *et al.*, 1995).

1.8.4 Prevention of colonisation: oral intubation

The preventative steps outlined so far aim to prevent aspiration of a pathogen. Preventative measures are also aimed at reducing colonisation with potential pathogens so as to reduce the bacteria at the sources of infections. The potential sites of colonisation are the sinuses, the oropharynx and the gastrointestinal tract. Patients can either be intubated via the nasal or oral routes. Nasal intubation has the advantage over oral intubation in that it is better tolerated in patients on less sedation and it removes the risk of patients biting the ETT. The main disadvantage however, is the risk of developing infective sinusitis, which could result in infective secretions bypassing the ETT. Reports are conflicting on the significance of nasal intubation as a risk factor for VAP. In a trial of 111 patients randomised to either nasal or oral intubation, sinusitis was diagnosed based on portable x-rays being performed of the head(Salord *et al.*, 1990). X-ray was performed before intubation, at day 3, day 7 and each week until extubation. Maxillary sinusitis occurred in 1.8% of the oral intubation group and in 43% of the nasal intubation group. This trial did not measure the influence on VAP. In a larger trial of 300 patients, patients were randomised to oral or nasal intubation(Holzapfel *et al.*, 1993). Patients were followed up for clinical signs of sinusitis including purulent nasal discharge and pyrexia. CT scans were performed every 7 days or if there were clinical signs of sinusitis. If an air-fluid level was present

on CT, a transnasal puncture was taken for culture. VAP was diagnosed based on microbiology data from PSB sampling. This trial did not find a significant difference in the rates of sinusitis (which were 19% in the nasal group and 17% in the oral group) or in VAP (11% of the nasal group and 6% in the oral group). Despite the inconsistency in the evidence, oral intubation is widely recommended in guidelines(Lorente, Blot and Rello, 2007).

1.8.5 Prevention of colonisation: oral antiseptics

The use of oral biocides, such as chlorhexidine, to reduce oropharyngeal colonisation is another strategy that has been widely investigated. The use of chlorhexidine oral care has become almost ubiquitous and has been recommended in national guidelines(NICE, 2008; Institute for Healthcare Improvement, 2012). Meta-analyses are conflicting and this requires a reassessment of these recommendations. In a meta-analysis by Chan *et al*, 11 RCT of oral decontamination were analysed(Chan *et al.*, 2007). These included 4 trials of oral antibiotics and 7 trials of oral antiseptics. There was no significant difference in incidence of VAP associated with oral antibiotics but there was with oral antiseptics with a RR of 0.56 (95% CI 0.39-0.81). In contrast to this, a meta-analysis of 7 RCTs of chlorhexidine did not demonstrate a significant reduction in VAP (RR 0.70, 95% CI 0.47-1.04)(Chlebicki and Safdar, 2007). The trials included in these meta-analyses incorporated trials in cardiac surgery patients and general/mixed ICU patients. The distinction between these two groups of patients is crucial in the emerging controversy surrounding this area. Cardiac surgery patients are generally mechanically ventilated for 24-48 hours whereas general ICU patients are often ventilated for longer periods. In a further meta-analysis of trials of the oral antiseptics chlorhexidine or povidone-iodine, the total risk ratio was 0.67 (95% CI 0.50-0.88) in favour of oral antiseptic(Labeau *et al.*, 2011). In the subanalysis, however, this was only significant in the cardiac surgery population with a risk ratio of 0.41 (95% CI 0.17-0.98). In the mixed ICU population the risk ratio was 0.77 (95% CI 0.58-1.02) and in the surgical/trauma population the risk ratio was 0.38 (95% CI 0.13-1.10). In this meta-analysis, two RCTs in cardiac surgery patients accounted for 37% of patients in the meta-analysis(DeRiso *et al.*, 1996; Houston *et al.*, 2002). Trials in this area are limited by inconsistent definitions of VAP and other methodological limitations. De Riso *et al* carried out a double-blinded placebo-controlled trial of 350 patients in a cardiac ICU(DeRiso *et al.*, 1996). The diagnosis of VAP was based on clinical findings and radiological signs. The duration of mechanical ventilation was not reported but the duration of ICU stay was 7.9 and 8.5

days in the chlorhexidine and control groups respectively. They demonstrated a reduction in all respiratory infections by 69%, which included criteria for VAP and tracheobronchitis. When subdivided between upper and lower respiratory tract infection, there were no significant differences. The trial by Housten *et al*(Houston *et al.*, 2002) also has a significant influence on the meta-analyses including 561 cardiac ICU patients. The authors report that few patients received more than two doses of chlorhexidine because of early extubation, however the average duration of mechanical ventilation is not reported, although clearly it was short. This trial did not find any difference in VAP rates between the two arms. Another large RCT in cardiac ICU, randomised 991 patients to chlorhexidine or placebo(Segers *et al.*, 2006). The mean duration of ICU stay was only 1.2 days. The primary endpoint was all nosocomial infections, which were significantly lower in the chlorhexidine group than the control group (19.8% vs 26.2%, absolute risk reduction 6.4%, 95% CI 1.1-11.7%). Lower respiratory tract infections, based on CDC criteria, were reduced with an absolute risk reduction of 6.5% (95% CI 2.3-10.7%).

Trials in a mixed ICU or surgical/trauma ICU setting have resulted in inconsistent results. In a trial of 385 patients from general ICUs from 5 hospitals in the Netherlands, patients were randomised to either chlorhexidine, chlorhexidine and colisitin, or placebo(Koeman *et al.*, 2006). BAL was encouraged for diagnosis but not mandated and VAP definition was based on clinical and radiological criteria. The trial was stopped early due to meeting stopping criteria for superiority of chlorhexidine over placebo. The daily risk of VAP was reduced by chlorhexidine by 65% (hazard ratio (HR) 0.352, 95% CI 0.160-0.791) and by chlorhexidine and colistin by 55% (HR 0.454, 95% CI 0.223-0.925). A number of other studies of lower methodological quality support this finding(Fourrier *et al.*, 2000; Seguin *et al.*, 2006; Özçaka *et al.*, 2012). In contrast however, other trials did not find a significant benefit from oral chlorhexidine, although a number of them have methodological limitations(Grap *et al.*, 2004; Fourrier *et al.*, 2005; Tantipong *et al.*, 2008; Panchabhai *et al.*, 2009; Scannapieco *et al.*, 2009; Berry *et al.*, 2011).

Two recent meta-analyses of chlorhexidine in non-cardiac surgery patients have produced very different findings from the earlier meta-analysis. In a meta-analysis of 16 trials, cardiac surgery patients accounted for 51% of patients in the population(Klompas *et al.*, 2014). There was significantly less VAP in cardiac patients receiving chlorhexidine (RR 0.56, 95% CI 0.41-0.77). In the non-cardiac patients the reduction in

VAP was not significant (RR 0.78, 95% CI 0.60-1.02) and the point estimate was higher in blinded studies (RR 0.88, 95% CI 0.66-1.16). In terms of mortality there was a non-significant trend towards increased mortality in non-cardiac patients receiving chlorhexidine with a RR 1.13 (95% CI 0.99-1.29). Price *et al* performed a meta-analysis to determine the role of selective decontamination of the digestive tract, selective oropharyngeal decontamination and topical chlorhexidine in the prevention of death on the general ICU population(Price, Maclennan and Glen, 2014). Although both SDD and SOD were associated with a reduction in mortality, chlorhexidine was associated with a significant increase in risk of death with an OR of 1.25 (95% CI 1.05-1.50). These two recent meta-analyses raise concerns over the widespread use of chlorhexidine outside of the cardiac surgical population.

1.8.6 Prevention of colonisation: selective decontamination

Selective decontamination of the digestive tract (SDD) and selective oropharyngeal decontamination (SOD) are two further approaches to reduce colonisation and thereby reduce the risk of VAP and HCAI more generally. This is the administration of topical, non-absorbable antibiotics, and in the case of SDD also a short (4 day) course of broad-spectrum antibiotic (eg. cefuroxime) to eradicate aerobic bacteria which are potentially pathogenic and so leave the gut colonised with anaerobic bacteria. In early animal work Van der Waaij *et al* found that mice who had been given antibiotics to eliminate gut flora remained resistant to colonisation by orally ingested *E. coli, P. aeruginosa* and *K. pneumoniae*(Van der Waaij, Berghuis-de Vries and Lekkerkerk-van der Wees, 1971). Furthermore if the healthy mice were contaminated with the flora of SDD-treated mice, they too became resistant to colonisation. The GI tract of these mice were colonised with anaerobic bacteria and it was concluded that the resistance to colonisation stemmed from this. The concept of selective decontaminaton of Gram-negative aerobes was first tested in ICU in a group of trauma patients(Stoutenbeek *et al.*, 1984). In this historical case control study, patients received systemic antibiotics until they were free of all potential pathogenic microorganisms. The mouth was decontaminated with 2% polymyxin E, 2% tobramycin and 2% amphotericin. The gut was decontaminated with polymyxin E, torbramycin and amphotericin. In the control group, 86% of patients had oropharyngeal colonisation by Gram-negative potentially pathogenic microorganisms (PPM) by day 15. In terms of intestinal colonisation, the pattern in the control group was that rates of *E. coli* colonisation remained constant but colonisation with other PPM aerobic Gram-negative bacteria increased, in particular *P. aeruginosa*, *Proteus* spp., and

Acinetobacter spp. In the SDD cohort, oropharyngeal colonisation decreased rapidly and was significantly lower by day 2. In the intestine, colonisation with *E. coli* decreased and colonisation with PPM aerobic Gram-negative bacteria was significantly reduced and occurred in only 14% of patients within 2 weeks. The authors found that the total infection rate decrease by 16%.

Although the initial description of SDD involved the use of systemic antibiotics for a short period in addition to non-absorbable antibiotics, the systemic component is often not used. SOD refers to the use of non-absorbable oral antibiotics only. The full SDD regimen is aimed at reducing the pool of Gram-negative aerobes and therefore aims to reduce any HCAI. SOD on the other hand is predominantly focusing on reducing VAP There have been 8 meta-analyses evaluating the trial evidence of SDD and SOD in terms of reducing VAP, HCAI and mortality(Vandenbroucke-Grauls and Vandenbroucke, 1991; Selective Decontamination of the Digestive Tract Trialist' Collaborative Group, 1993; Kollef, 1994; Chan *et al.*, 2007; D'Amico *et al.*, 2009; Silvestri *et al.*, 2009; Pileggi *et al.*, 2011; Price, Maclennan and Glen, 2014). These meta-analyses generally contain a range of definitions of respiratory tract infections including VAP and tracheobronchitis and therefore report reductions in respiratory tract infections (RTI) rather than VAP *per se*. Generally these meta-analyses have shown a consistent reduction in RTI(Selective Decontamination of the Digestive Tract Trialist' Collaborative Group, 1993; Kollef, 1994; D'Amico *et al.*, 2009; Silvestri *et al.*, 2009; Pileggi *et al.*, 2011; Price, Maclennan and Glen, 2014). A Cochrane review of 36 RCTs including 6914 patients found that there was a greater reduction in RTI with SDD that included systemic antibiotics, with OR 0.28 (95% CI 0.20-0.38), in comparison to topical antibiotics only with a OR of 0.44 (95% 0.31-0.63)(D'Amico *et al.*, 2009). The meta-analysis by Chan *et al* is an outlier in that it did not find a reduction in VAP for SOD(Chan *et al.*, 2007). The meta-analysis was of oral decontamination only and so included SOD and oral antiseptics. In the 4 trials of SOD included there was no reduction in VAP with a relative risk of 0.69 (95% 0.41-1.18). This meta-analysis was more robust in terms of ensuring that a definition of VAP was used rather than any RTI. In terms of antibiotic stewardship the distinction between VAP and RTI may be less important since patients are likely to receive antibiotics for either and so a reduction in RTI remains an important outcome.

In addition to a greater reduction in RTI, the use of the full SDD regimen was also associated with a reduction in mortality with an OR of 0.75 (95% 0.65-0.87)(D'Amico

et al., 2009). The number needed to treat (NNT) for the mortality benefit was 18. Silvestri *et al* performed a meta-analysis of trials that used the full SDD (ie. including systemic antibiotics) and found that there was a mortality benefit with SDD (OR 0.71, 95% CI 0.61-0.81)(Silvestri *et al.*, 2009). The subgroup analysis suggested the reduction in mortality was in late deaths rather than early deaths (OR 0.58, 95% CI 0.45-0.77), and also found a greater reduction when all patients in the ICU received both non-absorbable enteral and systemic antibiotics (OR 0.59, 95% CI 0.42-0.82). Furthermore, in the recent meta-analysis by Price *et al*, there was a significant mortality benefit for SDD with an OR 0.73 (95% 0.64-0.84) and a smaller benefit with SOD with an OR of 0.85 (95% CI 0.74-0.97)(Price, Maclennan and Glen, 2014). This mortality benefit has not been demonstrated in other meta-analyses(Vandenbroucke-Grauls and Vandenbroucke, 1991; Selective Decontamination of the Digestive Tract Trialist' Collaborative Group, 1993; Kollef, 1994; Chan *et al.*, 2007; Pileggi *et al.*, 2011).

A number of high quality trials of SOD and SDD have been included in the meta-analyses. De Jonge *et al* conducted a trial in two ICUs, with identical patient populations, within a single institution with each ICU randomly assigned to SDD or placebo(de Jonge *et al.*, 2003). The design was adopted to avoid cross contamination of flora between SDD and placebo patients. Their primary outcomes were colonisation by resistant strains of microorganisms, ICU- and hospital-mortality, and the trial included 934 patients. Both ICU- and hospital-mortality were reduced in the SDD group (relative risk 0.65, 95% CI 0.49-0.85 and 0.78, 95% CI 0.63-0.96 respectively). There was no overall difference in resistance in colonising organisms between the two groups although there was increased resistance in Gram-negative organisms in the control group. It should be noted, however, that resistance in this population was generally low with vancomycin-resistant *Enterococcus* (VRE) in 1%,and no cases of methicillin-resistant *S. aureus* (MRSA).

In another large Dutch trial, 5939 patients were randomised in a cluster-randomised, three arm trial of SDD, SOD and placebo(de Smet *et al.*, 2009). A cluster-randomised approach was used again to avoid cross contamination of flora between trial arms. In terms of the primary endpoint of 28-day mortality, there was a reduction in the risk of death in both the SOD and SDD arms with an OR of 0.86 (95% CI 0.74-0.99) and 0.83 (95% CI 0.72-0.97) respectively. This corresponds to a NNT to save one life at 28 days of 34 for SOD and 29 for SDD. This trial also found that antimicrobial resistance was low generally with no increase in resistance in the SOD and SDD groups. In fact in a

subsequent analysis of the data, there were fewer 'highly resistant organisms' in the SDD group in comparison to the SOD and placebo group, which may appear counter intuitive(de Smet *et al.*, 2011). Furthermore there was less colonisation of the respiratory tract with highly resistant organisms in both the SDD and the SOD groups in comparison to placebo (OR 0.58, 95% CI 0.43-0.78 and 0.65, 95% CI 0.49-0.87 respectively).

Despite the substantial evidence to support the use of SDD and SOD, it is not widely accepted(Walden, Bonten and Wise, 2012; Cuthbertson *et al.*, 2013). One of the issues is that much of the evidence in support of SDD comes from the Netherlands(Bergmans *et al.*, 2001; de Jonge *et al.*, 2003; de Smet *et al.*, 2009), which has low levels of endemic resistance. In contrast, trials from other countries have found inconsistent results. A reduction in respiratory tract infections was found in two Spanish trials(Sánchez García *et al.*, 1998; de La Cal *et al.*, 2005), a Belgian trial(Verwaest *et al.*, 1997) and an international trial(Stoutenbeek *et al.*, 2007). No benefit in reducing respiratory tract infections was found in other trials(Gastinne *et al.*, 1992; Hammond, 1992; Wiener *et al.*, 1995). In many of these smaller RCTs no mortality benefit was detected although these would be underpowered to detect such an effect(Gastinne *et al.*, 1992; Hammond, 1992; Wiener *et al.*, 1995; Verwaest *et al.*, 1997; Sánchez García *et al.*, 1998; de La Cal *et al.*, 2005; Stoutenbeek *et al.*, 2007).

Concern regarding emergence of antibiotic resistance is a major barrier to the uptake of SDD and SOD. Although resistance has not been associated with SDD in large trials(de Jonge *et al.*, 2003; de Smet *et al.*, 2009), there are trials which have found notable increases in resistance(Sánchez García *et al.*, 1998). In a meta-analysis of 35 trials with resistance outcomes, when comparing SDD or SOD to control, there was no significant increase in MRSA (OR 1.46, 95% CI 0.90-2.37) or VRE (OR 0.63, 95% CI 0.63-1.02)(Daneman *et al.*, 2013). Amongst Gram-negative bacilli there was no difference between SDD/SOD and control in aminoglycoside- or fluoroquinolone-resistance but there was a reduction in resistance to polymyxin-E or B (OR 0.58, 95% CI 0.46-0.72) and in third-generation cephalosporin resistance (OR 0.33, 95% CI 0.20-0.52). It should however be noted that this meta-analysis is heavily influenced by the large Dutch trials. In determining the effect of SDD/SOD over time, there was an increase in the prevalence of VRE for each month of trial duration in the SDD/SOD group with an OR 1.2 per month (95% CI 1.1-1.3). In a 5 year observational study of a single German ICU that routinely performed SDD, 4,597 isolates were compared against 46,346 isolates

from reference ICUs that did not routinely perform SDD(Heininger *et al.*, 2006). There was no difference in MRSA in the SDD ICU and aminoglycoside-resistant *P. aeruginosa* was lower compared to the reference ICU. There was, however, an increase in VRE, aminoglycoside-resistant *E. coli* and aminoglycoside-resistant *K. pneumoniae*. In a Spanish observational study in a single ICU over 5 years, the *Enterobacteriaceae* resistance to antimicobials in the SDD regimen was stable and *Pseudomonas* spp. resistance to the SDD regimen antibiotics reduced(Ochoa-Ardila *et al.*, 2011). There was a significant increase in *P. aeruginosa* resistance to ceftazidime, which was not temporally related to the increase in cephalopsporin use in the SDD regimen. There was an increase in imipenem resistance amongst *Pseudomonas* spp. associated with an increase in imipenem use. Overall there was not a significant increase in antimicrobial resistance over the 5 year period.

1.8.7 Prevention of colonisation: stress ulcer prophylaxis

Gastrointestinal (GI) bleeding is a recognised complication of being critically ill and acid-suppressive therapy is used to raise the stomach pH, which renders pepsin inactive and inhibit finbrinolysis of clot(Fennerty, 2002). The consequence of raising pH, however is that there is increased colonisation of stomach contents, which could act as a source of pathogens that will cause VAP(Bonten *et al.*, 1995). GI bleeding is generally reported as ‘overt bleeding’ when frank blood, melaena or ‘coffee grounds’ are reported or as ‘clinically significant bleeding’, when there is a fall in blood pressure, a drop in haemoglobin or the need for a blood transfusion. In a seminal multi-centre cohort study, Cook *et al* followed up 2252 patients in whom they encouraged physicians to withhold stress ulcer prophylaxis (SUP)(Cook *et al.*, 1994). Of these, 100 patients had overt GI bleeding and 87 of these were receiving SUP. Only 33 patients, out of the whole cohort, had ‘clinically important GI bleeding’ (1.5%, 95% CI 1.0-2.1). Respiratory failure and coagulopathy were identified as risk factors for GI bleeding and mortality was considerably higher in those with GI bleeding. Within the group of patients who are at risk of GI bleeding and in whom SUP is appropriate, there is the need to weigh up the effectiveness of different drugs against the risk of promoting VAP. In a RCT of 1200 patients in a blinded, placebo-controlled trial of sucralfate (a complex of aluminium hydroxide and sulfated sucrose that forms a physical barrier to protect gastric mucosa) versus ranitidine (a histamine H_2-receptor antagonist that reduces gastric acid production) there were fewer clinically important GI bleeds in the ranitidine group with a RR of 0.44 (95% CI 0.21-0.92) although the event rate was still low (1.7% in the

ranitidine group and 3.8% in the sucralfate group)(D. Cook *et al.*, 1998). There was no significant increase in mortality for VAP between the two groups. A number of meta-analyses have been carried out in this area which support the finding that ranitidine provides superior SUP over sucralfate(Cook *et al.*, 1996; Messori *et al.*, 2000; Huang *et al.*, 2010). The meta-analyses also demonstrated that ranitidine was associated with a higher incidence of VAP.

ICU practices are likely to have changed considerably since the earlier trials of SUP. In particular the use of early enteral feeding and the use of proton pump inhibitors (PPI) are in far more widespread use now. To determine the role of enteral feeding in SUP, Marik *et al* performed a meta-analysis of RCTs of histamine H_2-receptor blockers (H2RB) versus placebo and grouped patients into those with or without enteral feeding(Marik *et al.*, 2010). In this analysis H2RB SUP was found to reduce the risk of GI bleeding with an OR of 0.47 (95% CI 0.29-0.76). This effect was only observed in patients who were not receiving enteral feeding and there was no benefit for those who were enterally fed (OR 1.26, 95% CI 0.42-3.7). Furthermore the risk of VAP was not significant when considering all patients (OR 1.53, 95% CI 0.89-2.61) but for the subgroup of patients who were fed enterally the OR was 2.81 (95% CI 1.20-6.56). In a historical observational study of two 15 month periods in which SUP was used for the first 15 months and no SUP was used for the second, no difference was found in incidence of GI bleeding, VAP or mortality(Faisy *et al.*, 2003).

Proton pump inhibitors (PPI) (a parietal cell gastric proton pump blocker that reduces gastric acid) are increasingly used as first line SUP despite little evidence to support their use(Daley *et al.*, 2004). A meta-analysis of 7 RCTs including 936 patients found there was no difference between PPI and H2RB with a risk difference of -0.04 (95% CI -0.09-0.01)(Lin *et al.*, 2010). This was associated with moderate to high heterogeneity (I^2 66%). In the sensitivity analysis, removal of one trial reduced the heterogeneity (I^2 26%), and the effect remained non-significant. There was no difference in risk of VAP between PPI and H2RB either (risk difference 0.00 (95% CI -0.04-0.05). Despite the limited evidence for the use of PPI, their use has increase not only within the ICU but also amongst non-ICU patients(Heidelbaugh and Inadomi, 2006). There is no evidence base for the use of SUP in non-ICU patients and PPI use is associated with increased rates of HAP(Herzig *et al.*, 2009) and with community-acquired pneumonia(Sarkar, Hennessy and Yang, 2008). In a large scale pharmacoepidemiological study of 35,312 patients mechanically ventilated for more than 24 hours and receiving either a PPI or a

H2RB, PPIs were associated with a greater risk of GI bleeding (OR 2.24, 95% CI 1.81-2.76), pneumonia (OR 1.2, 95% CI 1.03-1.41) and *Clostridium difficile* infection (OR 1.29, 95% CI 1.04-1.64)(MacLaren, Reynolds and Allen, 2014).

Although these data do not provide conclusive evidence that enteral feeding is sufficient to protect against GI bleeding, it does suggest that it may be sufficient. These data also highlight that SUP is not without risk and it is used for the prevention of an infrequent complication of critical illness. The role of SUP needs to be reassessed in light of changing ICU practices.

1.8.8 VAP prevention bundles

Grouping the prevention strategies already discussed into ‘bundles’ of care has become widespread. VAP prevention bundles have not been tested in randomised trials and so are subject to the biases of non-randomised studies. When multiple interventions are delivered together as a bundle it is difficult to determine the contribution of individual components of the bundle. The Institute for Healthcare Improvement (IHI) has been a major driving force with its VAP prevention bundle(Institute for Healthcare Improvement, 2012). The bundle includes elevation of the head of the bed to between 30 and 45 degrees; DSI and SBT; SUP; deep venous thrombosis (DVT) prophylaxis; and daily oral care with chlorhexidine. Notwithstanding recent data that suggest the use of chlorhexidine is inappropriate, one can appreciate that this bundle is not solely aimed at prevention of VAP and reflects more a ‘ventilator care bundle’ by including DVT prophylaxis, which is not used for VAP prevention. In an observational study to determine the association between the occurrence of VAP and the use of the IHI bundle, 630 patients were followed up until the occurrence of VAP(Croce *et al.*, 2013). Overall compliance with the bundle averaged 77% and compliance with individual components ranged from 70.5-92.5%. There was no difference in bundle compliance between patients who developed VAP versus those who did not and in regression analysis, bundle compliance was not associated with prevention of VAP.

As part of the highly influential Michigan Keystone ICU project (see high profile paper on prevention of catheter-related blood stream infection(Pronovost *et al.*, 2006)), an intervention to improve compliance with VAP prevention measures was implemented in 127 ICUs, including 550,800 ventilator-days(Berenholtz *et al.*, 2011). The VAP prevention bundle included: SUP; elevation of head of bed; DVT prophylaxis; DSI; and SBT. This study reported significant reductions in VAP from a median of 5.5 cases per 1000 ventilator-days at baseline to zero at 16-18 months, which was maintained at 28-

30 months after intervention. There are significant limitations of this study. Primarily the study was not randomised and so was vulnerable to bias. The case definition of VAP was based on the CDC surveillance criteria, which has subjective elements such as CXR changes and the characteristics of sputum. Furthermore, cases of VAP were identified by hospital "preventionists", a group of specialists familiar with the CDC criteria and therefore felt to be less at risk of bias. However an alternative interpretation is that these individuals would be most vulnerable to bias in a non-randomised, non-blinded study.

In a European observational study in which VAP was diagnosed based on clinical and microbiology data by an independent adjudicator, the reduction in VAP rates was much more modest, falling from 15.5% to 11.7% after the implementation of a bundle(Rello *et al.*, 2013). The bundle comprised: oral care with chlorhexidine; handwashing; maintenance of ETT cuff pressure; sedation control measures; and not changing ventilator circuits regularly. Compliance with all 5 elements was only 20% and for individual elements compliance ranged from15-34%. While this result could be interpreted as suggesting that greater compliance could obtain an even greater reduction in VAP, it may well also suggest there is a significant effect from carrying out a non-randomised intervention.

1.9 Treatment of VAP

1.9.1 General considerations

Different diagnostic strategies, clinical versus invasive, result in different rates of VAP(Conway Morris *et al.*, 2011). There is the potential that a clinical strategy over-treats patients with *suspected* VAP with antibiotics or alternatively that the invasive strategy under-treats patients. In an invasive/microbiological strategy the assumption is that if the culture growth is below the threshold (10^4 cfu/ml for BAL, for example), it represents colonisation. There have been a number of reports describing that patients with culture growth below the threshold for VAP have equivalent outcomes to the baseline ICU population and that there may even be some benefit in with-holding antibiotics in these circumstances, in terms of reducing antibiotic resistance(Fagon *et al.*, 1996; Raman *et al.*, 2013).

Another possibility is that the patients have bronchitis rather than VAP. Ventilator-associated tracheobronchitis (VAT) is an area of controversy. There are varying clinical definitions used without a gold standard diagnosis. It has been previously defined as patients with fever, raised inflammatory markers and purulent tracheal secretions *without* CXR changes(Nseir *et al.*, 2014) or based on total sputum load from

endotracheal secretions(Palmer, 1995). Trials of antibiotic therapy for VAT are of low quality and firm conclusions are unable to be drawn from them(Nseir *et al.*, 2008; Palmer *et al.*, 2008). Recent observational data do suggest improved outcome and reduced progression to VAP with adequate antibiotic therapy(Martin-Loeches *et al.*, 2015).

The adequacy of treatment for VAP is fundamental to the outcome. In a prospective study of 107 patients Iregui *et al* demonstrated that a delay in appropriate antibiotic therapy (defined as ≥ 24 hours) was associated with a greater hospital mortality at 69.7% in comparison to the mortality rate in those with appropriate treatment at 28.4%, although the median time for initiation of antibiotics for appropriate therapy was 12 hours(Iregui *et al.*, 2002). Initial delay in appropriate antibiotic therapy was an independent risk factor for hospital mortality (OR 7.68, 95% 4.5-13.09). Luna *et al* demonstrated a similar outcome, in a prospective study of 508 patients(Luna *et al.*, 2006). The mortality rate with appropriate antibiotic therapy was 29.2%, with inappropriate therapy (antibiotics that did not provide sufficient coverage of pathogens) the mortality was 75% and for those with a delay in appropriate antibiotic therapy the mortality was 58.3%.

The consequences of inappropriate therapy are considerable as are delays in treatment. Therefore guidelines advise initial antibiotic therapy should be started promptly and aim to provide coverage of likely pathogens. Since infection with a multi-drug resistant (MDR) pathogen could lead to inadequate coverage, initial therapy is based on the risk of MDR pathogens(American Thoracic Society, 2005). Risk factors for MDR pathogens include previous antibiotics, prolonged mechanical ventilation (> 7 days), higher number of comorbidities and admissions from another healthcare facility(Trouillet *et al.*, 1998; Rello *et al.*, 1999; Depuydt *et al.*, 2008; Parker *et al.*, 2008).

The American Thoracic Society guidelines for the treatment of VAP(American Thoracic Society, 2005) recommend that patients with early-onset VAP and without risk factors for MDR pathogens should have coverage for *Streptococcus pneumoniae, H. influenzae,* methicillin-sensitive *S. aureus* and antibiotic-sensitive Gram-negative bacilli. The recommended antibiotics are: ceftriaxone; levofloxacin, moxifloxacin or ciprofloxacin; ampicillin/sulbactam; or ertapenem.

Considering patients with late-onset disease or risk factors for MDR pathogens, antibiotic coverage should also include cover for *P. aeruginosa*, *K. pneumoniae*, *Acinetobacter* spp, MRSA, and *Legionella pneumophila*. Combination therapy is

advised with (a) an antipseudomonal cephalosporin (cefepime, ceftazidime) or antipseudomonal carbepenem (imipenem or meropenem) or β-lactam/β-lactamase inhibitors (piperacillin-tazobactam); (b) an antipseudomonal fluoroquinolone (ciprofloxacin or levofloxacin) or an aminoglycoside (amikacin, gentamicin or tobramycin); and (c) linezolid or vancomycin.

In prospectively validating these guidelines, the guidelines accurately predicted the organisms for patients classed as having late-onset VAP or risk factors for MDR pathogen, correctly identifying 92% of pathogens(Ferrer *et al.*, 2010). However its prediction of pathogens for patients classed as having early-onset VAP or no risk factors for MDR pathogens was only 50%, with 10 patients (26%) having a potentially resistant pathogen. In a prospective, multi-centre cohort study aimed at improving compliance with the ATS antibiotic recommendations for patients at risk of MDR pathogens, 303 patients were included, of whom 129 received compliant treatment and 174 did not(Kett *et al.*, 2011). They found that 34% of compliant patients died in comparison to 20% of non-compliant patients (p=0.004) and the survival benefit of non-compliant therapy did not vary with key potential confounding factors such as duration of mechanical ventilation, VAP (as opposed to HAP), age >60, severe sepsis or APACHE 2 score, although the compliant group did have significantly higher APACHE 2 scores and more met criteria for severe sepsis. It should also be noted that the coverage of identified pathogens was similar in both the compliant and non-compliant groups at 81% and 85% respectively.

This study calls into question the previously accepted view that infections should be ‘hit hard and fast’. It suggests that there is a potential detrimental effect of multiple classes of antibiotics being used. In a quasi-experimental study, Hranjec *et al* challenged the traditional view of antibiotic prescribing(Hranjec *et al.*, 2012). In this before-and-after study, an aggressive approach to antibiotics was used in a single surgical ICU for 1 year during which, when infection was suspected, appropriate cultures were taken and empirical antibiotics were started. Antibiotics were stopped if cultures were negative. In the following year the ICU adopted a conservative approach. If infection was suspected appropriate cultures were taken but antibiotics were not started until objective evidence of infection was found. Physicians could start antibiotics empirically at their discretion if the clinical situation required such an approach. The incidence of infection was similar between the two time periods with 26.0 infections per 1000 patient-days in the aggressive period and 27.2 infections per 1000 patient-days in the conservative period.

There was a significant difference in the time from onset of fever to initiation of antibiotics, with a median of 11 (IQR 3-14) and 24 (IQR 9-44) hours for the aggressive and conservative periods respectively. The duration of treatment was significantly shorter in the conservative group (12.5 days versus 17.7 days) and the appropriateness of initial antibiotic therapy was higher in the conservative group (74% vs 62%). The all-cause mortality rate was 27% during the aggressive period versus 13% during the conservative period (p=0.015). A sub-group analysis was performed on patients with a mean arterial pressure (MAP) of <60mmHg and despite the time from blood culture to initiation of treatment being a median of 20 hours (IQR 8-39) in the conservative group in comparison to a median of 4 hours (IQR 3-12.5) in the aggressive group, the mortality was still lower in the conservative group at 26%, in comparison to the aggressive group (66%).

1.9.2 Duration of treatment

There are surprisingly few data on the appropriate duration of antibiotic therapy for VAP. Updated guidelines recommend 7 days of antibiotics(Kalil *et al.*, 2016). Expert opinion has previously recommended that a longer duration of therapy, 14-21 days is used for patients with multi-lobar involvement, malnutrition, cavitation, Gram-negative necrotising pneumonia or isolation of *P. aeruginosa* or *Acinetobacter* spp(Chastre and Fagon, 2002). Duration of antibiotic therapy in trials varies from 9-15 days(Brun-Buisson *et al.*, 1998; Betrosian *et al.*, 2008; Chastre *et al.*, 2008).

In a prospective study of 27 patients with VAP confirmed by semi-quantitative culture from BAL, Dennesen and colleagues monitored clinical signs and ETA cultures for resolution of disease(Dennesen *et al.*, 2001). Resolution was by a return to ‘normal’ in temperature, leucocyte count, PaO_2:FiO_2 and no more than 1+ of bacterial growth from ETA cultures. There was a fall in mean log cfu/ml of 5.4 on day 0 to 1.4 on day 15. Patients colonised with *P. aeruginosa* did not achieve eradication with appropriate antibiotic therapy and so ETA culture is an unreliable metric of resolution for this pathogen. The mean time to resolution of all parameters was 9 days and the mean time for resolution of clinical parameters only (ie. excluding colonisation criteria) was 6 days. The median duration of treatment was 13 days (range 7-14).

Duration of therapy was tested in an RCT of 8 versus 15 days of therapy(Chastre *et al.*, 2003). One hundred and one patients with confirmed VAP following BAL and who received empiric therapy within 24 hours of BAL, were randomly assigned to receive 8 or 15 days of therapy. The choice of antibiotics was left to the discretion of the clinical

team, although empiric treatment had to include an aminoglycoside or flouroquinolone and a broad-spectrum beta-lactam. The mean duration of mechanical ventilation before VAP was 13.4 days in the 8-day group and 13.8 days in the 15-day group, suggesting that this was predominantly a study of late-onset VAP patients. They demonstrated non-inferiority for 8 days of treatment in comparison to 15 days in their primary outcome measures of mortality (18.8% for 8-day and 17.2% for 15-day) and pulmonary infection recurrence rate (28.9% for 8 day and 26% for 15 day). The 8-day therapy group had more AFD than the 15-day group, with a mean of 13.1 (SD 7.4) versus 8.7 (SD 5.2) respectively. In the sub-group analysis, patients with non-fermenting Gram-negative bacteria (GNB) had a recurrence rate of 40.6% in the 8-day group and 25.4% in the 15-day group, with a between-group risk difference of 15.2% (95% CI 3.9%-26.6%). There was, however, no difference in mortality in this subgroup. A further trial of 8 versus 15 days of treatment in patients with early on-set VAP similarly found non-inferiority with an 8-day course(Capellier *et al.*, 2012).

1.9.3 Monotherapy versus combination therapy

Combination therapy is recommended in guidelines for patients with late-onset VAP and risk factors for MDR pathogens(American Thoracic Society, 2005; Kalil *et al.*, 2016). The predominant reason for this is to provide sufficiently broad empiric therapy to cover antibiotic-sensitive GNB, MDR GNB and MRSA. In addition, antibiotics have shown *in vitro* synergism when used in combination(Hallander *et al.*, 1982). Furthermore, using antibiotics in combination may reduce resistance to individual antibiotics(Drago *et al.*, 2005).

A meta-analysis compared β-lactam monotherapy with β-lactam and aminoglycoside in combination in trials of patients with severe infections(Paul *et al.*, 2004). There was no difference in mortality between the two groups with a RR of 0.90 (95% CI 0.77 – 1.06). A sub-analysis of patients with GNB infections (and specifically *P. aeruginosa* infection) also had no benefit from combination therapy. Furthermore there was an increase in nephrotoxicity in the combination therapy group.

In a further meta-analysis of monotherapy or combination therapy for GNB bacteraemia, 17 studies were included in which only 2 were prospective RCTs(Safdar, Handelsman and Maki, 2004). Only 2 cohort studies found a mortality benefit with combination therapy and the overall summary OR was 0.96 (95% CI 0.70 – 1.32). In contrast to the previously described meta-analysis, there was a significant mortality

benefit in patients with *P.aeruginosa* infection with combination therapy (OR 0.50, 95% CI 0.30 – 0.79).

In the context of VAP, no difference in the resolution of clinical parameters was found between combination and monotherapy(Damas *et al.*, 2006). In a 2x2 factorial design RCT, monotherapy and combination therapy(Heyland *et al.*, 2008) as well as diagnostic strategies for VAP were compared(Canadian Critical Care Trials Group et, 2006). Patients were randomised to receive meropenem and ciprofloxacin or meropenem alone. As previously discussed, this trial excluded patients colonised with *P. aeruginosa* or MRSA, since clinicians may feel unable to give monotherapy to a patient colonised with *P. aeruginosa* and the antibiotic choice does not provided sufficient cover for MRSA. The overall 28-day mortality rate was 18.7% and the risk ratio of death at 28-days was 1.05 (95% CI 0.78 – 1.42). There was no difference in secondary endpoints of duration of MV, ICU or hospital death. However there was a significantly higher rate of adequate therapy in the combination therapy groups over the monotherapy groups (93.1% and 85.1% respectively). In a subgroup analysis of patients with *Pseudomonas* spp, *Acinetobacter* spp and MDR GNB, the difference in adequacy of therapy was more pronounced (84.2% in combination group and 18.8% in monotherapy group). In this sub-group there was a trend towards improved outcome with combination therapy.

In a retrospective analysis, 183 episodes of VAP caused by *P. aeruginosa* were examined based on monotherapy or combination therapy(Garnacho-Montero *et al.*, 2007). These authors similarly found that rates of adequate therapy were considerably higher when combination therapy was used (90.5% for combination therapy and 56.7% for monotherapy). Mortality was 72.5% amongst patients with inadequate therapy in comparison to 33.6% in those with adequate therapy. In multivariate analysis, adequacy of treatment was an independent risk factor for death, rather than monotherapy or combination therapy.

1.10 Antibiotic resistance and the need for improved antibiotic stewardship

1.10.1 The scale of the problem

Antibiotics are arguably the greatest discovery of modern medicine. They have greatly reduced mortality from infectious disease and allowed medical advances such as transplantation and joint replacements. The rise in antimicrobial resistance (AMR) has reached a critical level and is a global concern. The World Health Organization (WHO) has described it as one of the top three threats to human health and warns of a return to a

pre-antibiotic era in which we do not have the means to treat infectious disease(World Health Organisation, 2014).

Determining the scale of the problem of AMR globally is difficult. The WHO has attempted to give a description of the global burden using existing data from surveillance programmes, as well as national and institution-based data(World Health Organization 2014). Not all countries have formal surveillance programmes and so the WHO data are limited in this regard. Furthermore surveillance programmes do not necessarily capture all AMR. For example the European Antimicrobial Resistance Surveillance Network (EARS-Net)(European Centre for Disease Prevention and Control, 2012a, 2014) performs surveillance on 8 bacteria of importance: *E. coli, K. pneumoniae, P. aeruginosa, Acinetobacter* spp*, S. pneumoniae, S. aureus, Enterococcus faecalis and Enterococcus faecium*. Only data from invasive isolates (from blood and cerebrospinal fluid) are included. If we consider that the prevalence of bacteraemia in the intensive care unit is 15%(Vincent *et al.*, 2009), then surveillance of these positive cultures reflects only a fraction of treated infections and antibiotics being prescribed. Notwithstanding the limitations of the data presented in the WHO report, it does demonstrate a worrying situation with resistance amongst common pathogens (*E. coli, K. pneumoniae* and *S. aureus*), being greater than 50% in many member states.

In Europe, resistance to Gram-positive bacteria has been relatively stable(European Centre for Disease Prevention and Control, 2012a, 2014). In contrast to Gram-positive bacteria, Gram-negative are intrinsically more resistant and are often implicated in HCAI. The rise in resistance amongst these pathogens is alarming. *E. coli* is the commonest cause of bloodstream infection and in Europe the mean rate of full susceptibility to all antibiotics was only 39.5%(European Centre for Disease Prevention and Control, 2012a). The prevalence of resistance to aminopenicillin was 57.1% and to third generation cephalosporins was 12%(European Centre for Disease Prevention and Control, 2014). 71-100% of resistant isolates to 3rd generation cephalosporins were attributable to extended-spectrum beta-lactamases (ESBLs). Although the mean prevalence of carbapenem resistance was <0.1%, the emergence of carbapenem resistance is a particular concern.

K. pneumoniae is a pathogen of importance in HCAI as a frequent cause of bloodstream infection and pneumonia. Its rising AMR is a serious public health concern particularly due to the rise of carbapenem resistance. More than a third of isolates are resistant to one antibiotic under surveillance(European Centre for Disease Prevention and Control,

2014). Resistance to 3rd generation cephalosporins was present in 28% (of which 85-100% were ESBLs), with the corresponding figures for flouroquinolones 27.4%, aminoglycosides 23.1% and carbapenems 7.3%. Resistance to a combination of 3rd generation cephalosporins, fluoroquinolones and aminoglycosides was present in 19.6%. Resistance to these antibiotics have all increased since the previous 2012 report(European Centre for Disease Prevention and Control, 2012a).

P. aeruginosa is an intrinsically resistant organism and is an opportunistic pathogen in hospitalised patients and a major pathogen in VAP. *P. aeruginosa* is intrinsically resistant due to the limited permeability of its outer membrane, efflux pump mechanisms and its ability to rapidly acquire resistance(Li, Zhang and Poole, 2000). 63.9% of isolates in Europe were fully susceptible in EARS-Net 2012 report and resistance rates have largely fallen in the 2014 report(European Centre for Disease Prevention and Control, 2012a, 2014). Resistance to piperacillin was 16.9%, ceftazidime 13.1%, fluoroquinolones 19.4%, aminoglycosides 14.8% and carbapenems 18.3%. Resistance to 3 or more classes of antibiotics occurred in 13.3% of isolates(European Centre for Disease Prevention and Control, 2014).

With low numbers of virulence factors, *Acinetobacter* spp is implicated in HCAI infections in the immunocompromised patient. Resistance in *Acinetobacter* spp varies widely across Europe with high rates of resistance in countries in the east and south of Europe. Resistance to fluoroquinolones was 29% in Denmark and 95.3% in Greece. Carbapenem resistance was zero in the Netherlands and 93.2% in Greece(European Centre for Disease Prevention and Control, 2014).

Gram-positive resistance shows a more stable picture in Europe. Resistance in *S. pneumoniae* has remained relatively stable with most countries reporting penicillin non-susceptibility below 10%. Macrolide resistance is higher than penicillin resistance and ranged from zero in Cyprus to 48% in Romania(European Centre for Disease Prevention and Control, 2014).

S. aureus has been a pathogen of intense political pressure in the UK since 2000. At its peak resistance was at 40% in 2000 and is now at 11.3% in the UK(Livermore, 2012; European Centre for Disease Prevention and Control, 2014). Preventative measures such as MRSA eradication therapy, hand hygiene measures and infection control measures have been credited with this decline. Average resistance across Europe is 17.4% with trends decreasing in Belgium, France, Germany, Ireland, Italy,

Luxembourg, Portugal and the UK but increasing trends in Denmark and Slovenia(European Centre for Disease Prevention and Control, 2014).

Enterococcus spp. are common gut colonisers of low virulence which have arisen as important pathogens in nosocomial infection(Health Protection Agency, 2011; European Centre for Disease Prevention and Control, 2012b). High-level aminoglycoside resistance in *E. faecalis* in Europe has seen a significant increase, occurring in 28.8% of isolates. Vancomycin resistance in *E. faecium* (VRE) is on average 7.9% which is considerably different to the USA where VRE is >80%(Arias and Murray, 2012).

There is a geographical variation in antibiotic resistance across Europe with a north-south and east-west gradient, with more resistance in southern and eastern countries. Countries such as Italy, Bulgaria, Romania and Poland have some of the highest rates of antibiotic resistance. Causes of this geographical variation are unclear but antibiotic consumption and infection control practices have been suggested as reasons(European Centre for Disease Prevention and Control, 2012a, 2014). The ECDC carries out the European Surveillance of Antimicrobial Consumption (ESAC) project(European Centre for Disease Prevention and Control, no date). There is considerable variation between European countries in antibiotic consumption. Antibiotic consumption is expressed as defined daily dose (DDD), which is an assumed average maintenance dose per day for a drug used in adults and expressed as DDD per 1000 inhabitants per day (DID). The lowest rate of consumption is found in the Netherlands at 11.8 DID and the highest in Greece at 34.0 DID. Data from non-EU eastern European countries show antibiotic consumption ranging from 15.3 DID in Armenia to 42.3 DID in Turkey(Versporten *et al.*, 2014).

These data give a European perspective but AMR is a global public health concern. In the USA surveillance of HCAI, predominantly in the ICU, is conducted through the National Healthcare Safety Network (NHSN), which monitors rates of HCAI and AMR. The pathogens of concern are similar to Europe with 82% of pathogens belonging to the following groups: *S. aureus, Enterococcus* spp., *E. coli*, coagulase-negative Staphylococci, *Candida* spp., *Klebsiella* spp., *P. aeruginosa* or *Enterobacter* spp(Sievert *et al.*, 2012). Resistance amongst pathogens isolated from central line-associated bloodstream infections (CLABSI) was high. Vancomycin resistance amongst *E. faecium* was 83.6%; carbapenem resistance amongst *Klebsiella* spp. and *P. aeruginosa* was 14.2% and 26.8% respectively; and carbapenem and multi-drug

resistance amongst *A. baumannii* was 64.6% and 69.7% respectively. Although the percentage resistance had been largely stable when compared with previous periods (2009-2010 vs 2007-2008), there was a 54.3% increase in *E. coli* extended-spectrum cephalosporin resistance and a 25.3% increase in *A. baumannii* resistance to carbapenems.

Data from the Asia-Pacific region similarly demonstrate concerning levels of Gram-negative resistance with *Enterobacteriaceae* susceptibility to ampicillin/sulbactam being only 41.4% overall for the region, cefotaxime only 68.0%, ceftriaxone 67.7%, ciprofloxacin 64.4% and levofloxacin 67.3%(Hsueh *et al.*, 2010). Resistance was particularly high in India and China. Furthermore a high proportion of *E. coli* and *K. pneumoniae* were ESBL producers with rates as high as 59.1% in China and 61.2% in India. A prospective surveillance study of AMR in respiratory pathogens in HAP and VAP carried out in Asia is valuable considering this is a major reason for antibiotic use (and considering that the EARS-Net only reports blood and CSF cultures)(Chung *et al.*, 2011). MDR isolates occurred in 60.7% of *S. aureus* and in 44.7% of *K. pneumoniae*. Carbapenem resistance was 27.2% for *P. aeruginosa* and as high as 56.9% in China. MDR rate for *P. aeruginosa* was 42.8% and extensively drug resistance rate was 4.9%. *Acinetobacter* spp had a very high rate of imipenem resistance at 67.3%. These are alarming rates of resistance in pathogens that cause HCAI in the most vulnerable group of hospitalised patients.

1.10.2 Antibiotic consumption and resistance

The link between antibiotic consumption and antibiotic resistance is widely accepted(Davies, 2011; Centers for Disease Control and Prevention, 2013). Bacteria produce a massive array (millions) of small organic molecules of which a small proportion have been identified and used as antibiotics(Davies and Ryan, 2012). Many of these small molecules are resistance factors that are necessary for the survival of bacteria. Many bacteria have efflux pumps that are a common mechanism of antibiotic resistance. These mechanisms have been evolutionarily selected in bacteria since the industrial revolution with the increased heavy metal wastes that have been discarded in the environment, which bacteria handle using efflux pumps(Davies and Davies, 2010). The antibiotic era has applied an evolutionary selective pressure on bacteria to produce resistance factors at an accelerated rate.

Mechanisms of resistance are complex and beyond the scope of this thesis but exposure of bacteria to antibiotics clearly results in increasing resistance(Drago *et al.*, 2005). In a

clinical context, antibiotic exposure is linked to increased antibiotic resistance. In an outpatient setting, large-scale, Europe-wide, surveillance data have demonstrated significant variations in antibiotic prescriptions, with the lowest rate in the Netherlands at 10 DDD per 1000 inhabitants daily and the highest rate in France at 32 DDD per 1000 inhabitants daily(Goossens *et al.*, 2005). There was a strong correlation between antibiotic use and antibiotic resistance (Spearman correlation for pencillin use and pencillin resistance 0.84 (95% CI 0.62-0.94)). Data from US surveillance of ICUs between 1994 and 2000 examined susceptibility of Gram-negative bacilli(Neuhauser, 2003). Overall there was a 10% chance of an infecting organism being resistant to a single antibiotic. There was a decrease in ciprofloxacin susceptibility by 10% over the period studied, which coincided with an increase in fluoroquinolone use. In fact the selective pressure of antibiotics can occur rapidly and one of the most alarming resistance patterns is the emergence of carbapenem resistance. In an observational study of 523 patients admitted to an ICU environment in which imipenem was widely used, imipenem-resistant Gram-negative bacilli resistance in intestinal flora, as measured by rectal swabs, increased from 5.6% in week 1 to 58.6% in week 2(Armand-Lefèvre *et al.*, 2013). Conversely studies in which antibiotic usage had been limited found a fall in antibiotic resistance(Rahal *et al.*, 1998; Singh *et al.*, 2000; Geissler *et al.*, 2003; Cook *et al.*, 2006).

1.10.3 The need for antibiotic stewardship

As antibiotic resistance increases, the discovery pipeline for new antibiotics is drying up. Most large pharmaceutical companies are no longer investing in their anti-infectives division(Boucher *et al.*, 2013) Bringing a drug through research and development can cost $800 million, which is a major disincentive for companies(Infectious Diseases Society of America, 2004). For an antibiotic, once brought to market, it will have its use restricted for a small number of patients and is likely to obsolete itself with its increased use. Compare this to development of drugs for chronic conditions or disease prevention, which millions of people will take each day for the rest of their lives. In a report from 2004, of all drug R&D programmes from the world's largest pharmaceutical and biotechnology companies registered with the United States Food and Drug Administration (FDA) database, only 6 out of 506 drugs were antibiotics(Spellberg *et al.*, 2004). None of these had novel mechanisms. A recent report suggests that there has been little improvement in this area, with two antibiotics developed since 2009 and at

the time of the study 7 were in development but still a falling trend in R&D for antimicrobials(Boucher *et al.*, 2013).

In addition to the urgent need for antibiotic drug development, there is a need to conserve the effectiveness of antibiotics that are in current use. Antibiotic stewardship is the judicious use of antibiotics to preserve their future effectiveness and involves optimising therapy for individuals; prevention of overuse, misuse and abuse; and minimising development of resistance at an individual and community level(Davies, 2011). An important limitation to improving antibiotic stewardship lies in diagnostics. Diagnosis of infection has in the main remained unchanged since the development of the Gram stain in the 19th century. Since microbiology culture takes up to three days to provide results, many patients will receive antibiotics based on clinical suspicion. This has implications in the outpatient setting when decisions regarding antibiotic prescription must be made on clinical suspicion. In the hospital setting it is of great importance that antibiotics are de-escalated or discontinued in the face of microbiology evidence. The need to develop novel rapid diagnostics to improve antibiotic stewardship is one of the challenges put forward in the 2011 report by the UK Chief Medical Officer on antimicrobial resistance(Davies, 2011).

1.11 Rapid diagnostics

Novel methods to rapidly detect infections can be divided between culture-independent molecular techniques to detect the pathogens and biological markers, biomarkers, that act as surrogates for detecting pathogens.

1.11.1 Molecular diagnostics

The three main techniques of molecular detection are polymerase chain reaction (PCR), matrix-assisted laser desorption ionisation-time of flight spectrometry (MALDI-TOF MS) and peptide nuclei acid-based fluorescence *in situ* hydridisation (PNA FISH). MALDI TOF MS and FISH are able to detect pathogens in small numbers directly from the clinical specimen, eliminating the time taken for them to the cultured to visible colonies. MALDI TOF MS has been shown to have high concordance with traditional blood cultures (greater than 90%) and rapidly identifying pathogens in less than 3 hours(Beal *et al.*, 2013; Dodemont *et al.*, 2014). Detection of pathogens in respiratory cultures by FISH has also been shown to be high with a sensitivity of 94% and specificity of 88%(Koncan *et al.*, 2015). The FISH assay returned results in approximately 10 hours. Although MALDI TOF MS and FISH can detect pathogens

against large databases, they cannot detect resistance genes. PCR on the other hand has the ability to detect resistance genes but does have other limitations. Firstly PCR techniques detect a limited range of pathogens predetermined by the panels used and so can only detect pathogens in those panels(Ost *et al.*, 2010; Rios-Licea *et al.*, 2010; Lacroix *et al.*, 2015). PCR is extremely sensitive to detecting bacterial DNA, which is both a strength and a weakness. Studies generally report high sensitivities for PCR when compared to traditional culture but often lower specificities, often due to detection of bacterial DNA when culture is negative(Dickson *et al.*, 2014; Lacroix *et al.*, 2015; Vincent *et al.*, 2015). Since PCR detects bacterial DNA, it will amplify the signal for non-viable bacteria as it will for live bacteria. The limitation of this is that it could lead to the overuse of antibiotics. The advantage of this could be in circumstances when there is a high index of suspicion of infection but the culture is negative. This could be due to the use of antibiotics or that the bacteria are difficult to culture. This has lead to the idea of 'salvage microbiology'(Farrell *et al.*, 2013). The use of this 'salvage microbiology' approach is understandable if the suspected infection is in a usually sterile location. The role of PCR in the respiratory samples is much more challenging because this is not a sterile site and the challenge for PCR will be to distinguish between viable and non-viable and infection and colonisation. In a large multi-centre observational study samples of blood, respiratory secretions (ETA and BAL) and sterile site fluid were obtained to compare culture with a novel technique of PCR followed by electrospray ionisation-mass spectrometry (PCR/ESI-MS)(Vincent *et al.*, 2015). The benefit of using ESI-MS after the PCR is that it allows detection of pathogens against a huge database of 800 pathogens. Again this study demonstrated an extremely sensitive assay that detected 228 positive pathogens in blood where only 68 were positive by culture. Amongst the respiratory tract samples, the discrepancy was not as large with 117 positive PCR/ESI-MS and 68 positive cultures (sensitivity 84% and specificity 53%).

Another approach to molecular detection is the detection of volatile organic compounds (VOC) that bacteria produce. Bacteria produce a characteristic signal of VOC that can be detected by gas chromatography and mass spectrometry (GC-MS)(Bos, Sterk and Schultz, 2013). VOCs are detectable in the exhaled breath of patients on mechanical ventilation and so have the potential to distinguish those with high bacterial burdens associated with infection. So far studies have been small proof-of-concept studies(Bos *et al.*, 2014; Chiu *et al.*, 2014; Filipiak *et al.*, 2015; Fowler *et al.*, 2015). In a single ICU derivation study mechanically ventilated patients, who were predominantly acute brain

injury patients, exhaled breath condensate was analysed by thermal desorption/gas chromatography/time-of-flight mass spectrometry(Fowler *et al.*, 2015). VAP was confirmed by semi-quantitative culture of blind-BAL samples. A clear separation in signal was observed between patients who were infected and those that were not.

These molecular methods hold significant promise in particular exhaled breath condensate analysis, that would allow for a minimally invasive method of detecting infection.

1.11.2 Biomarkers for bacterial infections

Biomarkers have the potential to meet the need for rapid diagnostics if they can act as accurate surrogates of infection while being able to be measured faster than routine microbiology cultures. For bacterial infections the most widely investigated is procalcitonin (PCT). PCT is a precursor to calcitonin and has been recognised to be released in response to bacterial infections(Assicot *et al.*, 1993; Dandona *et al.*, 1994). It was hoped that this could therefore be a marker to distinguish infective SIRS (sepsis) from sterile SIRS. However it has been shown to be raised in situations of sterile SIRS too(Carsin *et al.*, 1997).

The accuracy of PCT to discriminate sepsis from sterile SIRS has been the subject of three meta-analyses. The first of these was to determine its value in surgical or trauma patients(Uzzan *et al.*, 2006). Unfortunately this meta-analysis used any definition of sepsis and therefore is limited by the lack of a rigorous standard. Twenty-five studies were included, many of which were small and sample sizes ranged from 15-405 patients. Among the studies included, the sensitivity of PCT to distinguish sepsis from SIRS ranged from 42-97% and the specificity was 48-100%. The summary diagnostic accuracy of PCT had an OR of 15.7 (95% CI 9.1-27.1). The Q* value is a single summary statistic of the receiver operating characteristic curve (ROC) and is the point where the sensitivity and specificity are equal. The Q* for the study described was 0.78 (95% CI 0.71 – 0.84).

A further meta-analysis used inclusion criteria for trials that incorporated a rigorous definition of sepsis, but excluded trials with too narrow case mix, such as studies of abdominal sepsis(Tang *et al.*, 2007). This meta-analysis included 18 studies of which 14 were phase 2 diagnostic studies (diagnostic studies in patients with or without the disorder) and 4 phase 3 studies (real-life performance in patients suspected of having the disorder). A total of 2097 patients were included. Considering the 14 phase 2 studies the diagnostic OR was 7.79 (95% CI 5.86-10.35) with a Q* value of 0.73. Adding in the

4 phase three studies introduced significant heterogeneity (I^2 86.1%) and the overall result was similar with a Q* of 0.72. Furthermore, on inspection of the funnel plot the authors determined that there had been a publication bias and that missing studies were likely to fall to the left of the summary estimate, suggesting that the summary estimate was an overestimate of the effect.

Although the ROC curves for PCT between these two meta-analyses were quite similar, the authors drew quite contrasting conclusions with Uzzan *et al* suggesting the diagnostic value was high and Tang *et al* concluding the diagnostic value was low. A more recent meta-analysis aimed to update this area and also to overcome the shortcomings of these meta-analyses with respect to the reference definition of sepsis and the heterogeneity of studies(Wacker *et al.*, 2013). 30 studies with 3244 critical care patients were included. The authors reported pooled sensitivity and specificity. The pooled sensitivity was 77% (95% CI 72-81%), the pooled specificity was 79% (95% 74-84%) and the AUROC was 0.85 (0.81-0.88). Since the authors had taken an inclusive approach to trials included, the heterogeneity was high (I^2 96%). The authors report a positive PCT would give a post-test probability (PTP) of sepsis of 48%. On the other hand a negative PCT would give a PTP to exclude sepsis of 7%. The authors conclude that PCT is a helpful biomarker but cannot be used as a single biomarker of sepsis.

Despite the differing methods and conclusion made by the authors of these three meta-analyses, the diagnostic performance of PCT has been fairly consistent across the three meta-analyses. The PTP reported by Wacker *et al*(Wacker *et al.*, 2013) serves as a useful summary of the limitations of PCT as a single biomarker of sepsis. Despite this it has been used in a number of RCTs to influence patient outcome. In the high profile PRORATA trial, patients with suspected bacterial infection either on admission or during their ICU stay were randomised to either a PCT-guided antibiotic initiation and discontinuation algorithm or to standard care(Bouadma *et al.*, 2010). The primary outcomes were death from any cause 28 and 60 days and number of days without antibiotics at 28 days. 630 patients were randomised and of the 311 patients in the PCT group, antibiotic recommendations were not followed in 219 episodes, including episodes when antibiotics were started despite low PCT or not discontinued in light of low PCT. Despite this non-adherence, there were significantly fewer antibiotics used in the PCT group with an absolute difference of 2.7 days (95% CI 1.4-4.1). There was a non-inferior mortality difference between the two groups (0.8%, 90% CI -4.6-6.2). Another trial used PCT to reduce antibiotic exposure in patients with suspected

VAP(Stolz *et al.*, 2009). Patients with suspected VAP were randomised to daily PCT with recommendations to discontinue antibiotics with low levels of PCT, or to standard care. The primary outcome measure was the number of AFD alive at 28 days. One hundred and one patients were randomised and those in the PCT group had significantly more AFD than the control group with a median of 13 (IQR 2-21) in the PCT group and 9.5 (IQR 1.5-17) in the control group. The protocol of using PCT to rule out VAP is questionable since serum PCT has not been shown to have any diagnostic utility in VAP(Luyt *et al.*, 2005, 2008; Zielińska-Borkowska *et al.*, 2012).

In contrast to these trials, another group of investigators used PCT to escalate care when raised rather than to use it as a tool for de-escalation(Jensen *et al.*, 2011). Patients randomised to the PCT group had daily measurement of serum PCT and if ≥1 ng/ml the clinician received an alert triggering further investigation of potential infection and broadening of antibiotic spectrum. 1200 patients were randomised and as might be expected there were more broad-spectrum antibiotics used in the PCT group. The median duration of antibiotics was 6 days (IQR 3-11) in comparison to 4 days (IQR 3-10) in the control group. There was no significant difference in death at 28 days but the length of ICU stay was extended by 1 day in the PCT group and the relative risk of days with estimated glomerular filtration rate of $<60mL/min/1.73m^2$ was 1.21 (95% CI 1.15-1.27).

1.11.3 Biomarkers for VAP

The most widely investigated biomarkers in the context of VAP have been PCT and soluble triggering receptor expressed on myeloid cells (sTREM-1). Although PCT has had some acceptance for bacterial infections in general, in the specific context of VAP its value is much more questionable. All the studies assessing PCT utility for diagnosing VAP used semi-quantitative or quantitative culture to base the VAP definition on, although the sampling methods varied. In a study of 96 patients with suspected VAP in which BAL was performed using a blind method, there was no significant difference in alveolar PCT between VAP and non-VAP patients(Duflo *et al.*, 2002). However this study did find a diagnostic utility from serum PCT, with an AUROC of 0.79 and (at a cut-off of 3.9ng/ml) a sensitivity of 41% and a specificity of 100%, giving this a very strong ‘rule-in’ value. In a rigorous study in which BAL was performed to a clear standard operating procedure and samples excluded based on pre-defined quality definitions, no significant differences were found in serum or BAL fluid PCT or C-reactive protein (CRP) between VAP patients (n=50) and non-VAP patients

(n=66)(Linssen *et al.*, 2008). In two further studies no difference in serum or BAL PCT could be found between those who had VAP confirmed and those who did not(Luyt *et al.*, 2008; Jung *et al.*, 2010). PCT has also been evaluated for prognostic applications and falling levels over the course of the disease has been prognostic for survival from VAP(Luyt *et al.*, 2005; Seligman *et al.*, 2006), although in the context of biomarkers to improve antibiotic stewardship this has limited relevance.

The evidence for sTREM-1 has been limited by methodological inadequacies and inconsistencies. In a study of 148 mechanically ventilated patients in whom pulmonary infection was suspected, blind BAL was performed and pneumonia diagnosed based on semi-quantitative culture(Gibot *et al.*, 2004). 38 patients were diagnosed with community-acquired pneumonia, 46 patients with VAP and 64 as having no pneumonia. BAL fluid sTREM-1, TNF-α and IL-1β were raised in both pneumonias in comparison to those without pneumonia. On constructing ROC curves, the AUROC for sTREM-1 was 0.93 (95% CI 0.92-0.95) and a cut-off of 5 pg/ml had a sensitivity of 98% and a specificity of 90%. The AUROC for IL-1β and TNF-α were 0.69 (95% 0.67-0.72) and 0.64 (95% CI 0.62-0.69) respectively.

Significant diagnostic performance for sTREM-1 was derived from a small study of 28 patients, only 9 of whom had confirmed VAP(Determann *et al.*, 2005). Similarly in this study, a blind BAL technique was used and performed on alternate days while patients were mechanically ventilated. BAL sTREM-1 increased on the day of clinical suspicion of VAP and the AUROC to discriminate VAP from non-VAP was 0.83 (95% CI 0.65-1.00). A level of 200 pg/ml had a sensitivity of 75% and a specificity of 84%. The AUROC for TNF-α was 0.64 (95% CI 0.40-0.87), for IL-1β 0.66 (95% CI 0.42-0.89) and for IL-6 0.74 (95% CI 0.54-0.95). IL-1β, at cut-off of 1000 pg/ml had a sensitivity of 67% and a specificity of 69%. Furthermore, in a group of patients with bilateral infiltrates (rather than suspected VAP), sTREM-1 had sensitivity and specificity of 86% and 90% respectively for the presence of bacterial infection(Huh *et al.*, 2008)

In contrast, two studies in which BAL fluid was more rigorously obtained via bronchoscopy, no useful diagnostic value was found for sTREM-1. In one study there was no significant difference in sTREM-1 between the VAP and non-VAP groups(Horonenko *et al.*, 2007) and in the other, although there was a significant difference between the groups, when a ROC was constructed the AUROC was poor (0.58, 95% CI 0.50-0.65)(Oudhuis *et al.*, 2009).

In terms of prognostic value of sTREM-1, in a small study of 35 patients with suspected VAP, BAL was performed at enrolment and a further 2 BAL performed in the course of the illness(Wu *et al.*, 2011). In this study the rate of confirmed VAP was unusually high at 70%. sTREM-1 was significantly raised in culture positive patients in contrast to non-VAP patients at baseline and day 4-5. ROC curves were constructed for sTREM-1 at baseline, day 4-5 and day 7-9 to identify survivors versus non-survivors. At baseline the AUROC was 0.54 (NS), at day 4-5 the AUROC was 0.77 (95% CI 0.57-0.97) and at 7-9 days the AUROC was 0.89 (95% CI 0.72-1.07). A fall in sTREM-1 of 10 pg/ml at day 7-9 predicted survival with a sensitivity of 90% (non-survivors) and specificity of 87.5% (survivors).

Other biomarkers that have been investigated for VAP include elastin fibres (ETA)(el-Ebiary *et al.*, 1995), copeptin (serum)(Seligman *et al.*, 2008a), nitrated proteins (BAL)(Mathy-Hartert *et al.*, 2000), beta-d-glucan (serum)(Heyland *et al.*, 2011), pancreatic stone protein (serum)(Boeck *et al.*, 2011), midregional pro-atrial naturetic peptide (serum)(Seligman *et al.*, 2008b), pentraxin 3 (serum)(Lin *et al.*, 2013), Clara cell protein (BAL)(Vanspauwen *et al.*, 2011), leukocyte RNA profiles (blood)(Cobb *et al.*, 2009), leptin (serum)(Parmentier-Decrucq *et al.*, 2014) and gene expression (blood)(Textoris *et al.*, 2011). These studies have either found no diagnostic value or have demonstrated some prognostic value. They have all fallen short of a biomarker that can provide the crucial clinical need, a rapid rule-in or rule-out test for VAP.

1.11.4 Importance of innate immune response biomarkers in BAL

In a single-centre study Conway Morris *et al* measured a range of biomarkers of the innate immune response in the lung in BAL fluid(Conway Morris *et al.*, 2010). Seventy-two patients with suspected VAP were included in the analysis. Patients were included if VAP was suspected, and excluded based on criteria that predicted poor tolerance of BAL (eg. severe hypoxia, haemodynamic instability and unstable intra-cranial hypertension). VAP was confirmed by quantitative culture of BAL fluid with a pathogen growth of >10^4 cfu/ml. The biomarkers measured in BAL fluid were TNF-α, IL-1β, IL-6, IL-8, IL-10, granulocyte colony-stimulating factor (G-CSF), MIP-1α, sTREM-1 and monocyte chemo-attractant peptide (MCP)-1. Urea was measured in BAL and serum and used as a correction factor for dilution of ELF by BAL(Rennard *et al.*, 1986). 17 patients had confirmed VAP and 55 patients had culture growth below the threshold or had sterile culture and formed the non-VAP group. It is notable that no VAP was caused by *P. aeruginosa*. Significant differences were found between VAP

and non-VAP patients in BAL fluid IL-1β, IL-8, G-CSF and MIP-1α. ROC curves were constructed for these and IL-1β and IL-8 had AUROC with 0.81 (95% CI 0.71-0.91) and 0.83 (95% CI 0.74-0.95) respectively. The AUROC for G-CSF and MIP-1α were lower with 0.73 (95% CI 0.58-0.86) and 0.77 (95% CI 0.66-0.90) respectively. For IL-1β an optimal cut-point determined by the Youden index at 10 pg/ml had a sensitivity of 94% and a specificity of 64%. This corresponds to a negative predictive value of 97% to rule-out VAP.

In a further report from the same cohort of patients, BAL fluid was tested for a range of markers of neutrophil activity and neutrophil protease inhibitors(Wilkinson *et al.*, 2012). The neutrophil proteases measured were HNE, MMP-8 and MMP-9. Endogenous inhibitors of these proteases were also quantified, including alpha-1 protease inhibitors (α1-PI), secretory leucocyte protease inhibitor (SLPI) and elafin (all of which inhibit HNE) and tissue inhibitors of metalloproteinases (TIMPs, which inhibit MMP-8 and MMP-9). In addition the fibinolytic enzymes tissue-type plasminogen activator (t-PA) and urinary-type plasminogen activator (u-PA) were measured. All markers were quantified by ELISA with the exception of plasminogen activators, which were measured by semi-quantitative zymography. There were significant differences between the VAP and non-VAP groups in concentrations of HNE, MMP-8 and MMP-9. ROC curves were constructed and optimal cut-points calculated. For HNE the AUROC was 0.87 (95% CI 0.78-0.96) and at 670 ng/ml, the sensitivity was 93% and specificity was 79%. For MMP-8 the AUROC was 0.81 (95% CI 0.69-0.93) and at a cut-point of 13 ng/ml the sensitivity was 91% and the specificity 63%. MMP-9 had an AUROC of 0.79 (95% CI 0.66-0.92) and a cut-point of 22 ng/ml had a sensitivity of 82% and specificity of 63%.

The diagnostic performance for the 5 biomarkers with the greatest promise are outlined in **Table 7.**

	AUROC	Cut-point	Sensitivity	Specificity
IL-1β	0.81 (0.71-0.91)	10pg/ml	94%	64%
IL-8	0.83 (0.73-0.95)	2000pg/ml	81%	83%
HNE	0.87 (0.78-0.96)	670ng/ml	93%	79%
MMP-8	0.81 (0.69-0.93)	13ng/ml	91%	63%
MMP-9	0.79 (0.66-0.92)	22ng/ml	82%	63%

Table 7: Cut-points and diagnostic performance of 5 top biomarkers from derivation cohort. AUROC, area under ROC curve (95% CI).

1.12 Aim and Hypothesis

The aim of this work is to take forward the findings in the derivation cohort described in section 1.10.4.. There are two elements to this work, the first of which is to establish whether the diagnostic utility of IL-1β, IL-8, MMP-8, MMP-9 and HNE could be validated in a multi-centre observational study. The **hypothesis is that these will be successfully validated and that an optimum combination of biomarkers will be determined with sufficient value as a test of exclusion for VAP to be used in a clinical trial.**

The second element, dependent on the successful validation of the biomarkers, is to determine the clinical utility of these biomarkers in reducing antibiotic exposure in

patients with suspected VAP by prompting early discontinuation of antibiotics in cases of biomarker-excluded VAP. This will be measured by number of antibiotic-free days (AFD) in the days following BAL. The **hypothesis is that biomarker-guided decisions regarding antibiotic prescribing will reduce antibiotic use and thereby improve antibiotic stewardship**.

This work represents a significant part of the translational research process, bridging initial derivation to determining clinical utility. The two elements of this thesis are conducted in a highly complex and sensitive clinical setting and so the lessons learnt through this process are highly valuable. The potential clinical value is significant. I have highlighted the scale of impact that VAP, the scale of antibiotic use and need to reduce antibiotic use as part of a multi-faceted approach to tackling AMR. If this intervention demonstrates a reduction in antibiotic use, this would be a significant step in tackling this problem.

1.13 Thesis overview

Chapter 2 outlines the methods used in this work. Chapters 3 and 4 present the findings of a multi-centre validation study and a multi-centre RCT respectively. Final conclusions are presented in chapter 5. At the time of writing this thesis, the RCT was still in progress. To allow completion of this thesis, data from a subset of recruited patients were analysed to demonstrate the principles of the analysis. This subgroup analysis was performed at a time that it could not influence trial course and the chief investigator was unaware of the results until the close of the trial. The trial statistician provided assistance using blinded data, such that only I was aware of the unblinded results. This strategy was approved by the data monitoring and ethics committee (DMEC) and trial Sponsor.

Chapter 2. Methods

2.1 Introduction to chapter

This chapter will outline the methods used in the two studies reported in this thesis, a multi-centre validation study and a multi-centre RCT. I shall outline the design of these studies, the clinical elements, laboratory protocols and statistical considerations.

2.2 Design of studies

2.2.1 Design of validation study

The validation study was a prospective, multi-centre observational study conducted in the general ICUs of 9 NHS Trusts (12 ICUs in total). The participating sites were: Royal Victoria Infirmary (ward 18 and 32), Newcastle upon Tyne; Freeman Hospital, Newcastle upon Tyne; Sunderland Royal Hospital, Sunderland; Edinburgh Royal Infirmary, Edinburgh; Western General Hospital, Edinburgh; Heartlands Hospital, Birmingham; Preston Royal Hospital, Preston; Salford Royal Hospital, Manchester; Countess of Chester Hospital, Chester; Royal Victoria Hospital, Belfast; and Chelsea and Westminster Hospital, London. A flow diagram of patient recruitment is shown in **Figure 1**.

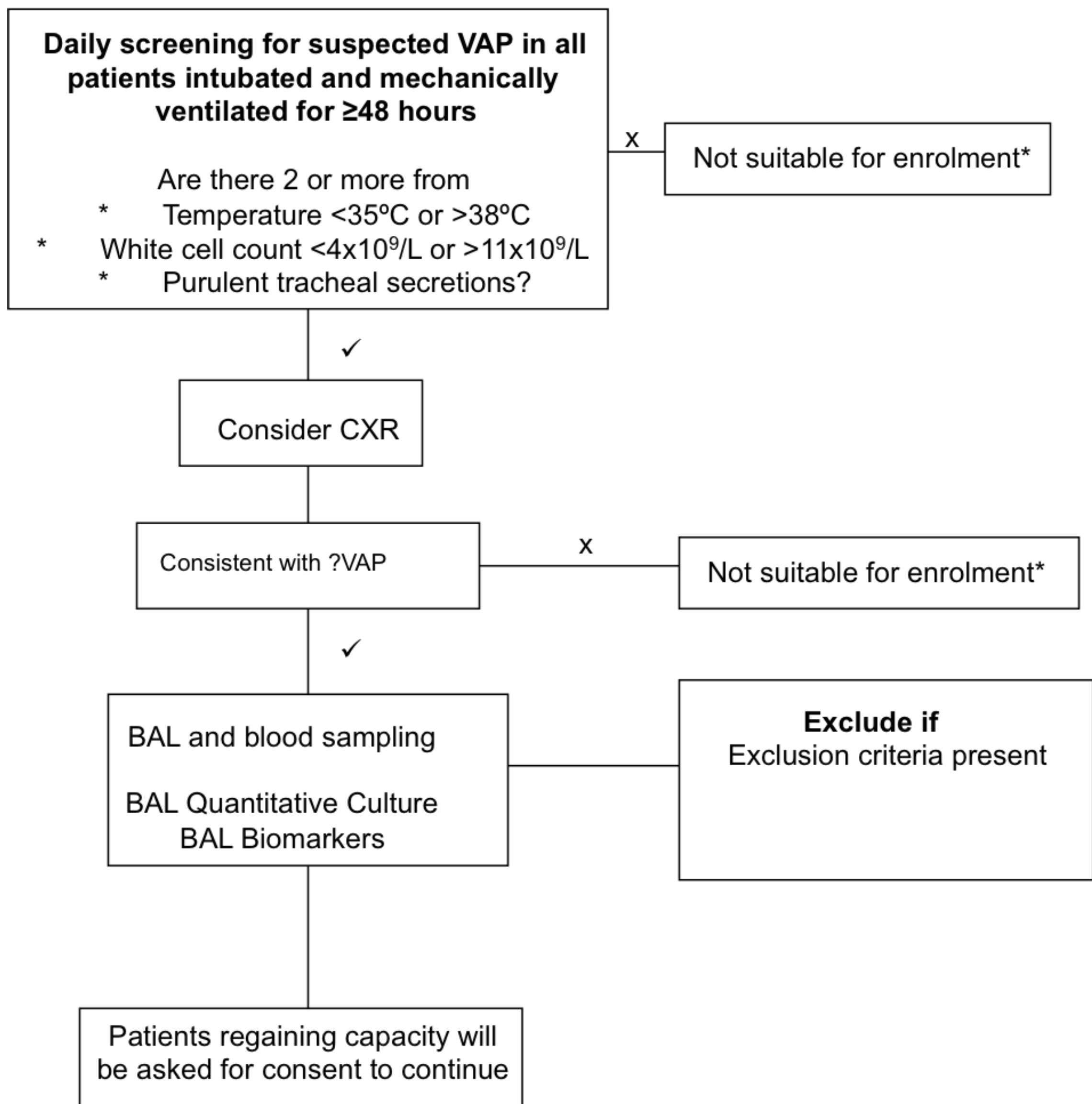

Figure 1: Flow diagram of validation study recruitment

2.2.2 Design of RCT

The RCT was a multi-centre, prospective, RCT in which patients with suspected VAP were randomised 1:1 to either a biomarker-based rule-out of VAP in addition to standard care (microbiological culture) or to standard care only. This trial adheres to the Consolidated Standards of Reporting Trials statement(Schulz, Altman and Moher, 2010) (**Figure 2**).

The RCT was conducted in 23 ICUs from 17 NHS trusts. In addition to the sites involved in the validation study, the RCT also included the following ICUs: North Tyneside General Hospital, North Shields; Wansbeck General Hospital, Ashington; University Hospital, Coventry; Queen Elizabeth Hospital, Gateshead; James Cook University Hospital, Middlesbrough; Royal Infirmary, Manchester; Sandwell Hospital, Birmingham; City Hospital, Birmingham; The Royal Liverpool University Hospital, Liverpool; City Hospital, Belfast; and Russell Hall Hospital, Dudley.

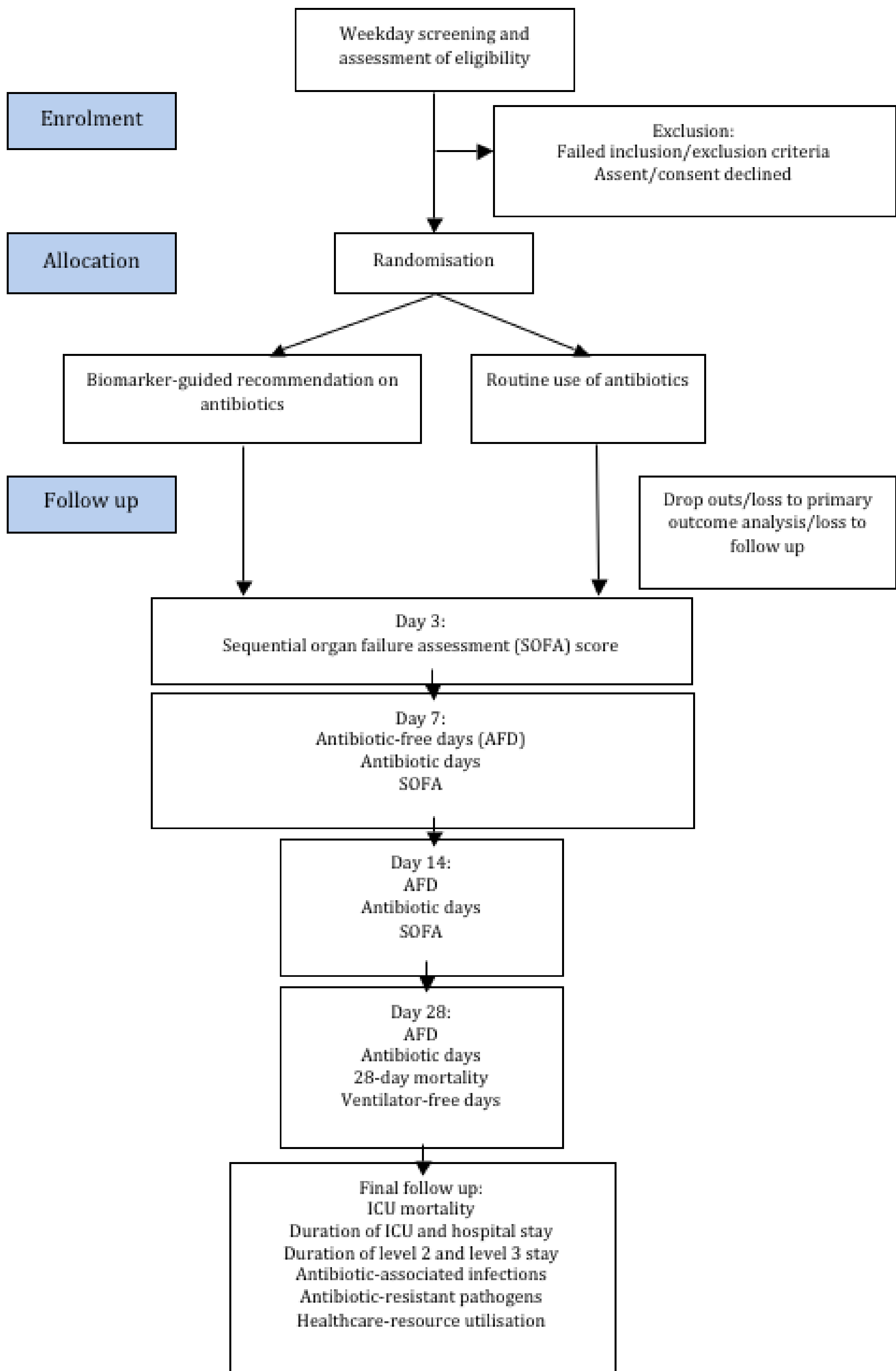

Figure 2: CONSORT diagram for RCT

2.3 Participants: inclusion and exclusion criteria

2.3.1 Validation study participants

Patients with suspected VAP were identified by the following inclusion criteria: Mechanically ventilated for ≥48 hours, ≥18 years of age (16 for Scotland) **and** new or worsening chest radiograph alveolar changes **and** at least 2 of the following criteria:

- temperature $<35^{O}$C or $>38^{O}$C,
- blood white cell count (WCC) $<4x10^9$/L or $>11x10^9$/L
- purulent tracheal secretions

Patients were excluded on the basis of criteria that predict poor tolerance of bronchoscopy and BAL(Meduri and Chastre, 1992). These criteria were:

- PaO_2<8kPa on FiO_2>0·7,
- Positive end-expiratory pressure >15cmH_2O,
- Peak airway pressure >35 cmH_20
- Heart rate > 140 beats per minute
- Mean arterial pressure <65mmHg
- Myocardial infarction in the last month
- Bleeding diathesis (including platelet count $<20x10^9$ per litre of blood or international normalised ratio (INR) >3)
- Poorly controlled intracranial pressure (> 20mmHg)
- ICU consultant assesses procedure not to be safe

Other exclusion criteria included a previous BAL as part of this study and if consent was declined. The exclusion of myocardial infarction in the past month was later removed in an amendment 6 months into the study.

2.3.2 RCT participants

The inclusion criteria used in the validation study were also used in the RCT with a number of alterations. Firstly, all patients were 18 years of age or over, including Scottish patients. Secondly radiological changes could include worsening CT scan changes. Thirdly there was the addition of the criterion that patients had to be

considered potentially suitable for early discontinuation of antibiotics. Considering that the primary outcome measure was AFD in the 7 days following BAL (see section 2.11.2) it was felt necessary to exclude patients who were receiving antibiotics for alternative (ie extra-pulmonary) sources of infection in whom the biomarker test could not influence the number of AFD. Patients who *could not* have antibiotics stopped due to a confirmed alternative source were excluded. Patients were not to be excluded based on clinician *willingness* to stop antibiotics due to the clinician's pre-test probability of VAP, judgement of severity of illness or perceived risk of stopping antibiotics. This criterion required on-going education of trial sites to ensure correct interpretation.

Patients were otherwise excluded on the same criteria as for the validation study. Co-enrolment was allowed with other RCTs after consideration of biological and statistical interactions and consideration of burden to patients and to patients' families. Once there was agreement between investigators to allow co-enrolment, an agreement was drawn up in keeping with the recommendations of the Intensive Care Society(Krige *et al.*, 2013). Co-enrolment was allowed with observational studies without prior agreement.

2.4 Screening and consent/assent process

All patients within the participating ICUs were screened on weekdays for eligibility. While weekday only screening was not mandated during the validation study, few ICUs had research support on weekends and only 1 patient was enrolled on a weekend. Weekday screening was only possible during the RCT as laboratories supporting the biomarker assay only provided weekday support. Potentially eligible patients were discussed with the ICU consultant to determine any safety concerns of performing a bronchoscopy and, for the RCT, to determine the appropriateness of potential early discontinuation of antibiotics.

Consent and assent procedures were in keeping with the legal framework of either England, Northern Ireland (Mental Capacity Act, 2005) or Scotland (Adults with Incapacity (Scotland) Act, 2000) for consent/assent of adults without capacity. In England and Northern Ireland assent is obtained, where possible, following discussion with the patient's next of kin (personal consultee). A nominated consultee provided assent for patients without a personal consultee. This was usually the ICU physician in charge of that patient, providing the individual was not also a member of the research team.

In Scotland the patient's relative or welfare attorney provides consent for incapacitated patients. If the patient's relative or welfare attorney were unavailable, consent was provided in a telephone conversation providing a second member of staff witnessed the discussion.

Patients recovering capacity were approached to provide retrospective consent. The decision as to whether the patient had regained capacity resided with the treating team.

2.5 Bronchoscopy (validation study, RCT)

A standard operating procedure was used based on previously published recommendations on a standardised approach to BAL in critically ill patients(Meduri and Chastre, 1992). All bronchoscopy was performed under the supervision of an ICU doctor (registrar or consultant) who managed the patient's anaesthesia and monitoring. Patients were ventilated with 100% oxygen prior to bronchoscopy. Frequently patients were also given a neuromuscular blocking agent in addition to sedation, although this was not mandated. Bronchoscopy for both studies was considered to be a clinical procedure (in addition to research purposes) and therefore was carried out by either the research team or the clinical team. The bronchoscope was passed through the ETT or tracheostomy tube to the lobe that corresponded to the area of new CXR change. If multiple areas were involved on CXR then the lobes were inspected at bronchoscopy and the area with the most prominent purulent secretions was lavaged. If the CXR demonstrated a 'white out' or no particular lobe had purulent secretions, the posterior segment of the right lower lobe was lavaged. Autopsy data have demonstrated that VAP has a predominance for the posterior segments of the lower lobes(Rouby *et al.*, 1992). BAL was performed by wedging the bronchoscope in the relevant subsegment and instilling 20mls of sterile 0.9% saline, which was aspirated and discarded as the 'bronchial sample'. A further 120mls of sterile 0.9% saline was then instilled in 40ml aliquots with aspiration and collection of BAL fluid between each 40ml aliquot into collection traps. The BAL fluid aspirated from each aliquot was pooled.

2.6 Biomarker assay

2.6.1 Reagents and equipment

All reagents and equipment for the project were supplied by BD Biosciences (with the exception of Spherotech Ultra rainbow beads (Spherotech, Lake Forest, Illinois). CBA assay diluent, CBA detection reagent diluent, CBA capture bead diluent, CBA wash buffer, human IL-8, IL-1β, IL-6, IL-12p70 and TNF-α flex set are commercially

available reagents (BD Biosciences, New Jersey, USA). Enhanced sensitivity IL-1β and IL-8 flex sets, and enhanced sensitivity master buffer kits, were also supplied by BD.

MMP-8 CBA beads ($6x10^6$ beads/ml), MMP-8 phycoerythrin (PE) CBA detection reagent (0.1mg/ml), MMP-9 CBA beads ($6x10^6$ beads/ml), MMP-9 PE CBA detection reagent (0.1mg/ml), HNE CBA beads ($6x10^6$ beads/ml) and HNE PE CBA detection reagent (0.1mg/ml) were custom made by BD Biosciences, San Diego.

Human recombinant MMP-8 (100ug/ml) and human recombinant MMP-9 (100ug/ml) were purchased from R&D systems (Minneapolis, USA). Recombinant HNE was purchased from Merck Millipore (Massachusetts, USA). 50ug was reconstituted in 500μl HNE buffer (sodium acetate 50mM-200mM NaCl pH5.5) to give a 100μg/ml stock solution.

Urea was measured using a QuantiChrom urea assay kit (Bioassays Systems, Hayward, USA).

CBA assays were performed on Accuri C6 flow cytometers. These were supplied to Newcastle University by BD for the validation study and then one to each of the 6 testing hubs for the RCT (Freeman Hospital, Newcastle; Royal Infirmary of Edinburgh, Edinburgh; Salford Royal Hospital, Salford; Heartlands Hospital, Birmingham; Royal Victoria Infirmary, Belfast; and Chelsea and Westminster Hospital, London). Accuri quality control (QC) procedures were carried out using BD cytometer setup and tracking beads (BD Biosciences, New Jersey, USA) and with Spherotech ultra rainbow beads (Spherotech, Lake Forest, Illinois) in keeping with Newcastle University's Flow Cytometry Core Facility standard operating procedures (SOP). QC data were monitored by the flow cytometry core facility during the study.

2.6.2 Processing BAL and blood (validation study and RCT)

During both the validation study and the RCT, BAL aliquots collected from the three instillations of 40mls of saline were pooled. A 2ml sample was sent to the hospital microbiology laboratory for semi-quantitative culture. In the validation study the remaining BAL was centrifuged at 700*g* for 10mins at room temperature. The supernatant was aspirated and stored at -80°C where possible and otherwise stored at -20°C. Samples were stored at the study sites and transported to Newcastle University at the end of the study period.

During the RCT, fresh BAL samples were transported from recruiting sites to testing hubs at 4°C for processing. Samples were centrifuged as above but prior to freezing, a

500μl aliquot was taken for immediate biomarker analysis in patients randomised to the intervention arm.

Serum tubes were centrifuged at 1500*g* for 30mins at 4°C. Serum was aspirated and stored as per the same SOP for BAL fluid.

2.6.3 Measurement of BAL biomarkers (validation study)

Concentrations of IL-1β, IL-8, MMP-8, MMP-9 and HNE were measured in BAL by CBA in a 5-bead multi-plex assay. All buffers were brought to room temperature and samples were thawed at room temperature before being stored on ice.

Preparation of CBA bead master mix

Beads were supplied at $6x10^{6}$ beads/ml and used at 6000 beads per test, corresponding to 1μl of each bead solution used per test. Bead stocks were vortexed for 30 seconds and a sufficient aliquot of each bead stock was pipetted into a 15ml falcon tube. 0.5ml of wash buffer was added to the bead mixture and then the beads were centrifuged for 5 minutes at 200*g* at room temperature. The supernatant was aspirated and the beads re-suspended in a volume of capture bead diluent (for serum) to give 50μl capture bead solution per test. 50μl of bead mixture was added to each labelled tube and incubated in the dark, at room temperature for 15 minutes to equilibrate.

Reconstitution of lyophilised standard spheres (IL-1β and IL-8)

Standards for BD flex sets are supplied as lyophilised spheres. These were reconstituted in 500μl of assay diluent (20 ng/ml) and allowed to equilibrate for 15 minutes at room temperature.

Preparation of dilution series of standard master mix

Recombinant MMP-8, MMP-9 and HNE standards were pre-diluted 1:10 (10μg/ml) in assay diluent. To make the master mix of standards, 62.5μl of reconstituted lyophilized spheres, 20μl of pre-dilution of MMP-8 and MMP-9 and 4μl of HNE, was made up to 500μl with assay diluent. The final concentration of IL-1β and IL-8 was 2500pg/ml, MMP-8 and MMP-9 was 400ng/ml and HNE was 80ng/ml. 1:2 serial dilutions were performed to give 10 standard concentrations with the lowest concentration for IL-1β and IL-8 being 5pg/ml, MMP-8 and MMP-9 being 0.78ng/ml and HNE being 0.16ng/ml.

Preparation of sample dilutions

Samples were centrifuged for 2 minutes at 10,000*g* at room temperature to spin down any aggregates prior to performing dilutions. Samples were prepared in dilutions of 1:5, 1:50 and 1:500 in a 96 well plate using assay diluent. In repeat assays, any sample in which the IL-1β in the 1:5 dilution fell below the standard range, was repeated as a neat sample.

Addition of standard or samples to beads

50µl of either standard or sample was added to the 50µl of bead mixture in each tube. Each sample was mixed thoroughly by pipetting. Samples were incubated at room temperature in the dark for 1 hour.

Preparation of PE detection reagent master mix

A master mix of PE detection reagent was made as per the bead master mix. 1µl per test of each detector was added to a 15ml falcon tube, with the exception of HNE PE, which was used at 2µl per test. The mix was made up to a volume of 50µl per test with detection reagent diluent.

Addition of PE detection reagent to tests

Once the beads had incubated with samples for 1 hour, 50µl of PE detector reagent was added to test tubes. The tests were incubated for a further 2 hours in the dark at room temperature.

Washing samples

1ml of wash buffer was added to each tube and then centrifuged at 200*g* for 5 minutes at room temperature. The supernatant was decanted and 200µl of wash buffer added.

Analysis

Samples were immediately analysed on an Accuri C6 flow cytometer. The cytometer laser configuration was FL1 530/30, FL2 585/40, FL3 780/60 and FL4 675/25 (2 blue, 2 red). Results were analysed using FCAP array V3.0 software (Soft Flow, Pecs, Hungary).

2.6.4 Measurement of urea and concentration correction (validation study)

Urea was measured using the QuantiChrom urea assay kit (BioAssay Systems, Hayward, USA). This method uses a chromogenic reagent that binds to urea to form a coloured complex. The colour intensity is directly proportional to the urea

concentration. The assay has a linear detection range of 0.08mg/dL to 100mg/dL and is performed in a clear bottom 96-well plate.

All reagents were brought to room temperature before use. Urea standard was supplied at 50mg/dL. Serum samples were assayed neat. 5µl of water (blank) and 5µl of standard (50mg/dL), in duplicate, were pipetted to wells as the assay control. 5µl of sample, in duplicate, were placed into the remaining wells on a 96 well plate. Working solution was added at 200µl and allowed to incubate for 20 minutes before reading the optical density (OD) at 520nm.

For the low urea concentrations of BAL the assay was modified as per the manufacturer's instructions by transferring 50µl of water, 50µl of standard (5µl of stock diluted into 50µl of water to give 5mg/dL) and 50µl of sample in duplicate. These were incubated for 50 minutes before reading OD at 430nm.

Urea concentration (mg/dL) was calculated by the following formula:

$[Urea] = \{(OD_{SAMPLE} - OD_{BLANK}) / (OD_{STANDARD} - OD_{BLANK})\} \times n \times [STD]$

Where OD_{SAMPLE}, OD_{BLANK} and $OD_{STANDARD}$ are the OD values for sample, water and standard respectively. *n* is the dilution factor (1 for serum, 50 for BAL) and [STD] is the urea standard concentration (50mg/dL for serum assays and 5mg/dL for BAL assays).

ELF biomarker concentration was determined by correcting the BAL biomarker concentration by the following equation:

$Volume_{ELF} = ([Urea_{BAL}] \times Volume_{BAL}) / [Urea_{PLASMA}]$(Rennard *et al.*, 1986)

$Dilution\ factor = Volume_{BAL} / Volume_{ELF}$

$Dilution\ factor = [Urea_{SERUM}] / [Urea_{BAL}]$

$[Biomarker_{ELF}] = [Biomarker_{BAL}] \times ([Urea_{SERUM}] / [Urea_{BAL}])$

2.6.5 Measurement of serum biomarkers (validation study)

Biomarkers in serum that have diagnostic utility in patients with suspected VAP would be of great value, as it would avoid the need to perform a BAL. Serum biomarkers were measured in the original derivation cohort and no difference was found between the VAP and the non-VAP groups(Conway Morris *et al.*, 2010). Considering the potential value of a serum biomarker, this was attempted in this study also. A pilot study of IL-1β, IL-8, IL-6, TNF-α, IL-12p70, HNE, MMP-8 and MMP-9 was conducted in 20 validation study serum samples to determine which of these 8 biomarkers should be tested on the full validation cohort and which dilutions should be used in the main

cohort. Samples were diluted using assay diluent in concentrations of 1:4, 1:20, 1:100 and 1:500.

From this, a multiplex of IL-8, MMP-8, MMP-9, HNE and IL-6 were tested on the full cohort of validation study samples in dilutions of 1:4, 1:20 and 1:100. The CBA procedure was the same as the assay outlined in the above section. The standard for IL-6 was handled in the same way as IL-8 and the top standard was also 2500pg/ml.

2.6.6 Enhanced sensitivity assay (validation study)

IL-1β and IL-8 were also tested using an enhanced sensitivity assay. The standard range for the enhanced sensitivity flex set is 274-200,000 fg/ml and so has improved detection of low concentration biomarkers.

All reagents were brought to room temperature and samples thawed at room temperature before being stored on ice prior to testing. The enhanced sensitivity assay is identical to the CBA for stages preparing the CBA bead master mix, preparation and dilution of standards. The samples were similarly centrifuged at 10,000*g* prior to diluting 1:3, 1:30 and 1:500. Beads were incubated with sample or standard for 2 hours in the dark at room temperature (as compared with 1 hour in the standard assay).

During this time the detection reagents part A and part B were prepared. Reagent part A was prepared as per standard PE detector reagent with 1µl of each reagent (IL-1β and IL-8) per test being added to a volume of detector reagent diluent to give a final volume of 20µl per test. Reagent part B is provided as a lyophilised cake that is reconstituted in 0.55ml of detection reagent. This is incubated in darkness at room temperature for 15 minutes. 530µl of the reconstituted part B reagent was added to 4770µl of detection reagent diluent and mixed by vortexing. After the initital 2-hour incubation period, 20µl of detection reagent part A was added to the samples and further incubated for 2 hours. After this incubation period, the samples were washed by adding 1ml of wash buffer and centrifugation at 200*g* at room temperature. The supernatant was decanted off before 100µl of detection reagent part B was added and gently mixed. The samples were incubated for a further 1-hour period in the dark at room temperature. A final wash was performed (as previously described) before 200µl of wash buffer was added and then samples analysed on the Accuri C6.

2.6.7 Biomarker assay protocol for RCT

Collaborators at BD made a number of modifications to standard flex set CBA assay for the RCT in comparison to the validation study protocol. The intra-assay variation is

greatest at the extremes of the standard curve and so the 5pg/ml point was removed and 9 points on the standard curve, with the lowest point being 10pg/ml, were used for IL-1β and IL-8.

There were two controls built into the assay to ensure robustness of the results. Since some patients will have low IL-1β levels or even no detectable IL-1β, there are concerns about the robustness of detecting very low levels of an analyte. IL-8 acts as a control in this regard since IL-8 was present in all but 2 samples from the validation cohort. The 2 samples with undetectable IL-8 had globally low or undetectable biomarkers, suggesting low quality BAL. Therefore the IL-8 acts as a control to ensure that there is measureable protein in the sample. In addition to the IL-8 control, an IL-1β and IL-8 positive control was incorporated into the assay and this had to fall within predetermined limits for the result to be reliable. This controlled for assay quality including correct solubilising of lyophilised standard, correct reagent temperature and timing of incubation periods. Internal control limits were initially determined from 17 independent experiments for IL-8 and 8 independent experiments for IL-1β (performed by myself and BD scientist). These were later expanded based on CBA assays from the RCT to include a total of 26 experiments for IL-1β and 35 experiments for IL-8.

Samples were tested neat, 1:5, 1:50 and 1:500 dilutions. The positive control was diluted 1:40. As previously described, samples were measured on the Accuri C6 and results analysed using FCAP software. An analysis template (Excel file) was created to guide the technicians through the steps of checking the IL-8 and positive control quality control steps and then determining which sample dilutions should be included to calculate average IL-1β and IL-8 values. The template incorporated a locked cell that contained the logistic regression equation to calculate the final result and a logical formula to state if it was ‘VAP’ or ‘non-VAP’. This was to reduce the chance of error at this interpretation point.

2.7 Microbiology (validation study and RCT)

Culture of BAL fluid followed the same SOP in both the validation study and the RCT. VAP was defined as growth of a potentially pathogenic organism at $>10^4$ cfu/ml during the validation study, in keeping with widely accepted thresholds(Chastre *et al.*, 1995). For practical purposes the threshold was changed to $\geq 10^4$cfu/ml for the RCT. This change was made as it was felt, given the semi-quantitative nature of quantification,

laboratory reporting and clinical interpretation would not distinguish between >10^4 and ≥10^4 cfu/ml in a practical sense.

All microbiology samples were handled in a NHS or Health Protection England (HPE) (previously Public Health England) microbiology laboratory. BAL was handled following a SOP in accordance with the UK Standards for Microbiology Investigation(Health Protection Agency, 2012). BAL fluid samples did not undergo Gram staining unless specifically requested by the clinical team or if this was routine for the laboratory. Each specimen was mixed well by vortexing and then using a sterile 10μl loop the following plates were inoculated with fluid (**Table 8**):

Media	Atmosphere	Temp	Duration	Target organism
Chocolate blood agar + Bacitracin	5-10% CO_2	35-37°C	40-48 hrs	*H. influenza* *M. catarrhalis* *S. aureus* *S. pneumoniae*
BA+ Optochin disc	5-10% CO_2	35-37°C	40-48 hrs	*S. pneumoniae*
Fastidious anaerobic agar	AnO_2	35-37°C	40-48 hrs	Anaerobes
CLED or MacConkey	Air	35-37°C	40-48 hrs	Enterobacteriaceae Pseudomonads
Sabouraud's agar (x2)	Air	30 & 35-37°C	40-48 hrs	Fungi

Table 8: Culture conditions for semi-quantitative cultures. CLED, cysteine lactose electrolyte deficient.

The number of colonies were counted on the plate and interpreted as cfu/ml. Less than 10 colonies was interpreted as <10^3 cfu/ml, 10-100 colonies was interpreted as 10^3-10^4 cfu/ml and >100 colonies was interpreted as >10^4 cfu/ml.

During the validation study, all bacteria isolated at >10^4 cfu/ml were identified to species level. This proved a barrier in the labs when moving to the RCT and so only potentially pathogenic organisms isolated at >10^4 cfu/ml were identified to species level. All yeasts were identified to species level and filamentous fungi were sent to an appropriate mycology lab for speciation. Antimicrobial susceptibility testing was performed according to local practice.

An important consideration for culture results is the previous administration of antibiotics. The derivation study excluded patients who had had a change in prescribed antibiotics in the previous 3 days(Conway Morris *et al.*, 2010). Previous studies have shown that the development of suspected VAP while receiving antibiotics for another infection, does not influence the diagnosis of VAP since the emerging infection will be resistant to the current antibiotic treatment(Montravers *et al.*, 1993; Timsit *et al.*, 1995). The recent initiation of antibiotics for suspected VAP can, however, have a significant impact on the subsequent culture results and lead to false negative results(Fabregas *et al.*, 1996; Souweine *et al.*, 1998). In both the validation study and the RCT, the decision was made to allow recruitment of patients who had antibiotics started within 48 hours. Although this was not ideal, a pragmatic decision was made to ensure sufficient patient accrual.

2.8 Hub set up and training methods/materials (RCT)

The biomarker assay was carried out in 6 laboratories that acted as 'hubs' for the recruiting ICUs. All sites were within an approximate travel time of 1 ½ hour from the hub to avoid samples having excessive transit times.

The set up of the hub was dependent on local arrangements and generally the biomarker test was hosted in microbiology labs, since these labs were already engaged in the project. Salford, Belfast and Heartlands incorporated the biomarker assay within a NHS microbiology lab. Newcastle (Freeman Hospital) and Edinburgh utilised the academic microbiology labs. Chelsea and Westminster performed the test in an academic immunology lab. All labs were within NHS hospitals.

With the exception of Chelsea & Westminster, which used scientists with flow cytometry experience, all other sites used scientists with a microbiology background and limited flow cytometry experience. Essential training in the Accuri C6 and CBA assay was provided. Training material was designed for a user with limited flow cytometry experience. The Accuri C6 is a flow cytometer that is designed for its ease of use. The fluorescence detectors are pre-optimised and so adjustment of detector voltage is not necessary. It has minimum set up time and when using software templates, it is close to a 'plug-and-play' flow cytometer. It also has a small bench footprint and is easy to move if necessary which proved valuable in laboratories with limited bench space.

Training was provided to the technicians in two, 2-day sessions in London (Chelsea & Westminster) or Newcastle. Training material was developed in collaboration with BD Biosciences scientists and a manual was given to technicians. Training for the Accuri included an overview of the Accuri C6; templates; set-up and shut-down; maintenance; QC; and lasers and filters. Other topics related to the trial itself and included an overview of the trial; sample transport and handling; randomisation process; biomarker assay protocol; processing samples on Accuri C6; analysing Accuri data on FCAP software; final data sorting and interpretation; and reporting results to the clinical team. On site training was provided for Salford and Belfast in the same format as the centralised training.

2.9 Randomisation and blinding (RCT)

Randomisation was performed using a web-based randomisation service hosted by Newcastle Clinical Trials Unit (NCTU). Patients were randomised in a 1:1 ratio by variable size permuted blocks and stratified by site. Since randomisation determined by which means the BAL fluid was analysed and up to that point all patients undergo the same study procedures of bronchoscopy and BAL, the technicians performed the randomisation. To perform the randomisation the technician contacted NCTU and the patient's details were entered into the online randomisation service. The automated message instructed that the patient was randomised to 'biomarker-guided recommendation on antibiotics, analyse sample on arrival' or to 'routine use of antibiotics, do not analyse sample on arrival'. This instruction was emailed to the technician.

The clinical team were blinded to allocation for the first 6 hours of trial involvement until the results were called back. The technicians reported the results to the clinical team according to standard scripts. To ensure consistent unblinding, the clinical team

were informed if the patient was randomised to standard care after a similar interval (approximately 6 hours). The instructions were:

If the biomarker result was below threshold:

"Biomarker result below cut-off. The negative predictive value is 1 and a diagnosis of VAP is very unlikely. Consider discontinuation of antibiotics."

If the biomarker result was above the threshold:

"Biomarker result above the cut-off. VAP cannot be excluded, consider continuing antibiotics."

If the assay did not meet quality control standards the clinical team were advised to default to standard care. If the assay did not meet the internal control check-point the clinical team were advised:

"Biomarker assay has not met internal control standard. Unable to return biomarker result. Await microbiology result and default to standard care".

If the assay did not meet the IL-8 check-point, the clinical team were advised:

"IL-8 undetectable in BAL. The quality of BAL is uncertain. Unable to return biomarker result. Default to standard care."

2.10 Trial intervention (RCT)

Since all included patients had suspected VAP, they all underwent the sample study procedures of bronchoscopy and BAL. It was anticipated that all patients would have antibiotics started at the point of suspicion, around the time of BAL, although this would not always be the case depending on the clinical index of suspicion. Those who underwent biomarker testing would have a result in approximately 6 hours and those who had VAP ruled out would be able to have antibiotics discontinued. In comparison, those in the 'routine use of antibiotics group' would ordinarily be first considered for discontinuation of antibiotics when microbiology results return, typically 48-72 hours after BAL.

2.11 Outcome measures

2.11.1 Validation study outcome measures

The primary outcome measure of the validation study was the presence of confirmed VAP based on a potential pathogen at $>10^4$ cfu/ml. This provided the standard against which the BAL fluid biomarkers were measured.

Secondary outcome measures were biological measures (ie. biomarkers), safety and clinical measures. Biological measures included IL-1β, IL-8, MMP-8, MMP-9, HNE and urea in BAL fluid. In serum, the markers were IL-1β, IL-8, IL-6, TNF-α, IL-12p70, HNE, MMP-8, MMP-9 and urea. Safety measures included SaO_2, heart rate, BP, PaO_2:FiO_2 ratio, before and after the procedure. Clinical measures included demographics, all-cause ICU mortality and hospital mortality.

2.11.2 RCT outcome measures

The primary outcome measure for the RCT was the frequency distribution of AFD in the 7 days following BAL. This time-course was felt most appropriate since this is the typical duration of a course of antibiotics for VAP in the UK(Browne *et al.*, 2014). Days of antibiotic treatment were counted in whole numbers of days. Durations of antibiotic prescribing was counted from the date of the BAL procedure to date of treatment termination (or censoring from death or discharge), with the proviso that any courses started and stopped on the same day will be classed as one day of antibiotic use. Antibiotics that were not administered for active clearance of infection (e.g. those given as "prophylaxis" against infection or those used for an alternative indication such as a pro-motility function in the gastrointestinal tract) were not counted in the analysis.

Secondary outcome measures included antibiotic days and AFD, expressed as continuous variables, at day 7, 14 and 28; ventilator-free days at 28 days; 28-day all-cause mortality and critical care mortality; sequential organ failure assessment (SOFA) at days 3, 7 and 14 with modification for non-neurological assessment(Vincent *et al.*, 1996); duration of critical care, level 2-, level 3- and hospital-stay; the presence of *Clostridium difficile* and MRSA infections up to hospital discharge, death or 56 days; and number of antibiotic-resistant pathogens cultured up to hospital discharge, death or 56 days

VFD is defined as the number of days from enrolment up to 28 days that the patient breathes unassisted. A common situation in the ICU is that the patient will have a trial without assisted breathing and if the patient fails this trial, mechanical ventilation is restarted. For this reason the period of unassisted breathing begins when assisted breathing is discontinued for a period that is >48hrs. If the patient receives a further period of mechanical ventilation once unassisted breathing has been achieved, this is subtracted from the VFD. Death within the 28-day period will be assigned zero VFD. Mechanical ventilation for surgical procedures is not counted. Unassisted breathing is defined as:

- Extubated breathing on supplemental oxygen or room air.
- Open T-tube breathing.
- Tracheostomy mask breathing.
- Continuous Positive Airway Pressure (CPAP) <5 cmH_2O without pressure support.

2.12 Clinical data collection (validation study and RCT)

In the validation study clinical data were collected on age, sex, admission category (medical or surgical), Acute Physiology and Chronic Health Evaluation (APACHE) 2 score, use of organ support (renal replacement, vasopressors), corticosteroid use, presence of ARDS, temperature on day of BAL, white cell count on day of BAL, $PaO_2/PaCO_2$/pH on day of BAL, ICU length of stay, hospital length of stay, ICU mortality, hospital mortality, antibiotic use on the day of BAL and in the 3 days preceding the BAL, and antibiotic use in the 7 days following BAL. Safety measures were collected in relation to the BAL including oxygen saturations, heart rate, blood pressure, and $PaO_2:FiO_2$ ratio.

Clinical data collection for the RCT was similar to the validation study. Baseline data were collected on the day of enrolment (day 0). In addition to the above clinical data, reasons for ICU admission were collected, along with functional co-morbidities index score(Groll *et al.*, 2005), neutrophil count, platelet count, CRP, positive end-expiratory pressure, peak airway pressure, tracheal secretion character, CXR findings, pulse, mean arterial pressure, intracranial pressure, and indications for antibiotic use at the time of BAL and in the preceding 3 days. Patients were followed up on days 3, 7, 14 and 28, and the final follow-up was at death, discharge from hospital or at 56 days. Clinical data to assess primary outcome and secondary outcomes were collected.

2.13 Statistical methods

2.13.1 Validation study statistical considerations

The sample size for the validation study was based on validating the negative post-test probability (PTP) of VAP (ie. exclusion of VAP) using the IL-1β threshold determined in the derivation study(Conway Morris *et al.*, 2010). The derivation study stated that BAL IL-1β below 10pg/ml excluded VAP with a PTP of 2.8% (95% CI 0.1-15.9). A 95% confidence interval of 0-8% around a PTP of 3% was felt to be tight enough for clinical use. Estimating the prevalence of VAP to be 24% based on the derivation

cohort, 140 patients were required to achieve this and accounting for an approximately 15% dropout rate, we planned to recruit 160 patients.

Statistical analysis was performed using SPSS version 19 (Chicago, Illinois, USA), Prism 6 (La Jolla, California, USA) and R 3.0.0(Team, 2013). Comparisons of non-parametric continuous data were performed using Mann-Whitney U test and parametric data using the Student's T-test. Proportions were analysed by Chi-squared test. To determine diagnostic utility of the biomarkers the population was dichotomised into a VAP and non-VAP group based on the BAL culture thresholds ($>10^4$ cfu/ml = VAP). ROC curves were constructed for individual biomarkers. Biomarkers were tested in combination by $\log_{10}$ transformation of biomarkers with the addition of a constant of one before being entered into a logistic regression model. ROC curves were constructed from the output of the logistic regression. The AUROC was calculated and optimum cut-points determined. The pre-specified method for this was to use the Youden index(Youden, 1950). This statistic takes the optimal balance of sensitivity and specificity from the ROC curve. In a post-hoc analysis, cut-points were determined by fixing a minimum NPV of 95% to ensure a high rule-out performance. The diagnostic rules, performance measures and 95% confidence intervals were derived using the OptimumCutpoints library in R3.0.0(Lopez-Ranton and Rodriguez-Alvarez, 2013). Post-hoc sub-groups analyses were performed to investigate the effect of antibiotic use on the biomarker performance, to investigate differences in medical versus surgical patients and to investigate differences in VAP, sub-VAP and sterile culture.

2.13.2 RCT statistical considerations

As stated earlier, at the time of writing this thesis the RCT was still on going. A sub analysis was performed of a group of recruited patients who had a considerable amount of follow up data collected. This was not a formal interim analysis, as it was not used to inform RCT direction. In fact the results of this analysis was not made available to the chief investigator until the trial had closed. This strategy was approved by the trial sponsor and by the DMEC.

In relation to the RCT, the primary outcome measure of measuring a change in the frequency distribution of AFD in the 7 days following BAL was reached after a series of considerations. Firstly, although AFD is a continuous variable, it is bounded at its lower and upper values, 0 and 7 AFDs, and a non-trivial proportion of patients will have AFD values at the boundaries. This has implications for power calculations and for analysis based on location-shift models (ie. a change in mean, median or mode) since

these data are not normally distributed(Lesaffre *et al.*, 1993). For this reason, this outcome cannot be handled as a simple continuous variable.

The initial solution was to use a proportions model. Two groups were created, 0-2 AFD and 3-7 AFD and sample size estimated based on the number of patients required to detect an increase of 50% in those with 3-7 AFD. Initial estimations were based on derivation study data(Conway Morris *et al.*, 2010). Baseline proportions based on those data were that 59% of patients had 0-2 AFD and 41% of patients had 3-7 AFDs. To detect a 50% increase in the proportion of patients with 3-7 AFD with 80% power and an alpha of 0.05, 93 patients were required per trial arm. Allowing for a 15% drop out rate, 214 patients were initially estimated. This estimation was, however, based on data from a single centre. The completion of the validation study mandated a review of the power calculation based on the validation cohort AFD data. In the validation cohort 78.1% of patients had 0-2 AFD and 21.9% had 3-7 AFD. The difference between the AFD proportions had significant consequences for the sample size. A 50% increase from 22% to 33% would require 276 per arm of the trial. That would have been too large in terms of project budget and expected recruitment period.

One approach to reducing sample size was to power to a larger effect size. An increase in the proportion in the 3-7 AFD group from 22% to 44% (a 100% increase) required a sample size of 80 patients per group. Although this was an attractive sample size in terms of patient accrual, it is a large effect size. The biomarker sensitivity and specificity profile also made this impractical. A specificity of 44% means that 44% of non-VAPs will have a biomarker result below the threshold, but that is 29% of the *whole* population of *suspected* VAP. It then becomes apparent that a small percentage of the patients must have a sufficient effect from the intervention to detect the change in the whole trial arm. In fact an increase in the proportion of 3-7 AFD from 22% to 44% would have required 100% compliance in antibiotic guidance, which was considered an unrealistic expectation.

Using a binary outcome such as proportions in one of two groups misses much of the information around change in the primary outcome and large sample sizes are required. An alternative approach was to use a change in the frequency distribution of AFD. AFD were handled as ordinal values with patients being in 1 of 8 categories, 0-7 AFD. Using ordinal outcomes over dichotomous outcomes gives more statistical power, allowing a smaller sample size, and captures more of the information around the changes in the outcome measure(McHugh *et al.*, 2010). The baseline distribution, established from the

validation study, was skewed to the left with the majority of patients having 0-2 AFD (0 AFD = 57.6%, 1 AFD = 8.6%, 2 AFD = 11.9%). Models were created to demonstrate a shift in distribution towards the right representing a move towards more patients having more AFD. These models are demonstrated in **Table 9**. The effect sizes are the sum of the squared differences between the proportions for each AFD category.

	Proportion of AFD (%)								N per arm	Effect Size
	0	1	2	3	4	5	6	7		
Standard Care (from validation study)	55	10	10	5	5	5	5	5		
Model 1	40	20	15	5	5	5	5	5	215	0.033
Model 2	35	20	15	10	5	5	5	5	138	0.052
Model 3	30	20	15	10	10	5	5	5	96	0.075
Model 4	25	20	20	10	10	5	5	5	68	0.106

Table 9: Models of different frequency distributions of AFD. Standard care distribution is based on data obtained from validation cohort. The different models demonstrate increasing shifts in the frequency distribution towards more AFD in the sample. These distributions are illustrative and different

The changes in distribution in the models represent clinically significant changes in antibiotic use since any shift towards patients having more AFD is relevant in a clinical environment where antibiotic use is so high and antibiotic resistance is such a concern. A sample size of 90 patients per arm would have 80% power to be able to detect an effect size of 0.0797 (slightly higher than model 3). With approximately 15% predicted drop out rate, 210 patients were required. By way of illustration the change in distribution from baseline to model 4 is illustrated in **Figure 3**.

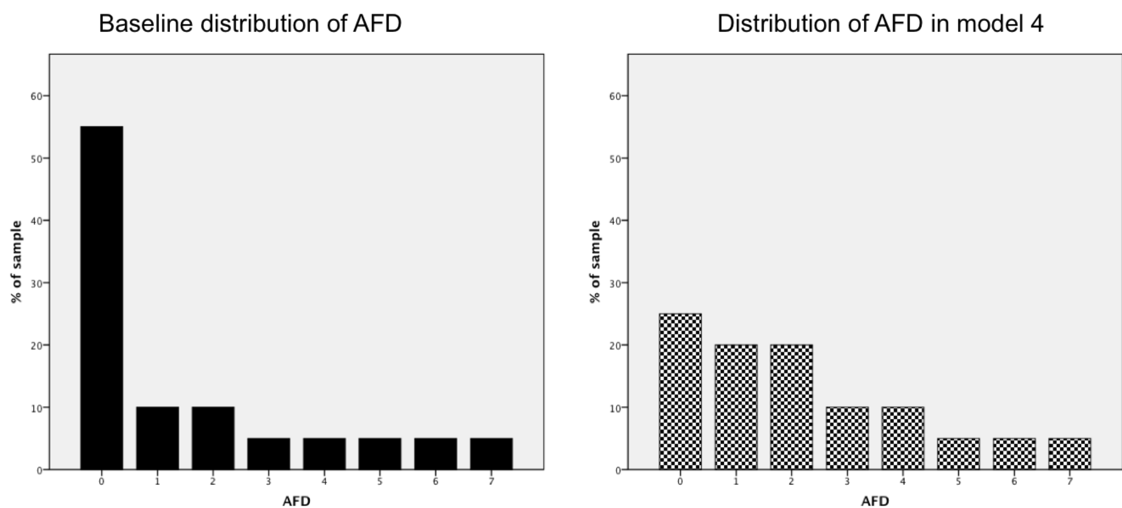

Figure 3: Shift in distribution of AFD from baseline to model 4.

The analysis plan for the RCT aimed to present baseline clinical characteristics using summary statistics without formal tests of equality. The primary outcome, handled as an ordinal variable, was analysed by chi-squared test in a 2x8 contingency table. AFD were censored to death or hospital discharge.

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Secondary outcomes measures were compared between the trial arms by Chi-squared, Mann-Whitney U or T-test depending on the nature of variable and its distribution.

Data analysis was performed on an intention-to-treat basis. Exploratory per-protocol analyses were planned *a priori* , in particular accounting for patients randomised to the intervention arm but in whom the biomarker assay was not reported for technical reasons and so defaulted to standard care.

2.14 Statement of significant contributions by others

The acknowledgements section outlines the contributions of many individuals who assisted in the delivery of two complex studies. This section aims to layout with more detail elements of the work that was carried out either entirely by others or with a significant contribution by others. This is largely with respect to the statistical elements of the research.

The power calculations for the validation study and the RCT were entirely performed by the trial statistician (Niall Anderson). For the validation study analysis, I carried out the analysis of demographics and constructed ROC curves for individual biomarkers. The statistician calculated optimum cut-points using R and performed the logistic regression analysis for the combination of biomarkers (including generation of graphs). I ensured that I was able to replicate this process with SPSS, to generate my own logistic regression output and graphs.

The urea testing of serum and BAL fluid as well as the CBA testing of serum (not including the pilot testing), was entirely performed by Jonathan Scott, laboratory technician.

Chapter 3. Results: Validation study

3.1 Introduction to chapter

This chapter reports the results of a multi-centre validation study. The purpose of the validation study was to determine whether BAL fluid biomarkers could effectively exclude VAP when tested in a multi-centre setting. The validation study was a preparatory study for the RCT and so the results reported here and considerations discussed are framed in the context of their implications for the RCT.

3.2 Screening and recruitment

Patients were recruited from 12 ICUs from 9 NHS Trusts between February 2012 and February 2013. This was slower than the expected timeline of 6 months (**Figure 4**).

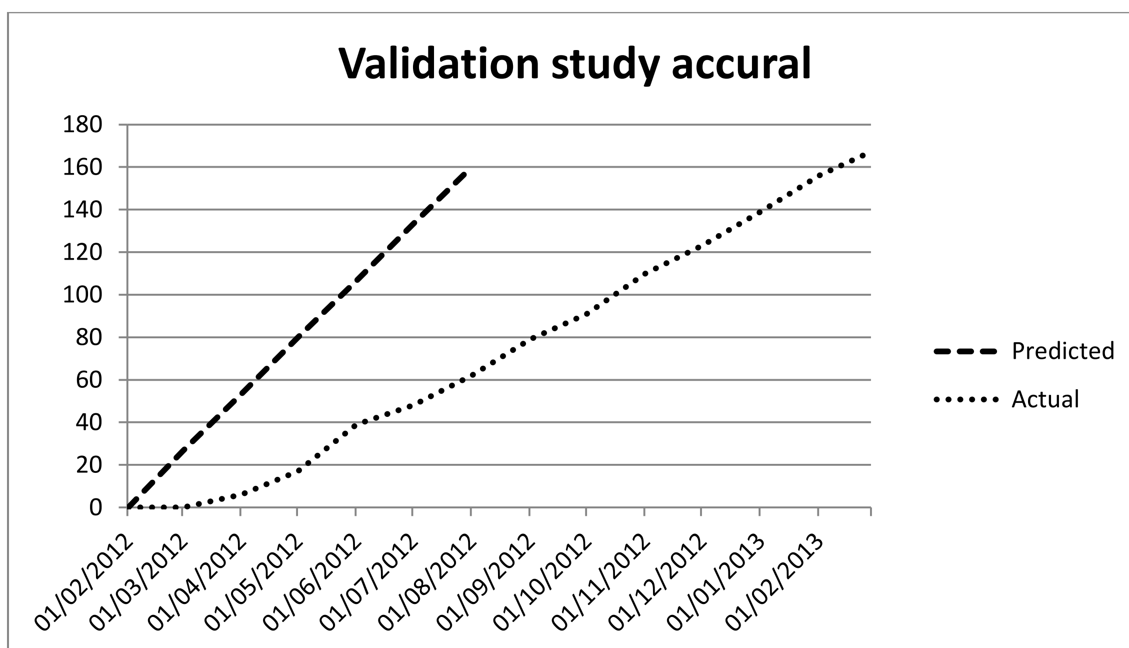

Figure 4: Validation study accrual. Actual versus predicted accrual rate.

Patients were recruited from a large number of general ICUs. Although admission diagnosis was not recorded, it would be expected that this is representative of the general UK ICU population. Although screening logs were kept on each site, these were not monitored through the NCTU since this was an observational study rather than a trial. Some sites recorded number of patients screened whereas other sites recorded

screening episodes. Therefore the total number of patients screened is an approximation. It is estimated that 1385 patients were screened and of these 415 patients met the inclusion criteria. Of these patients 248 were excluded and reasons were recorded for 220 patients. 87 (21%) patients were excluded due to exclusion criteria or due to safety concerns. These included 14 patients with high oxygen requirements, 18 patients with high intracranial pressure, 20 with recent MI (this was removed as an exclusion criterion during the validation study), 5 with abnormal coagulation and 2 with high airway pressure. In addition 28 patients were excluded based on consultant assessment that the patient was too unstable for BAL. Patients were also excluded when, despite meeting the inclusion criteria, the clinical team did not suspected VAP (ie. either hospital- or community-acquired pneumonia) (10 patients), lack of availability of BAL (6), suspension of recruitment (5), previous BAL outwith study recruitment (5), patient improving and weaning (10), consent declined (15), planned withdrawal of care (21), planned extubation (19), lack of availability of research staff (4), administration of new antibiotics for VAP episode for >72hrs (5), co-enrolment issues (3) and other miscellaneous reasons (8). A further 21 patients were excluded based on a consultant decision distinct from the group where patients were classed as 'too unstable'. The screening logs were not detailed enough to establish the reason but included factors such as safety, diagnostic issues, clinical indication for BAL and acceptability of BAL.

One hundred and sixty seven patients were consented and of these 17 patients dropped out. Five patients were withdrawn by clinicians or relatives prior to performing BAL, 4 patients deteriorated following consent making BAL unsafe, 7 samples were mishandled in the laboratory (either semi-quantitative culture not performed or sample incorrectly stored) and 1 patient improved between consent being obtained and BAL being performed.

One hundred and fifty patients with paired semi-quantitative microbiology results and biomarker results were entered into the analysis. VAP was confirmed in 53 patients (35%) with cultures $>10^4$ cfu/ml. The remaining 97 (65%) with sterile culture (54) or culture growth $\leq 10^4$ cfu/ml (43) were classed as non-VAP.

The Standards for Reporting of Diagnostic Accuracy (STARD) diagram is presented in **Figure 10** (Section 3.8 Biomarker combinations) after rationale for biomarker selection.

3.3 Demographics and clinical data

Demographics were similar between the VAP and non-VAP groups (**Table 10**). There was a predominance of surgical patients in the VAP group. There was no mortality difference between the VAP and non-VAP groups and the crude mortality rates were consistent with that expected for a sample of patients with suspected VAP(Chastre and Fagon, 2002; Bekaert *et al.*, 2011).

Characteristic	**VAP (N=53)**	**Non-VAP (N=97)**	***p* value**
Age (years) – mean ± SD	56.3 ± 17.7	55.1 ± 17.3	0.48
Male – n (%)	42 (79.2)	69 (71.1)	0.28
Time from ICU admission to consent (days) – median (IQR)	5 (4-8)	7 (4-11)	0.43
APACHE II score on admission* - mean ± SD	17.3 ± 6.0	19.1 ± 7.8	0.15
Surgical admission – n (%)	31 (58.5)	41 (42.3)	0.06
Medical admission – n (%)	22 (41.5)	56 (57.7)	0.06
Hospital mortality – n (%)†	19 (38.8)	31 (33)	0.49
ICU mortality – n (%)‡	13 (24.5)	23 (23.7)	0.68
Hospital LOS (days) – median (IQR)‡	39 (25-65)	36 (20-51)	0.26
ICU LOS (days) – median (IQR)§	17 (14-34)	17 (11-31)	0.21
Renal replacement therapy – n (%)¶	5 (9.4)	12 (12.5)	0.57
Vasopressors – n (%)§	12 (23.1)	35 (36.5)	0.10
ARDS criteria – n (%)//	8 (16)	19 (21.3)	0.44
Receipt of corticosteroids – n (%)*	7 (13.2)	21 (22.8)	0.16

Table 10: Demographics of VAP and non-VAP groups. IQR – Interquartile range; APACHE - Acute Physiology and Chronic Health Evaluation; ICU - Intensive Care Unit; LOS - length of stay; ARDS - Acute Respiratory Distress Syndrome. Data missing for: * 5 patients; † 7 patients; ‡ 21 patients; § 2 patients; ¶ 1 patient; // 11 patients.

Table 11 outlines the clinical/laboratory data for the VAP and non-VAP groups. There was a significant difference in WCC between the two groups but with higher WCC in the non-VAP group.

Parameter	VAP (N=53)	Non-VAP (N=97)	*p* value
Temperature – mean ± SD (^{o}C)	38.0 ± 0.8	37.7 ± 1.1	0.16
White cell count – mean ± SD (x10^9/L)	13.1 ± 6.3	16.4 ± 7.9	0.01
PaO_2:FiO_2 – median (IQR)	27.6 (20.6-33.9)	28.6 (22.3-39.0)	0.275
Partial pressure CO_2 – mean ± SD	5.4 ± 1.0	5.4 ± 1.3	0.81
pH – mean ± SD	7.44 ± 0.06 (n=42)	7.32 ± 0.82 (n=83)	0.33
H^+ - median (IQR)	38.1 (33.6-45.4) (n=10)	32.6 (30.6-37.4) (n=12)	0.12

Table 11: Laboratory data for VAP and non-VAP groups. Missing data: Temp = 0, WCC = 4, PaO_2 = 2, $PaCO_2$ = 2

3.4 BAL and safety data

The recruitment schedule identified patients during a morning screening round and patients generally underwent BAL in the afternoon. The most common time that BAL was sent to the laboratory was 15:30, with the earliest time 10:05 and latest 19:45. The frequency of pulmonary anatomical areas in which lavage was performed was: the right

lower lobe in 70 (47%) patients, the left lower lobe in 40 (27%) patients, the right middle lobe in 12 (8%) patients, the left upper lobe in 11 (7%) patients and right upper lobe in 6 (4%) patients. In addition 4 patients had bilateral BAL performed, 5 patients were recorded as having BAL performed on 'right' and 2 patients were recorded as having BAL performed on 'left'. The volume of saline most commonly instilled was 120mls, in keeping with the BAL SOP. The minimum volume used was 40mls and the maximum volume was 320mls. The mean volume retrieved from BAL was 32.0mls (SD 21.5).

18 patients had a drop in oxygen saturations of greater than 5% of the starting saturations during bronchoscopy and BAL. Amongst these 18 patients the mean that the saturations dropped to was 88% (range 79-94%). This fall in oxygen saturations, within 5% of starting saturations, persisted for 3.7 minutes on average once the bronchoscope was removed (range 1-15 minutes). For 9 patients the saturations fell below 90% during the BAL, which persisted for 1-3 minutes. Two hours after bronchoscopy patients were followed up with regard to oxygen saturations and oxygen requirements. The mean oxygen saturation was 97% (range 90-100%) with the lowest recorded at 90%. The mean FiO_2 was 0.52 (range 0.21-1.0). Comparisons were made of pre-BAL oxygen saturations and FiO2 and 2 hours post- BAL saturations and FiO_2. Sixty-one patients had an increase in FiO_2 of greater than 0.1. However not all of these patients had a fall in oxygen saturations (ie. some patients had an increase in saturations and therefore could have had FiO_2 reduced). Nineteen patients had a FiO2 increase of greater than 0.1 associated with any fall in percentage oxygen saturations and of these the drop in saturations was greater than 3% in 7 patients. In addition a total of 15 patients had a fall in oxygen saturations of greater than 3% but only 7 of these patients had their FiO_2 increased, suggesting a fall within an acceptable clinical range that did not necessarily prompt an increase in FiO_2.

3.5 Microbiology

The microbiology results took on average 3.27 (SD 2.8) days to be reported to the clinical team. The most common time which results were reported was midday. The organisms that were cultured at $>10^4$ cfu/ml are shown in **Table 12**. Gram negative bacteria accounted for 60% of identified organisms, Gram positive organisms 30%, and fungi 10%. The majority of VAP was caused by a single pathogen (39 patients (73%)). Polymicrobial VAP occurred in 14 (26%) patients with 12 patients having two micro-organisms and two patients had three micro-organisms cultured at $>10^4$ cfu/ml.

Organism	N
Methicillin-sensitive *Staphylococcus aureus*	14
Escherichia coli	8
Pseudomonas aeruginosa	7
Klebsiella pneumoniae	5
Haemophilus spp	6
Candida spp	5
Enterobacter aerogenes	2
Enterobacter cloacae	1
Acinetobacter spp	2
Coliform	2
Moraxella catarrhalis	2
Upper respiratory flora*	2
Streptococcus pneumoniae	2
Proteus mirabilis	2
Serratia marcescens	1
Citrobacter koseri	1
Stenotrophomonas maltophilia	1
Streptococcus pyogenes	1
Methicillin-resistant *Staphylococcus aureus*	1
Peptostreptococcus spp.	1
Yeasts	2
Streptococcus group C	1

Table 12: Organisms cultured at >10^4 cfu/ml.
***in both cases normal flora growth was in addition to another organism at >10^4 cfu/ml. N = the number of patients in whom the micro-organism in question was isolated from bronchoalveolar lavage fluid at >10^4 cfu/ml.**

3.6 Antibiotics and AFD

Data were collected on antibiotics received in the 72 hours before BAL and antibiotics received in the 7 days following BAL. One hundred and ten patients were receiving antibiotics at the time of BAL with significantly more in the non-VAP than VAP groups (82.5% vs 56.6%, p=0.001). The proportion of patients with new antibiotics started in the 72 hours before BAL was not significantly different between the two groups (VAP 15.1%, non-VAP 27.8%, p=0.08).

The antibiotic days in the 7 days post-BAL was similar in the VAP and non-VAP groups with a median (inter-quartile range (IQR)) of 7 (5-7) for the VAP group and 6.5 (5-7) for the non-VAP group. The median AFD for the VAP group was 0 (0-2) and for the non-VAP group was 0 (0-3). The distribution of AFD is illustrated in **Figure 5**.

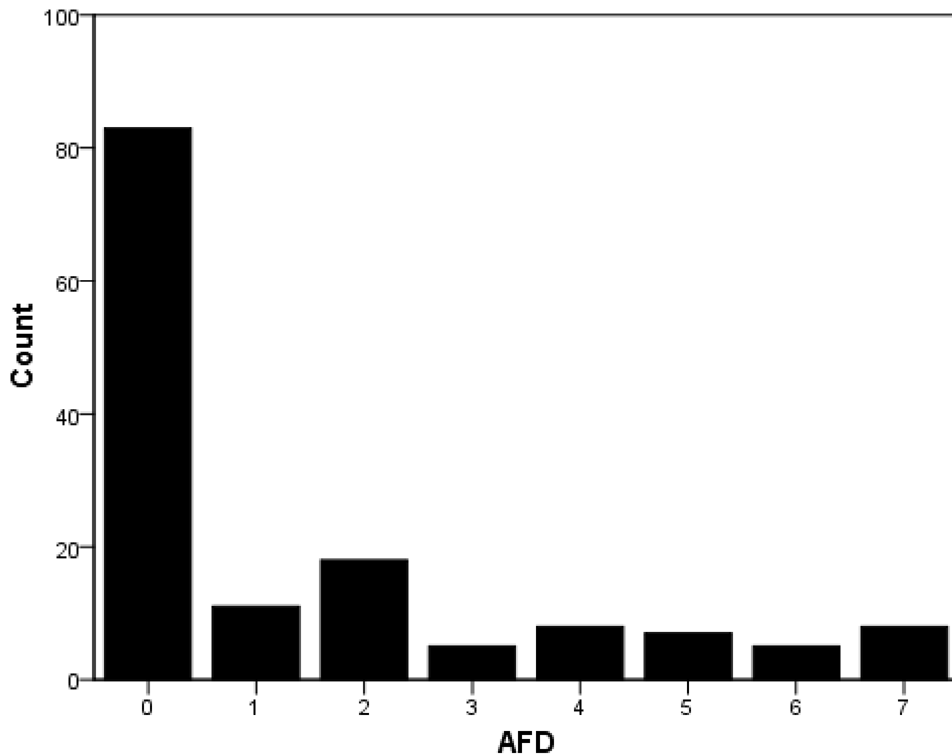

Figure 5: AFD expressed as integer value, n=145. Data missing for 5 patients

3.7 Biomarker results (standard flex sets)

3.7.1 Uncorrected biomarker data

IL-1β, IL-8, MMP-8, MMP-8 and HNE were measured in BAL fluid of 150 patients. There were significant differences between the VAP and non-VAP groups across all 5 biomarkers (p<0.001) (**Table 13**)

Biomarker	VAP	Non-VAP	*p* value
IL-1β *pg/ml*	712 (112-1999)	29 (3-184)	<0·001
IL-8 *pg/ml*	7546 (1987-23050)	1401 (369-4422)	<0·001
MMP-8 *ng/ml*	734 (113-2792)	66 (11-325)	<0·001
MMP-9 *ng/ml*	6840 (1721-22221)	491 (106-3146)	<0·001
HNE *ng/ml*	3882 (710-11183)	349 (96-1473)	<0·001

Table 13: Uncorrected BAL fluid biomarker concentrations for VAP and non-VAP groups. Data are expressed as median and IQR.

Since biomarker concentrations were significantly higher in the VAP group than the non-VAP group, diagnostic utility was determined by constructing ROC curves. The area under the curve reflects the overall ability of the test to discriminate between patients with the disease and those without. The ROC curves and AUROC is shown in **Figure 6**.

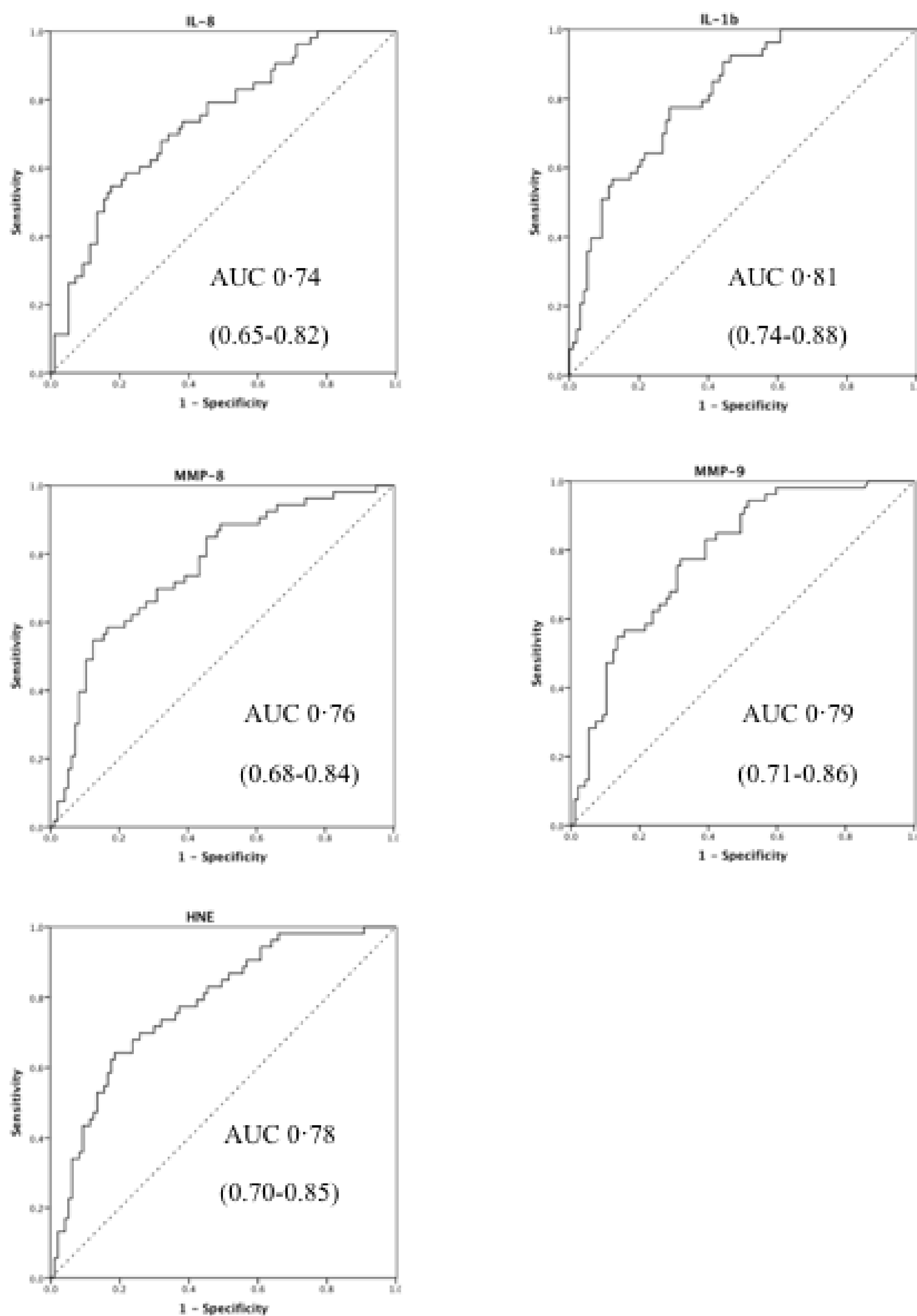

Figure 6: ROC curves and AUC with 95% CI for uncorrected biomarker results.

A number of strategies were used to determine the optimum cut-point on the ROC for the diagnostic performance. The Youden index is the point that has the optimum balance of sensitivity and specificity. This diagnostic performance using the Youden index to derive a cut off value is shown in **Table 14**.

Biomarker	**Sensitivity (95%CI), %**	**Specificity (95% CI), %**	**PPV (95% CI)**	**NPV (95% CI)**	**+LR (95% CI)**	**-LR (95% CI)**	**PTP (95% CI), %**
IL-1β *110 pg/ml*	77.4 (65.0-87.2)	71.1 (61.6-79.5)	0.59 (0.48-0.71)	0.85 (0.76-0.92)	2.68 (1.90-3.78)	0.32 (0.19-0.53)	14.8 (7.1-22.6)
IL-8 *6800 pg/ml*	54.7 (41.3-67.7)	82.5 (74.1-89.1)	0.63 (0.49-0.77)	0.77 (0.68-0.84)	3.12 (1.90-5.13)	0.55 (0.40-0.75)	23.1 (15.0-31.2)
MMP-8 *619 ng/ml*	54.7 (41.3-67.7)	87.6 (80.1-93.2)	0.71 (0.56-0.83)	0.78 (0.70-0.85)	4.42 (2.47-7.93)	0.52 (0.38-0.71)	22.0 (14.2-29.8)
MMP-9 *1675 ng/ml*	77.4 (65.0-87.2)	68.0 (58.4-76.8)	0.57 (0.45-0.68)	0.85 (0.76-0.91)	2.42 (1.75-3.35)	0.33 (0.20-0.56)	15.4 (7.4-23.4)
HNE *2078 ng/ml*	64.2 (50.8-76.2)	81.4 (72.9-88.3)	0.65 (0.52-0.77)	0.81 (0.72-0.88)	3.46 (2.18-5.49)	0.44 (0.30-0.64)	19.4 (11.6-27.2)

Table 14: Diagnostic performance at Youden index for uncorrected BAL fluid biomarkers. PPV, positive predictive value; NPV, negative predictive value; +LR, positive likelihood ratio; -LR, negative likelihood ratio; PTP, post-test probability.

The ideal diagnostic test would be able to effectively rule-in and rule-out a disease. Although the sensitivites and specificities are generally high, they are insufficient for any useful diagnostic purpose. Furthermore, since the purpose was to validate a rule-out test, the negative post-test probabilities (PTP) at the Youden index are not informative for this purpose. An alternative strategy was to set a minimum negative predictive value (NPV) at 95%. The cut-points at this level and the corresponding diagnostic performance are shown in **Table 15**.

Biomarker	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	-LR (95% CI)	PTP (95% CI), %
IL-1β *17 pg/ml*	96.2 (87.2-99.0)	43.3 (33.9-53.2)	0.48 (0.39-0.58)	0.96 (0.85-0.99)	1.70 (1.41-2.04)	0.09 (0.02-0.35)	4.5 (3.1-5.1)
IL-8 *382 pg/ml*	98.1 (90.1-99.7)	24.7 (17.2-34.2)	0.42 (0.33-0.50)	0.96 (0.80-0.99)	1.30 (1.16-1.47)	0.08 (0.01-0.55)	4.0 (0.7-19.5)
MMP-8 *160 pg/ml*	100 (93.3-100)	5.2 (1.7-11.6)	0.37 (0.29-0.45)	1.0 (0.57-1.0)	1.05 (1.01-1.10)	0.0 (NE)	0.0 (0.0-0.43)
MMP-9 *296 ng/ml*	96.2 (87.0-99.5)	43.4 (33.3-53.7)	0.48 0.38-0.58)	0.96 (0.85-0.98)	1.70 (1.41-2.04)	0.09 (0.02-0.35)	4.5 (3.9-15.3)
HNE *161 ng/ml*	98.1 (89.9-99.9)	34.0 (24.7-44.3)	0.45 (0.36-0.54)	0.97 (0.85-0.98)	1.49 (1.28-1.72)	0.06 (0.01-0.39)	2.9 (1.9-15.0)

Table 15: Diagnostic performance at cut-point with minimum NPV of 95% for uncorrected BAL fluid biomarkers. NE, Not evaluable.

Setting the minimum NPV at 95%, IL-1β and MMP-9 have the best profile of sensitivity and specificity. To further illustrate the rule-out performance of this cut-point for IL-1β, **Figure 7** plots VAP and non-VAP values on a logarithmic scale with the cut-point marked. This plot highlights the proportion of the population of *suspected* VAP who could be correctly identified as not having VAP, these are the non-VAP patients who fall below the cut-point line (bottom right, 'True negatives'). Patients with VAP who fall below the line are 'false negatives' (bottom left) and setting a minimum NPV of 95% aims to minimise this number.

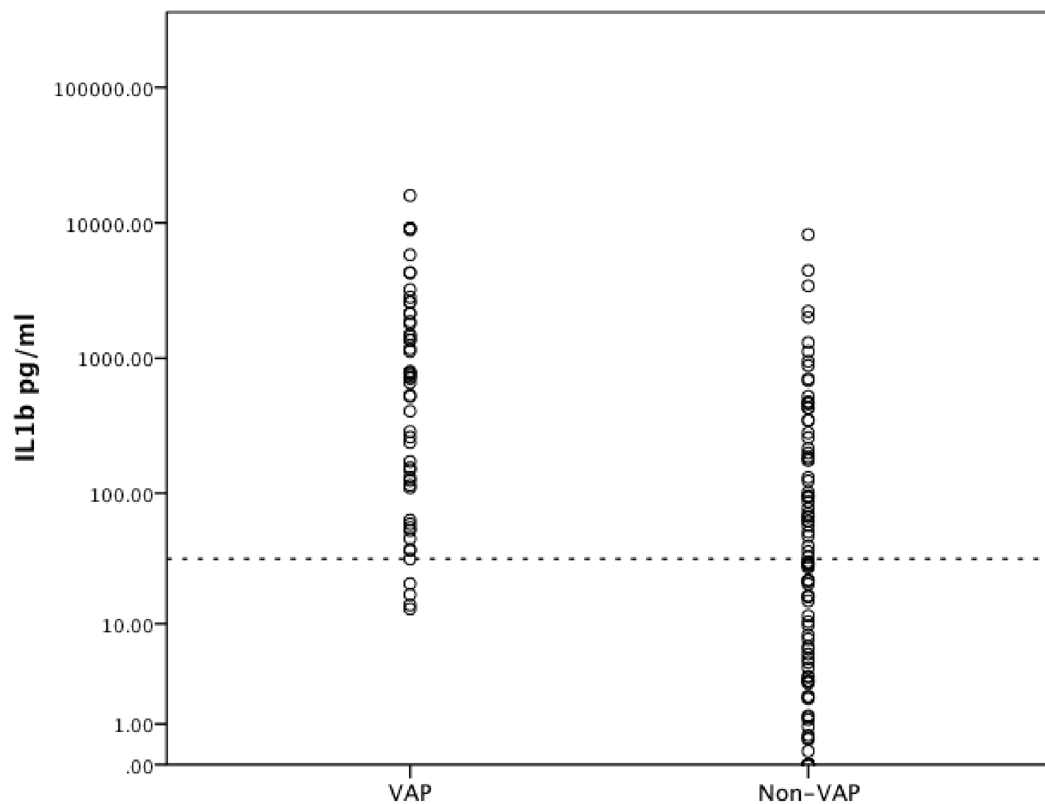

Figure 7: Scatterplot of IL-1β on a logarithmic scale in the VAP and the non-VAP groups. The horizontal dotted line represents 17pg/ml. 43.3% of non-VAP patients and 3.8% of VAP patients fall below this level.

3.7.2 Urea values and urea-corrected biomarkers

Urea was measured in BAL and serum and used to calculate a dilution factor for correction of biomarker levels. The mean serum urea was 40.9mg/dL (SD 21.9) and the mean BAL urea was 4.1mg/dL (SD 4.8). The mean dilution factor was 39.0 (SD 81.5), but with a minimum value of 0.9 and a maximum value of 709.4 the range was very high. Five patients could not have a dilution factor calculated because the BAL urea was unrecordable, therefore this section analyses 145 patients (94 non-VAP and 51 VAP).

A significant difference between the VAP and non-VAP groups remained after urea-correction but levels are considerably higher than uncorrected values (**Table 16**). ROC curves were constructed (**Figure 8**) and cut-points determined using the same strategy as with the uncorrected values using the Youden index (**Table 17**) and using a minimum NPV threshold of 95% (**Table 18**).

Biomarker urea-corrected	VAP	Non-VAP	*p* value
IL-1β *pg/ml*	8100 (3056-21255)	521 (55-3731)	<0·001
IL-8 *ng/ml*	100.5 (56.4-222.1)	30.7 (7.9-8.5)	<0·001
MMP-8 *ng/ml*	8633(4119-31234)	1550 (286-7738)	<0·001
MMP-9 *mg/ml*	81.3 (34.2-261.7)	13.7 (2.6-65.1)	<0·001
HNE *mg/ml*	47.8 (21.0-116.5)	8.0 (2.0-28.0)	<0·001

Table 16: BAL fluid urea-corrected biomarker concentrations for VAP and non-VAP groups. Data are expressed as median and IQR. Note change in units from uncorrected biomarker concentrations. n=145.

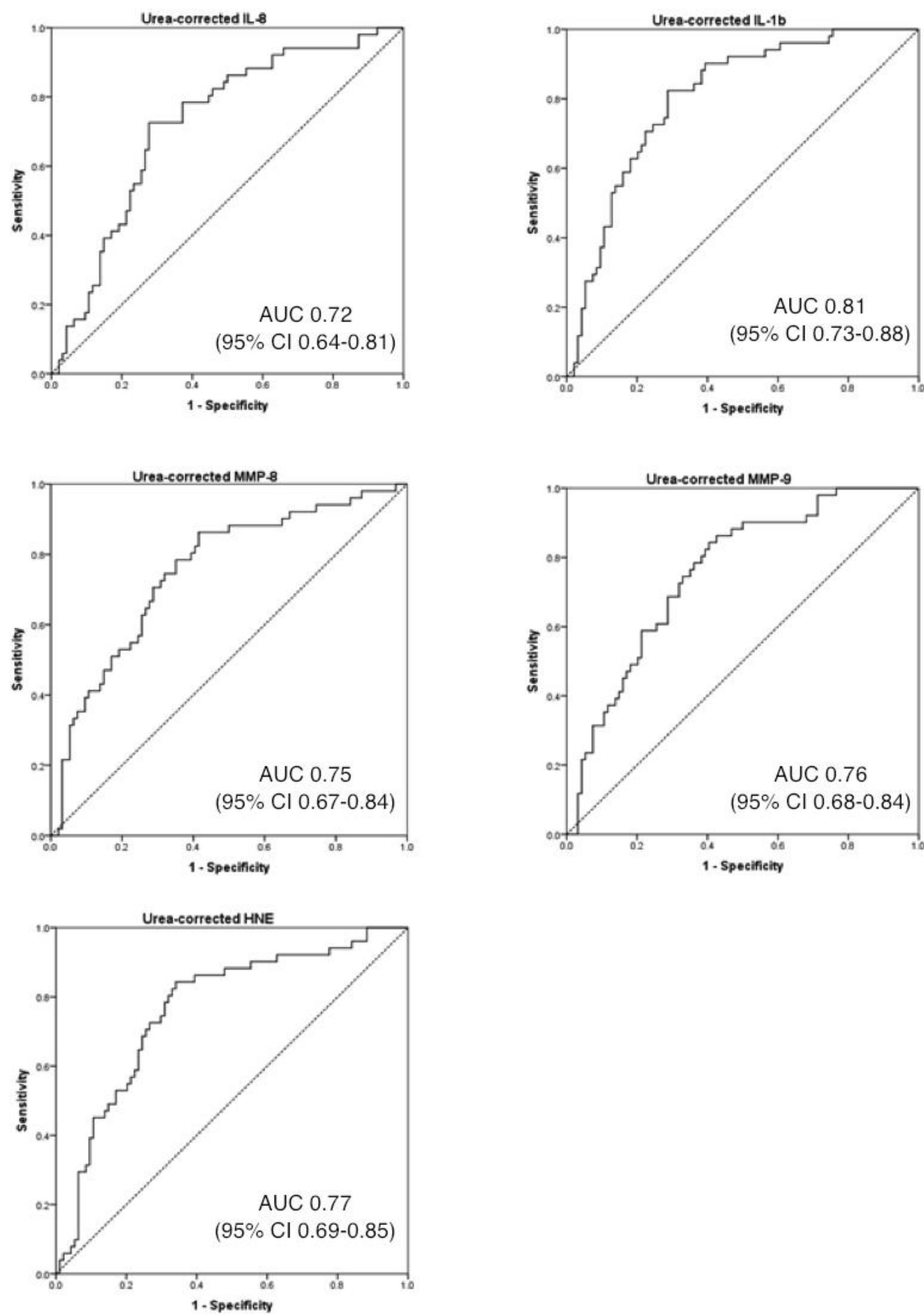

Figure 8: ROC curves and AUC with 95% CI for urea-corrected biomarker results.

Biomarker (urea-corrected)	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	-LR (95% CI)	PTP (95% CI),%
IL-1β *2566 pg/ml*	82.3 (72.9-93.1)	71.3 (62.1-80.2)	0.61 (0.50-0.72)	0.88 (0.81-0.95)	2.87 (2.06-4.02)	0.25 (0.13-0.44)	11.8 (4.5-18.6)
IL-8 *67806 pg/ml*	72.5 (59.6-83.8)	72.3 (63.3-81.1)	0.59 (0.46-0.70)	0.83 (0.74-0.90)	2.62 (1.79-3.70)	0.38 (0.25-0.61)	17.1 (9.5-25.7)
MMP-8 *2519 ng/ml*	86.3 (77.7-95.9)	58.5 (49.0-68.6)	0.53 (0.43-0.64)	0.89 (0.81-0.97)	2.08 (1.62-2.73)	0.24 (0.11-0.46)	11.3 (3.3-18.6)
MMP-9 *25619 ng/ml*	84.3 (75.3-94.5)	59.6 (50.0-69.6)	0.53 (0.43-0.64)	0.88 (0.80-0.96)	2.09 (1.62-2.76)	0.26 (0.13-0.49)	12.5 (4.8-20.0)
HNE *16886 ng/ml*	84.3 (75.2-94.5)	66.0 (56.6-75.4)	0.57 (0.47-0.69)	0.89 (0.82-0.96)	2.48 (1.85-3.37)	0.24 (0.12-0.44)	11.4 (3.9-18.4)

Table 17: Diagnostic performance at Youden index for urea-corrected biomarkers

Biomarker (urea-corrected)	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	-LR (95% CI)	PTP (95% CI), %
IL-1β *41 pg/ml*	100 (100-100)	24.5 (16.1-33.3)	0.42 (0.33-0.51)	1.0 (1.0-1.0)	1.32 (1.19-1.49)	0.0 (NE)	0 (0-0)
IL-8 *1161 pg/ml*	100 (100-100)	7.4 (2.1-12.4)	0.37 (0.29-0.45)	1.0 (1.0-1.0)	1.08 (1.02-1.14)	0.0 (NE)	0 (0-0)
MMP-8 *400 pg/ml*	100 (100-100)	3.2 (0-6.5)	0.36 (0.28-0.44)	1.0 (1.0-1.0)	1.03 (1.0-1.07)	0.0 (NE)	0 (0.0-0.0)
MMP-9 *3376 ng/ml*	98.0 (94.5-100)	28.7 (19.9-37.9)	0.43 (0.34-0.52)	0.96 (0.90-1.0)	1.38 (1.21-1.57)	0.07 (0.01-0.47)	3.6 (0.0-10.1)
HNE *581 ng/ml*	100 (100-100)	11.7 (5.0-17.7)	0.38 (0.30-0.46)	1.0 (1.0-1.0)	1.12 (1.05-1.21)	0.0 (NE)	0 (0-0)

Table 18: Diagnostic performance of urea-corrected biomarkers setting minimum NPV at 95%. NE, Not evaluable

The AUROC of urea-corrected values remains close to the uncorrected values. Furthermore the diagnostic performance at the cut-point of the Youden index is similar, if not better, than for the uncorrected values. However when setting a minimum NPV of 95%, the specificities of the urea-corrected cut-points become unacceptably low.

3.8 Biomarker combinations

Biomarkers were tested in combination using uncorrected biomarker values. Urea-corrected biomarker values did not offer greater discriminatory value and were associated with significant logistical challenges (see 3.12 Discussion). Therefore urea-corrected values were not tested further in the biomarker combinations. Combinations of biomarkers were modelled after log10 transformation with the addition of a constant of 1 (to remove zeros). All biomarkers were highly correlated and IL-1β had the greatest prognostic value, as reflected by the p-value of the IL-1β covariant in the equations below. Therefore combinations of biomarkers were all tested in combination with IL-1β.

The equations for the logistic regressions tested are shown below (**Table 19-22**):

IL-1β and IL-8:

$-2.738 + 1.463 \times \text{Log10}(1+\text{IL1}\beta) - 0.272 \times \text{Log10}(1+\text{IL8})$

IL-1β/IL-8	B	SE	Wald	p-value
Log_{10} (1+IL-1β)	1.463	0.352	17.287	0.000
Log_{10} (1+IL-8)	-0.272	0.371	0.538	0.463
Constant	-2.738	0.915	8.953	0.003

Table 19: Variables in logistic regression. IL-1β/IL-8. B = coefficient, SE = standard error, Wald = Wald chi-squared.

IL1β, IL-8 and MMP-9:

$-2.806 + 1.397 \times \text{Log10}\ (1+\text{IL1}\beta) - 0.314 \times \text{Log10}\ (1+\text{IL8}) + 0.109 \times \text{Log10}\ (1+\text{MMP9})$

IL-1β/IL-8/MMP-9	B	SE	Wald	p-value
Log_{10} (1+IL-1β)	1.397	0.457	9.352	0.002
Log_{10} (1+IL-8)	-0.314	0.417	0.569	0.451
Log_{10} (1+MMP-9)	0.109	0.476	0.053	0.819
Constant	-2.806	0.972	8.327	0.004

Table 20: Variables in logistic regression. IL-1β/IL-8/MMP-9. B = coefficient, SE = standard error, Wald = Wald chi-squared.

IL-1β, IL-8, MMP-9 and MMP-8:

-2.999 + 1.445 x Log10(1+IL1β) – 0.266 x Log10(1+IL8) + 0.274 x Log10(1+MMP9) – 0.266 x Log10(1+MMP8)

IL-1β/IL-8/MMP-9/MMP-8	B	SE	Wald	p-value
Log_{10} (1+IL-1β)	1.445	0.478	9.147	0.002
Log_{10} (1+IL-8)	-0.266	0.435	0.375	0.540
Log_{10} (1+MMP-9)	0.274	0.608	0.203	0.652
Log_{10} (1+MMP-8)	-0.266	0.615	0.187	0.666
Constant	-2.999	1.093	7.529	0.006

Table 21: Variables in logistic regression. IL-1β/IL-8/MMP-9/MMP-8. B = coefficient, SE = standard error, Wald = Wald chi-squared.

IL-1β, IL-8, MMP-9, MMP-8 and HNE:

-3.151 + 1.417 x Log10(1+IL1β) – 0.286 x Log10(1+IL8) + 0.206 x Log10(1+MMP9) – 0.392 x Log10(1+MMP8) + 0.263 x Log10(1+HNE)

IL-1β/IL-8/MMP-9/MMP-8/HNE	B	SE	Wald	p-value
Log_{10} (1+IL-1β)	1.417	0.484	8.584	0.003
Log_{10} (1+IL-8)	-0.286	0.442	0.420	0.517
Log_{10} (1+MMP-9)	0.206	0.643	0.103	0.748
Log_{10} (1+MMP-8)	-0.392	0.739	0.281	0.596
Log_{10} (1+HNE)	0.263	0.710	0.137	0.711
Constant	-3.151	1.191	6.993	0.008

Table 22: Variables in logistic regression. IL-1β/IL-8/MMP-9/MMP-8/HNE. B = coefficient, SE = standard error, Wald = Wald chi-squared.

From these predictive models ROC curves were constructed as with the single biomarkers and once again coordinates were selected to give a minimum NPV of 95%. The cut-points and the corresponding diagnostic parameters are shown in **Table 23** below.

Biomarker combination	Sensitivity (95% CI), %	Specificity (95% CI), %	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	-LR (95% CI)	PTP (95% CI), %
IL-1β/IL-8 -1.7616	100 (93.2-100)	44.3 (34.2-54.8)	0.50 (0.39-0.59)	1.0 (0.92-1.0)	1.80 (1.50-2.15)	0.0 (NE)	0.0 (0.0-9.2)
IL-1β/IL-8/ MMP-9 -1.7172	98.1 (89.9-100)	44.3 (34.2-54.8)	0.49 (0.39-0.60)	0.98 (0.88-0.98)	1.76 (1.47-2.11)	0.04 (0.01-0.30)	2.3 (1.5-11.9)
IL-1β/IL-8/ MMP-8/MMP-9 -1.7015	100 (93.3-100)	46.4 (36.2-56.8)	0.51 (0.41-0.60)	1.0 (0.92-1.0)	1.87 (1.55-2.24)	0.0 (NE)	0.0 (0.0-7.8)
IL-1β/ IL-8/ MMP-8/MMP-9/ HNE -1.6464	98.1 (90.0-100)	46.4 (36.2-56.8)	0.50 (0.40-0.60)	0.98 (0.89-0.99)	1.83 (1.52-2.21)	0.04 (0.01-0.29)	2.2 (1.4-11.5)

Table 23: Diagnostic performance for biomarker combinations at cut-points from predictive model. NE, Not evaluable

As can be seen from the combinations of markers, there is little improvement in diagnostic performance beyond IL-1β as this is the predominant component of all regression equations.

The cut-points used in the single biomarkers correspond to a specific concentration. This is less clear in the combinations as the cut-point is derived from the linear predictive scale. The cut-point for IL-1β as a single biomarker is 17pg/ml, which is above the second point on the standard curve against which it is measured. It was necessary to determine the IL-1β level at the cut-point in the combinations to ensure it fell within the standard curve.

To determine the IL-1β level at the cut-point, plots were constructed of the linear predictor against Log_{10} (1+IL1β) with the cut-point marked. The plot below for the combination of IL-1β and IL-8 shows that the cut-point corresponds to an IL-1β level that is approximately 15pg/ml (**Figure 9**). Cut-points of other combinations correspond with a similar level of IL-1β ranging between 16-19pg/ml. (A plot equivalent to Figure

9 could also be constructed to display the contribution of IL-8 to the overall prediction; however, the range of IL-8 values observed was far above the limit of detection, therefore the same concern around prediction from values below the standard curve did not exist for this biomarker and this plot is not shown.)

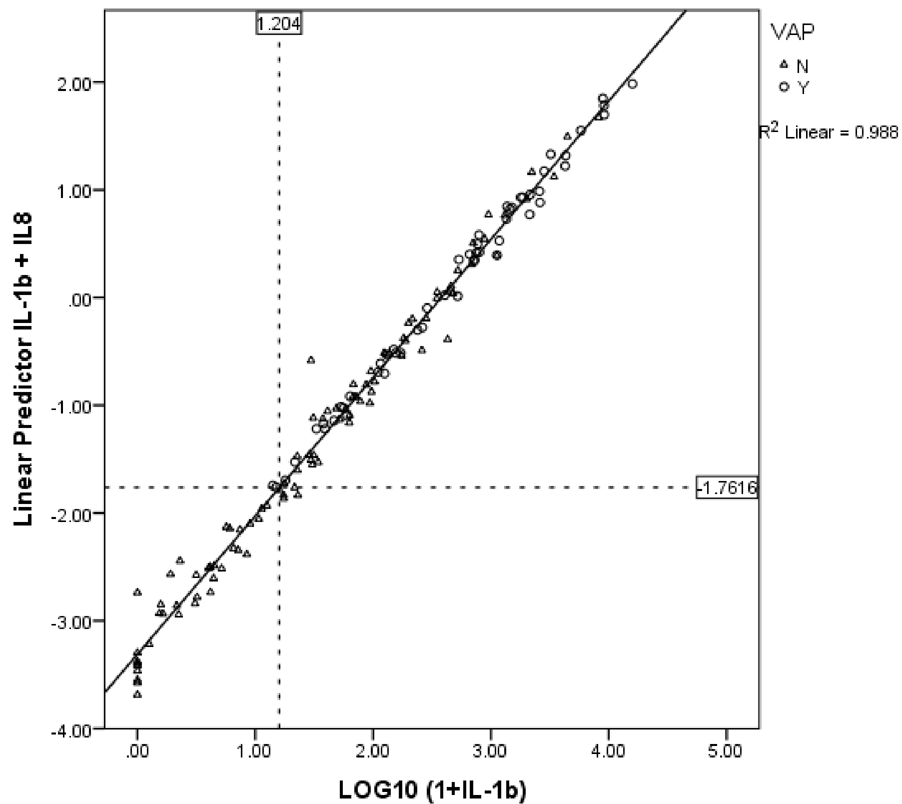

Figure 9: Plot of linear predictor of IL-1β and IL-8 against Log10(1+IL-1β). Horizontal dotted line represents the cut-point and vertical line marks the point that this intersects with the diagonal line and therefore the IL-1β level at the cut-point.

The ‘best’ biomarker combination had to be identified to take forward into the RCT phase. All of these combinations of biomarkers make minor changes to diagnostic performance in comparison to IL-1β as a single biomarker. Although the four biomarker combination of IL-1β, IL-8, MMP-8 and MMP-9 has the highest specificity and sensitivity, limitations in terms of assay consistency and cost did not favour this combination (see 3.12 Discussion). Therefore the IL-1β and IL-8 combination was taken forward to the RCT and its diagnostic performance is demonstrated in the following STARD diagram (**Figure 10**) and 95% CI are provided below (**Table 24**).

Biomarker combination	**Sensitivity** *(95% CI)*	**Specificity** *(95% CI)*	**PPV** *(95% CI)*	**NPV** *(95% CI)*	**+LR** *(95% CI)*	**-LR** *(95% CI)*	**PTP** (%) *(95% CI)*
IL-1β/IL-8 -1.7616	100% *(93.2-100.0)*	44.3% *(34.2-54.8)*	0.50 *(0.39-0.59)*	1.0 *(0.92-1.0)*	1.80 *(1.50-2.15)*	0.0 (NE)	0.0 *(0.0-9.2)*

Table 24: Diagnostic performance for combination of IL-1b and IL-8 with 95% CI. NE, Not evaluable

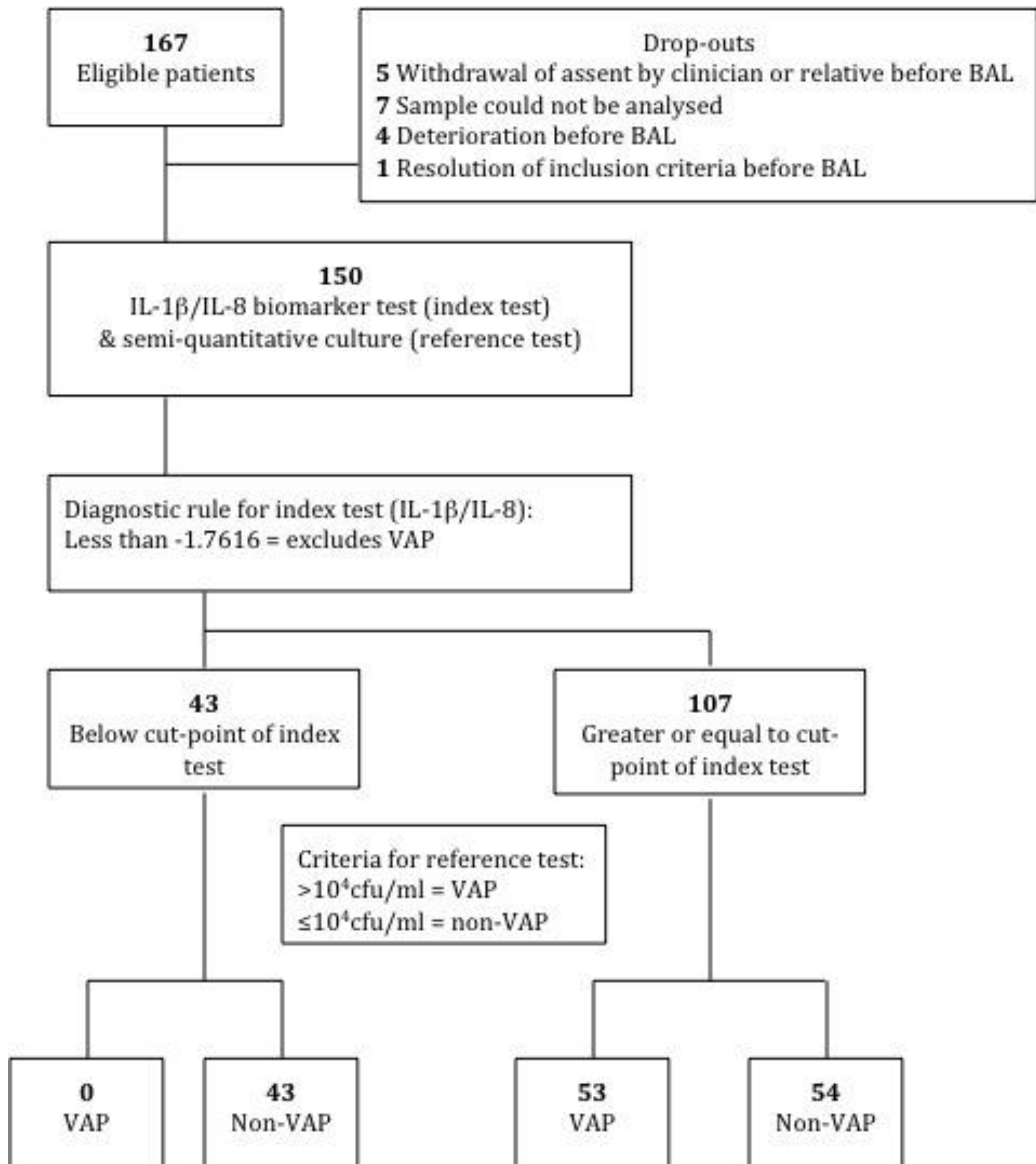

Figure 10: STARD diagram for combination of IL-1β and IL-8

By way of additional 'validation' of this diagnostic rule, the regression equation was applied to the cohort of patients from the original derivation study(Conway Morris *et al.*, 2010). Applying the same threshold -1.76 the sensitivity was 94% (95% CI 71-99%) and specificity was 58% (95% CI 44-71%). These results are reassuringly close to the main analysis.

3.9 Biomarker results (enhanced sensitivity)

The lowest point on the standard curve for IL-1β is 5pg/ml and since the cut-point for IL-1β was 17pg/ml, this falls between the second and third standard curve point. The area of the standard curve that has the lowest intra-assay variability is the linear segment of the curve. Therefore IL-1β and IL-8 were also measured using enhanced sensitivity CBA flex sets. The standard range for the enhanced sensitivity flex set is 274-200,000 fg/ml. The cut-point of 17pg/ml would fall within the linear segment of this standard curve.

A total of 143 samples (50 VAP, 93 non-VAP) were available to be tested by the ES assay. Biomarker concentrations using ES assay closely replicated the standard CBA (**Table 25**).

Biomarker (ES assay)	VAP	Non-VAP
IL-1β *pg/ml*	527.3 (79.6 – 1981.9)	25.0 (5.7 – 164.7)
IL-8 *pg/ml*	6957 (1688-21551)	1347 (277 – 5858)

Table 25: Concentrations of IL-1β and IL-8 when measured with ES CBA assay for VAP and non-VAP group. Concentrations expressed as median and IQR.

ROC curves were constructed for ES IL-1β and ES IL-8 and these closely reflected the standard CBA kit results (**Figure 11**). For ES IL-1β a cut-point of 22.5 pg/ml had a sensitivity of 96.0% and a specificity of 47.3%. For ES IL-8 a cut-point of 313.2 pg/ml

had a sensitivity of 98.0% and a specificity of 26.9%. Again these results closely reflected the results of the standard CBA assay.

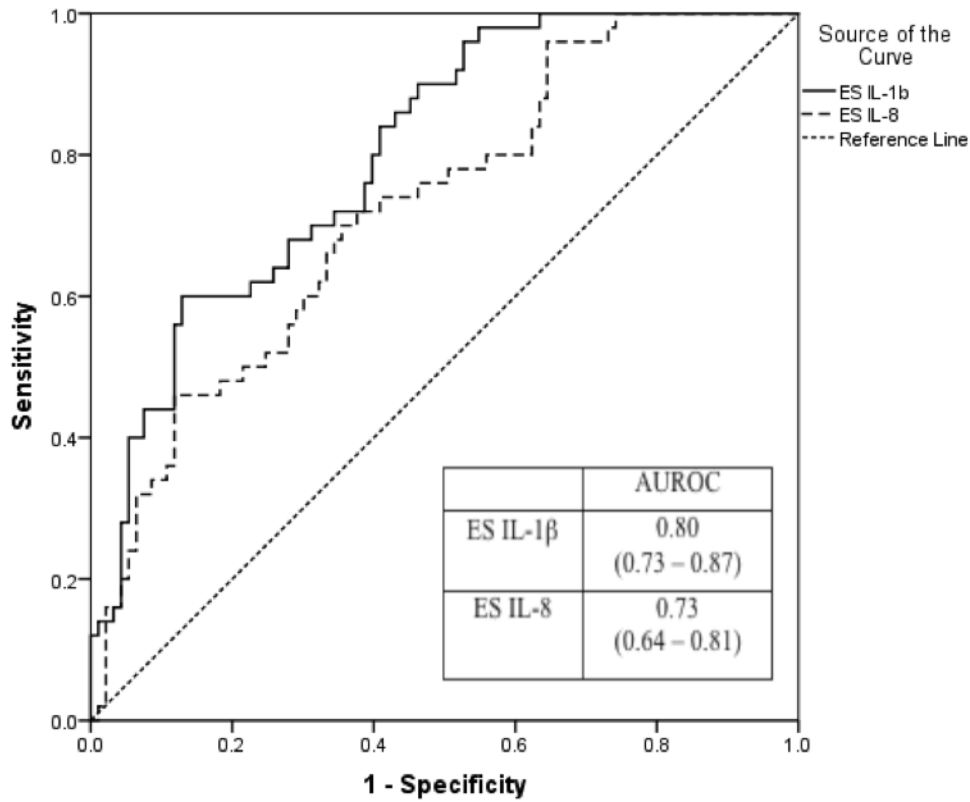

Figure 11: ROC and AUROC for ES IL-1b and ES IL-8

A combination of ES IL-1β and ES IL-8 were modelled by logistic regression. The predictive model was given by the equation:

$-5.480 + 1.606 \times \text{Log10}(1+\text{ES IL1}\beta) - 0.507 \times \text{Log10}(1 + \text{ES IL8})$

The AUROC curve was 0.81 (95% CI 0.74 - 0.88). Taking a cut-point that corresponds to the performance of the standard, uncorrected biomarkers, a cut-point of -1.7045 had a sensitivity of 100% and a specificity of 41.9%.

The biomarker diagnostic profile from the ES assay, as single biomarkers and in combination, closely reflected the standard CBA assay but did not provide greater diagnostic performance. This offered reassurance of the initial results, in that they were so closely replicated in a different assay.

3.10 Serum biomarkers

A pilot group of 20 serum samples (RVI 1-22, RVI 2 and 10 not included in analysis) were analysed with the primary purpose of determining which biomarkers were detectable in serum. No comparisons were made at this stage between VAP and non-VAP (**Table 26**).

Biomarker (n=20)	Median (IQR)
IL-8 pg/ml	51.1 (34.5-137.6)
IL-1β pg/ml	0.0 (0.0-0.0)
MMP-8 ng/ml	71.0 (12.2-108.4)
MMP-9 ng/ml	1616.4 (594.5-4384.2)
HNE ng/ml	680.4 (329.2-907.5)
IL-6 pg/ml	82.6 (57.1-200.7)
TNFα pg/ml	12.1 (2.8-18.0)
IL-12p70 *pg/ml*	0.0 (0.0-0.0)

Table 26: Levels of 8 biomarkers measured in serum of a pilot of 20 patients

This pilot data suggested that IL-1β, IL-12p70 and TNFα would not be detectable in any meaningful concentrations in serum of patients with suspected VAP. Therefore IL-6, IL-8, MMP-8, MMP-9 and HNE were tested in the cohort of validation study patients (n=150).

There were no significant differences between the VAP and non-VAP patients (**Table 27**), and consequently the AUROC curves did not demonstrate any diagnostic utility.

Biomarker	VAP (n=52)	Non-VAP (n=97)	*p* value	AUROC
IL-6 *pg/ml*	103.4 (62.95-238.6)	101.5 (29.11-237.3)	0.299	0.552
IL-8 *pg/ml*	75.32 (36.33-166.3)	71.12 (36.26-195.6)	0.669	0.479
MMP-8 *ng/ml*	70.00 (29.18-129.2)	73.07 (23.29-120.6)	0.802	0.512
MMP-9 *ng/ml*	1380 (720.6-2042)	1033 (485.6-1770)	0.069	0.591
HNE ng/ml	797.3 (449.0-1146)	783.1 (353.8-1751)	0.892	0.507

Table 27: Serum biomarker concentrations in VAP and non-VAP patients.

3.11 Sub-group analysis

Post hoc sub-analyses were performed to determine the effect of antibiotic use on the BAL biomarker analysis, to explore differences between medical and surgical patients and the effect of sterile culture or sub-threshold pathogen growth in comparison to confirmed VAP.

3.11.1 Effect of antibiotic on diagnostic performance

There was high use of antibiotics at the time of BAL with a greater proportion of non-VAP than VAP patients being on antibiotics at the time of BAL (82.5% vs 56.6%, *p*=0.001). The concern was that patients might be inappropriately assigned as 'non-

VAP' due to falsely negative culture due to the antibiotics. It was reassuring that the proportion of patients having new antibiotics started in the 72 hours prior to BAL was similar in both groups (VAP 15.1%, non-VAP 27.8%, p=0.08)

It is conceivable that patients with VAP and therefore local pulmonary inflammation but who are falsely classed as non-VAP, could still have a local inflammatory profile that reflects VAP (ie. high biomarkers). If this was the case, the effect of antibiotics would be to reduce the diagnostic performance of the biomarkers. The biomarker analysis was performed with respect to IL-1β, initially including patients that were not in receipt of any antibiotics and then in those that did not have any *new* antibiotics started in the 72 hours before BAL.

Patients who were not in receipt of any antibiotics were few and this therefore limits this analysis. 23 patients with VAP and 17 patients with non-VAP were included and significant differences were demonstrated in IL-1β concentration, with a median of 796pg/ml in the VAP group and 40pg/ml in the non-VAP group (p=0.0012). A ROC curve was constructed and the AUROC was 0.80 (95% CI 0.65-0.94). The specific diagnostic performance is of limited value given the small numbers but at the threshold close to the main analysis of 17.9pg/ml, the sensitivity is 91% (95% CI 72-99%) and specificity is 29% (95% CI 10-56%). The wide CI highlights the limitations of these small numbers.

The sub-group of patients who had no new antibiotics started in the 72 hours prior to BAL was larger with 46 VAP patients and 69 non-VAP patients included. Again there were significant differences in the concentration of IL-1β with a median in the VAP group of 590pg/ml and a median of 30pg/ml in the non-VAP group (p <0.0001). The AUROC was 0.78 (95% CI 0.70 – 0.87). At a cut-point of 17pg/ml the sensitivity was 93% (95% CI 82-98%) and the specificity was 39% (95% CI 27-52%).

The overall trend of this sub-group analysis was reassuring in that it closely reflected the results of the main analysis and in fact the diagnostic performance was actually worse in this sub-group analysis. Although it does not entirely determine whether antibiotics could have influenced individual patients' biomarkers, it does suggest that there was not a significant effect in the overall analysis.

3.11.2 Sub-group analysis of medical and surgical patients

It is of interest to determine whether the diagnostic rule has the same diagnostic performance in different patient groups and a sub-group analysis was performed on medical and surgical patients.

72 surgical and 68 medical patients were analysed separately. Considering individual biomarkers, the AUROC were greater for surgical patients (**Table 28**).

Biomarker	AUROC Surgical	AUROC Medical
IL-8	0.80 (0.70-0.90)	0.66 (0.52-0.79)
IL-1β	0.84 (0.75-0.93)	0.76 (0.65-0.87)
MMP-8	0.80 (0.69-0.90)	0.71 (0.58-0.84)
MMP-9	0.82 (0.72-0.92)	0.74 (0.62-0.87)
HNE	0.84 (0.74-0.93)	0.71 (0.58-0.84)

Table 28: AUROC for the 5 biomarkers for medical and surgical categories

Applying the IL-1β and IL-8 predictive model and applying the cut-point of -1.7616, the sensitivity in surgical patients was 100% and specificity was 22%. Amongst medical patients the sensitivity was 100% and specificity was 41%. Importantly the sensitivity of the cut-point is high in both groups, which maintains its value to rule-out VAP but the specificities vary, which may result in different rates of false positives in different patient groups.

3.11.3 Sub-group analysis of VAP, sub-VAP and sterile culture

The threshold used to define VAP at $>10^4$ cfu/ml, while derived from autopsy studies(Chastre *et al.*, 1995) and although widely accepted, is contentious. Measurement of biomarkers of the local inflammatory response could add clarity in determining whether, in terms of the inflammatory response, this threshold is discriminatory. Since the cultures taken were semi-quantitative rather than fully quantitative cultures, it is not

possible to plot inflammatory response against culture growth, which potentially could allow for a biomarker-based threshold to be determined.

Biomarkers were compared between those who had confirmed VAP, those who had sub-threshold culture, and those who had a sterile culture (**Table 29**).

Comparisons across all groups were significant by Kruskal-Wallis test (**Figure 12**). Dunn's multiple comparison *post hoc* tests were performed between all groups. There were significant differences between VAP and both the sterile and the sub-VAP groups across all 5 biomarkers. There were no significant differences between the sterile and sub-VAP groups for any biomarker.

Biomarker	Sterile (n=54)	Sub-VAP (n=44)	VAP (n=52)
IL-1β pg/ml	12.4 (1.1-136.2)	62.0 (12.8-452.1)	685.9 (110.8-2065)
IL-8 pg/ml	1061 (246.7-4283)	2241 (916.3-5710)	7634 (1956-23505)
MMP-8 ng/ml	42.9 (5.29-209.7)	152.2 (28.3-477.4)	684.7 (113.4-2910)
MMP-9 ng/ml	247.5 (82.5-1848)	1151 (243.7-4847)	6442 (1698-21011)
HNE ng/ml	214.4 (64.5-1305)	596.4 (176.7-2006)	3836 (680.3-11210)

Table 29: Biomarker levels between VAP, sub-VAP and sterile culture. Data reported as median and IQR.

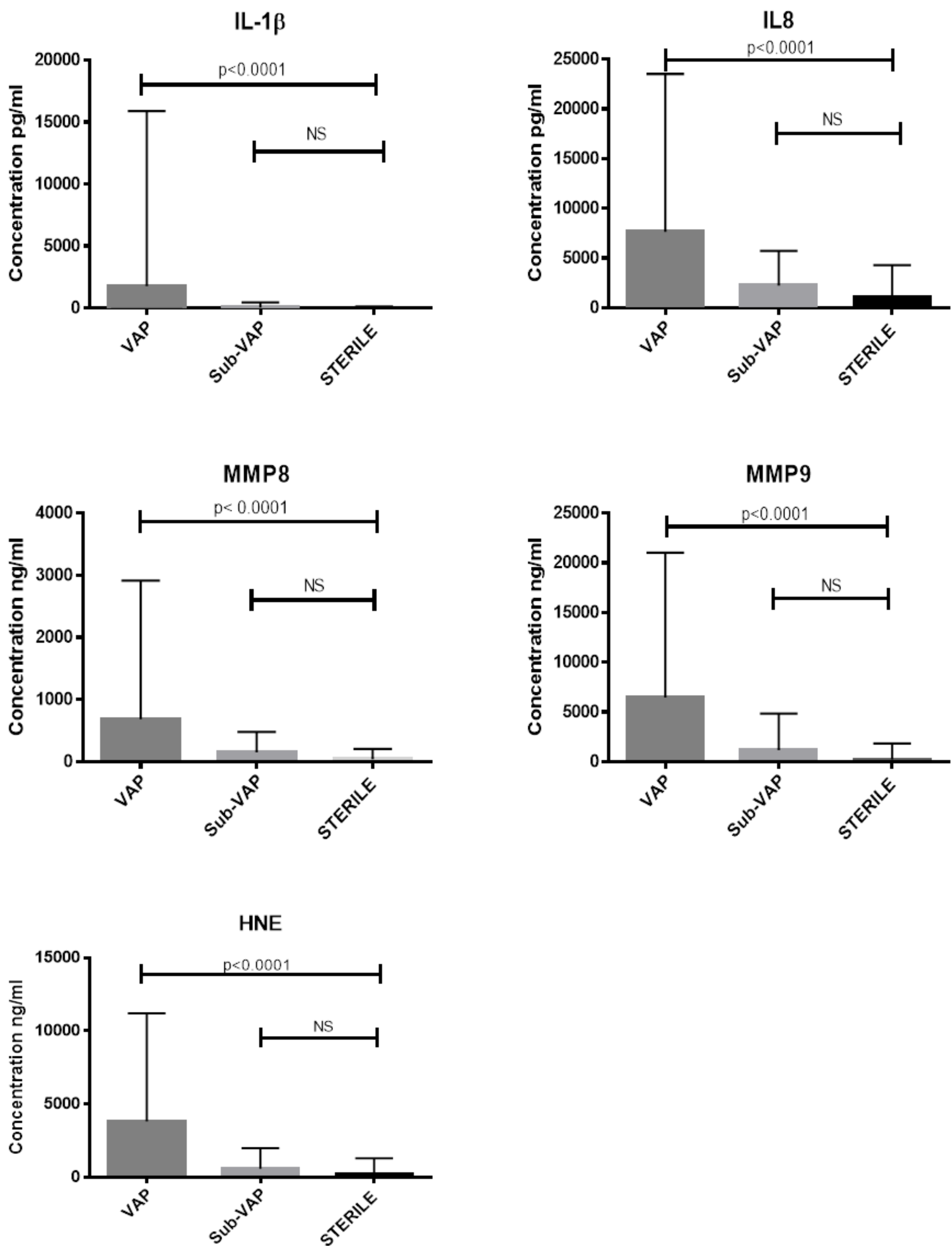

Figure 12: Comparison of biomarker levels between VAP, sub-VAP and sterile culture. Significant differences across all 5 biomarkers by Kruskal-Wallis test (p<0.0001). Significant differences between VAP, sub-VAP and sterile groups across all biomarkers by Dunn's multiple comparison

The significant difference between the VAP and the sub-VAP group does suggest that the diagnostic threshold of $>10^4$ cfu/ml is associated with a difference in immunological signal that can be detected. This suggests that this culture threshold does represent different inflammatory states.

3.12 Discussion

This validation study has successfully demonstrated that BAL fluid biomarkers, in particular IL-1β, can effectively rule-out VAP, when tested in a multi-centre setting. As one might expect in a validation study, the diagnostic performance using the same statistical method as the derivation study, the Youden index, did not deliver as high sensitivity and specificity. Using the Youden index, the cut-point of 110pg/ml for IL-1β had a sensitivity of 77% and a specificity of 71%. These values are nonetheless high and of themselves they do successfully validate the previous diagnostic utility of IL-1β. These values are however not sufficiently discriminatory to be used as a diagnostic rule for clinical application. Therefore a minimum NPV of 95% was set to ensure sufficient 'rule-out' performance. The Youden index represents a point on the ROC curve with the optimum balance of sensitivity and specificity, taking alternative points on the ROC curve will change the balance of sensitivity and specificity. Opting to achieve a minimum NPV of 95% to achieve a rule-out profile, the increase in sensitivity comes with a compromise in the specificity.

It is noticeable that the cut-points determined in the validation study are considerably higher than the derivation study, particularly considering that the derivation study reported urea-corrected biomarkers. One explanation could be that the volumes of saline instilled for the BAL was different with 120mls in this study and 200mls in the derivation study.

The selection of biomarker combination to take forward from the validation study to the RCT involved a number of important considerations. The primary consideration was the statistical performance of the biomarker test and the balance of sensitivity and specificity. Of the combinations tested the combinations of IL-1β/IL-8 (sensitivity 100%, specificity 44.3%) and IL-1β/IL-8/MMP-8/MMP-9 (sensitivity 100%, specificity 46.4%), had the best profile. In fact with the high NPV of 1 both of these combinations represent the near perfect rule-out test profile

A further consideration was that the biomarker assay used in the RCT would have to be robust enough to be consistent in a pragmatic trial. IL-1β and IL-8 were supplied as

commercial kits whereas MMP-8, MMP-9 and HNE were custom made for the validation study. In practical terms handling the IL-1β and IL-8 reagents is logistically easier. For example kits are supplied with a lyophilised standard. In contrast the custom made beads were supplied with aliquots of standard that were reconstituted and stored at -80°C. The commercial kits performed in a more consistent manner during the validation sample testing. There was concern regarding the complexity of reagent handling, storage and consistency for the custom made kits. In addition there were cost implication to increasing numbers of biomarkers when considering the difference between the IL-1β/IL-8 combination and the IL-1β/IL-8/MMP-8/MMP-9 combination had only marginal statistical differences. Based on these considerations, the combination that was taken forward to the RCT phase was IL-1β/IL-8.

Beyond the combination of biomarkers to be used in the RCT, these data also informed the RCT planning in terms of the biomarker assay to be used. Again, important considerations were the practicalities of utilising the assay in a large multi-centre RCT, aiming to minimise logistical barriers and ensure robustness of the assay in that setting.

The first was regarding urea-correction of biomarker levels. The urea-corrected values did not deliver a biomarker performance of sufficient discriminatory value to be used in a RCT. In addition to this, some BAL had unrecordable urea, which made urea-correction impossible in these patients. Since urea-correction is of doubtful value in this regard(Ward *et al.*, 1992, 2000), it was felt to be a level of complexity that could be abandoned.

The enhanced sensitivity assay was also considered. This was an attractive alternative since the IL-1β cut-point would fall on the linear section of the standard curve with expected lower intra-assay variability. Despite this potential benefit, there were practical barriers to using the enhanced sensitivity kit in the RCT. Bronchoscopy and BAL were often not performed until mid or late afternoon and since biomarkers were measured in real-time in the RCT, the assay would not commence until late afternoon. The standard flex set assay takes approximately 5 hours to complete and the enhanced sensitivity assay approximately 7 hours. This difference was important in relation to buy-in from the laboratories and willingness of technicians to work after hours. This also has implications for delivery of the trial intervention and future implementation into clinical service. Further to this, the enhanced sensitivity assay had not undergone the same derivation and validation process that the standard flex sets had undergone and so taking

it straight to RCT would have been questionable. Therefore the standard CBA assay was used in the RCT.

The BAL method used reflects both a strength of this study and a weakness. To my knowledge, this is the first study to successfully validate BAL-based diagnostics for VAP in a multi-centre study. Previous studies have used a wide range of diagnostic methods including BAL (with different procedures), blind-BAL and ETA(el-Ebiary *et al.*, 1995; Mathy-Hartert *et al.*, 2000; Duflo *et al.*, 2002; Gibot *et al.*, 2004; Determann *et al.*, 2005; Horonenko *et al.*, 2007; Huh *et al.*, 2008; Linssen *et al.*, 2008; Luyt *et al.*, 2008; Oudhuis *et al.*, 2009; Jung *et al.*, 2010; Vanspauwen *et al.*, 2011; Wu *et al.*, 2011). It has already been highlighted that the differences in BAL methods between the derivation study and the validation study could account for quite considerable differences in biomarker concentrations. However the differences in BAL and blind-BAL or ETA, could be expected to be more considerable as these could reflect entirely different anatomical regions (lung parenchyma vs bronchial area). Notwithstanding the differences in BAL instillation volume used, both the derivation study and the validation study used a similar BAL SOP and this consistency could well contribute to the successful validation of these biomarkers. The limitation of this is that these findings are bounded by the methods used. The approach to VAP diagnosis in the UK is highly variable(Browne *et al.*, 2014) and BAL is not used consistently in ICUs across the UK. For ICUs that are not routinely performing BAL for management of VAP, the use of these biomarkers would require a change in routine practice, although arguably for the better.

Considering the invasive nature of BAL testing, identifying biomarkers in serum would be highly desirable. Interestingly some markers of inflammation (IL-1β, IL-12p70 and TNFα) were not identifiable in the pilot study of 20 samples. Furthermore no significant differences were found between VAP and non-VAP patients in any of the biomarkers tested in serum. It is also noteworthy that the concentrations measured in serum were considerably lower than in BAL. This is in keeping with the previously suggested paradigm of lung infection as a compartmentalised inflammatory response(Boutten *et al.*, 1996; Millo *et al.*, 2004).

Of patients screened, 40% who met the inclusion criteria were included in the study. The level of detail obtained in the screening logs was insufficient to have a clear understanding as to why such as large proportion of patients were not eligible for the study. This limitation of data collection in the screening log is due to the fact that this

was an observational study and this was not mandated as it would be for a RCT. This raises concerns about the external validity of these results but some practical considerations mitigate against this risk. Understandably ICU clinicians had to give final approval for a patient to be enrolled in the study and therefore undergo BAL. The inclusion criteria were quite broad and anecdotally, a significant number of patients met these initial inclusion criteria based on temperature, white cell counts and purulent secretions (not necessarily CXR changes). Often if clinicians did not suspect VAP based on the wider clinical picture, no CXR was pursued but these patients may have been recorded as meeting inclusion criteria when they strictly did not without having a CXR. Furthermore clinicians could exclude patients if they felt that patients would not tolerate BAL outside of the exclusion criteria. This study has been performed in a large number of ICUs in which ICU clinicians have probably had a significant influence on including patients in whom they feel that this was a relevant clinical query for the patient (ie *they* suspected VAP) and therefore reflects a pragmatic strength of the study.

Another potential limitation is the high level of antibiotics at the time of BAL and in particular the imbalance between the VAP and non-VAP groups. Our definition of VAP was based on microbiological strategy with a culture $>10^4$ cfu/ml. The use of antibiotics could potentially result in a falsely low cfu/ml count in a patient with VAP that is partially treated and incorrectly classes as 'non-VAP'. This could lead to two possibilities. The first is that the lung still has on going inflammation and so the biomarkers are high. This would undermine the analysis, as the microbiology and biomarker levels would be discordant. The second possibility is that the inflammation is also resolving with antibiotic treatment and so the culture growth and biomarker levels are lower due to partial treatment with antibiotics.

In an effort to address this, sub-group analyses were performed for patients who had either no antibiotics and for patients who had no new antibiotics started in the 72 hours prior to BAL being performed. Reassuringly the AUROC curves were close to those generated by the main analysis, and in fact at the cut-point the specificity was lower than in the main analysis, where one might expect this to be higher if there were significant numbers of falsely negative non-VAP patients. Although this does not give confirmation on the effect that antibiotics have on culture growth or more importantly, on biomarker levels, it does suggest that antibiotic use has not had a large effect on determining the diagnostic utility of these biomarkers.

Another potential limitation is to class *Candida* spp. as VAP. Guidelines recommend that Candida should not be treated as a VAP(American Thoracic Society, 2005). The view was taken in this study that significant levels of *Candida* spp. may well be treated by physicians with anti-fungals and therefore should be classed as VAP. This is supported by a previous autopsy study that has demonstrated VAP caused by invasive *Candida* spp.(Corley *et al.*, 1997). Furthermore, in addition to the reported sub-analyses, biomarker levels were examined based on causative pathogens. This analysis is limited due to small numbers (5 patients with *Candida* spp.) and hypothesis testing is of limited value. Notwithstanding this limitation, it is interesting that there was a signal of higher biomarker levels in *Candida* spp. in particular for IL-8, MMP-9 and HNE in comparison to the non-VAP group (data not shown).

Data on antibiotic use in the 7 days following BAL demonstrated a high level of antibiotic use across both patients with confirmed VAP and those in the non-VAP group, with 57.6% of patients having zero AFD. These data were collected in order to inform the power calculation for the RCT primary outcome. Therefore data were collected on number of AFD only and not the indication for antibiotic use. It is unknown if patients, particularly in the non-VAP group, were receiving antibiotics for suspected VAP or a non-pulmonary infection. It does however, go some way to highlight the burden of the potential overuse of antibiotics in patients with suspected VAP. The overuse of antibiotics has been framed in the context of the growing issue of AMR(Davies, 2011). However there is evidence to suggest a potential detrimental impact to the individual patient. Strategies to improve compliance with multi-antibiotic empiric treatment or PCT-triggered escalation of antibiotics, which resulted in increased antibiotic use, have been associated with an increase in mortality, increased length of ICU stay and organ-related harm(Jensen *et al.*, 2011; Kett *et al.*, 2011). In contrast to this a more conservative approach to antibiotics has resulted in a lower antibiotic burden, a lower all-cause mortality and reduced length of ICU stay(Hranjec *et al.*, 2012). A biomarker approach to antibiotic de-escalation of antibiotics, using PCT, has resulted in more AFD both in patients with suspected bacterial infections and in patients with suspected VAP(Stolz *et al.*, 2009; Bouadma *et al.*, 2010).

The successful validation of these biomarkers in a large, multi-centre study represents a significant step forward in diagnostics for VAP. In terms of the potential impact that this test could have on antibiotic prescribing, consider that in this population with *suspected* VAP, VAP was confirmed in approximately 1/3 of patients with 2/3 of

patients making up the non-VAP group. With a NPV of 1, all VAP patients will be correctly identified as being *above* the threshold. With a PPV of 0.5, half of the non-VAP group will fall *below* the threshold. Therefore in a population of *suspected* VAP, this biomarker test could allow for 1/3 of the population to have antibiotics discontinued on the day of suspicion of infection. This is in comparison with conventional microbiological antibiotic de-escalation, where antibiotics could be stopped after 3 days, once the culture results are finalised. However this assumes that antibiotics are de-escalated in the face of negative cultures. Previous trials have reported or even insisted that antibiotics are not stopped if cultures are negative(Ruiz *et al.*, 2000; Canadian Critical Care Trials Group et, 2006). A negative biomarker result on the day of suspicion of VAP could be a strong impetus to discontinue antibiotics early rather than mid-way through an antibiotic course.

The high antibiotic use in this study does also set the scene for the scale of the antibiotic burden that the biomarker-test is aiming to alter. Since the reason for the antibiotic use is unknown from these data, we do not know whether these are antibiotics for suspected VAP, amongst which we could have an impact on or antibiotics for another reason that we cannot alter.

In conclusion this study has demonstrated that BAL fluid IL-1β can effectively exclude VAP when tested in patients with suspected VAP, in a multi-centre study. In combination with IL-8, a test with confident rule-out performance has been identified. The next stage is to determine the clinical effectiveness of a biomarker-based diagnostic strategy to reduce antibiotic use in a clinical trial conducted in a highly complex and high antibiotic-use clinical environment. The interim results of this trial will be presented in Chapter 4.

Chapter 4. Results: Randomised controlled trial

4.1 Introduction to chapter

The RCT extends from the validation study and aims to determine whether the biomarker-based exclusion of VAP has clinical effectiveness to reduce antibiotic use amongst patients with suspected VAP. At the time of writing this thesis the RCT was in the later stages of patient recruitment. Therefore for the purposes of this thesis, a subgroup analysis was performed on 140 patients for whom there was close to complete data collection around the time of my submission deadline. This analysis presents preliminary results to demonstrate the principles of analysis and give insights into what the completed trial may demonstrate.

4.2 Screening and recruitment

The projected recruitment period for the trial underwent a number of revisions. The initial projected recruitment duration was to recruit 210 patients (see Chapter 2, 2.13.2) between July 2013 and the end of January 2015. The trial did not commence until November 2013 and the first patient was recruited on the 18th December 2013. Revising the end date to the actual start date resulted in a target of May 2015. Due to logistical or regulatory reasons, not all study sites commenced screening and recruitment at the opening of the trial. The trial started with only two sites (Freeman Hospital and Royal Victoria Infirmary) in November and December 2013. A further 6 sites joined in January 2014 (Chelsea and Westminster, Salford Royal, Sunderland, Belfast, Heartlands, North Tyneside General). Coventry joined in Feb 2014, Chester and Preston in March 2014 and Wansbeck General, Edinburgh Royal Infirmary and Western General Hospital, Edinburgh in May 2014.

At a research steering group (RSG) review of progress in July 2014, 60 patients had been randomised with recruitment considerably behind schedule. The two main reasons were the staggered start for the recruiting sites and the slower than expected rate of recruitment. At this stage, the combined sites were recruiting an average of 8.65 patients per month. It was estimated that at this recruitment rate, the trial would complete in January 2016 and this was set as the revised target. Additional sites were added at this stage including the Queen Elizabeth, Gateshead; James Cook, Middlesbrough; Sandwell and City Hospitals, Birmingham; Royal Liverpool Hospitals, Liverpool; Manchester Royal Infirmary, Manchester; City Hospital, Belfast; and Russells Hall Hospital,

Dudley (for summary of sites see Methods, 2.2). These sites were brought on between October 2014 and January 2015.

A further RSG was held in December 2015, just prior to the planned completion date of the trial. At this stage, 175 patients had been randomised and the milestone was further extended to the end of September 2016.

This analysis reports on the first 140 patients randomised between November 2013 and the end of July 2015. Recruitment up to July 2015 is shown on **Figure 13** against the three different projected trial end dates.

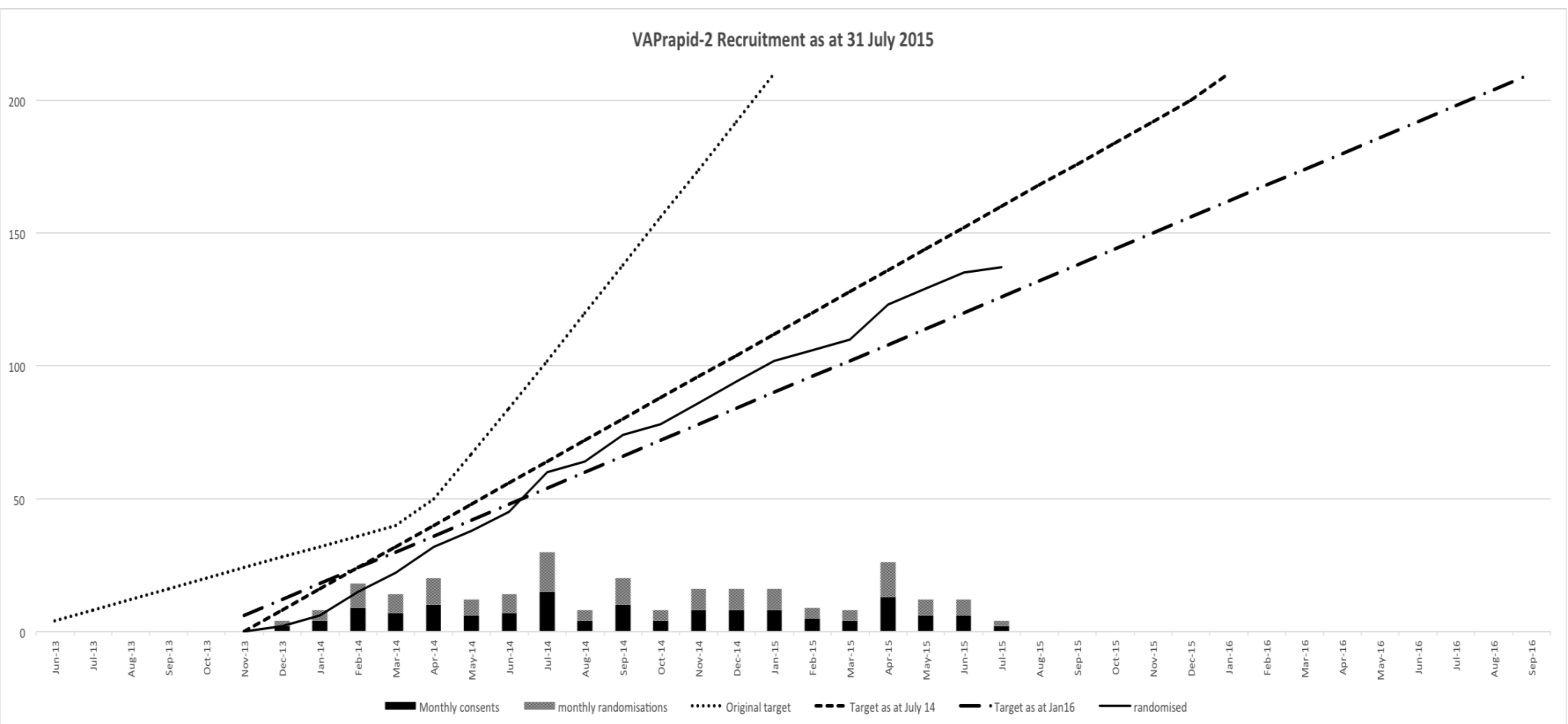

Figure 13: Recruitment of patients to trial. Actual recruitment with projected recruitment and revised recruitment targets.

Screening logs were maintained although precise details of all exclusions were not recorded. Between November 2013 and July 2015, 511 patients were screened. Of these, 296 patients met the inclusion criteria for suspected VAP (see Methods, 2.3). One hundred and twenty nine patients were not eligible including 90 patients who were not suitable for early discontinuation of antibiotics (inclusion criterion) and 39 patients who met exclusion criteria (**Figure 14**). A further 26 patients were eligible based on inclusion and exclusion criteria but not consented without a reason recorded. Of those eligible patients, 141 were consented and of these 140 were randomised (1 patient was consented but not randomised as there was no laboratory cover to process the sample). Of these, 4 patients were not included in this analysis for the following reasons: SUN001 was randomised but deteriorated and did not undergo BAL; MAN012 was randomised in error and did not undergo BAL; RLH003 was randomised but the sample was not processed as the lab had insufficient reagents; and CHS006 was randomised but the sample arrived too late in the lab to be processed and so was rejected. These patients were excluded because they had too many missing data points. For the purposes of this thesis, I set the threshold for intention to treat (ITT) analysis as having had a sample that had undergone its laboratory handling.

Of the 136 patients included in the analysis 68 were randomised into each trial arm and included in the ITT analysis. Eleven patients in the biomarker-guided arm had a failure of the biomarker test and therefore for these patients, the clinical teams were given the instruction to default to 'standard care'. These 11 patients were excluded in the per-protocol analysis. The recruitment and follow up of patients is outlined a CONSORT diagram (**Figure 14)**. The number of patients followed up reflects the patient having follow-up data recorded for that visit but does not reflect completeness of those data.

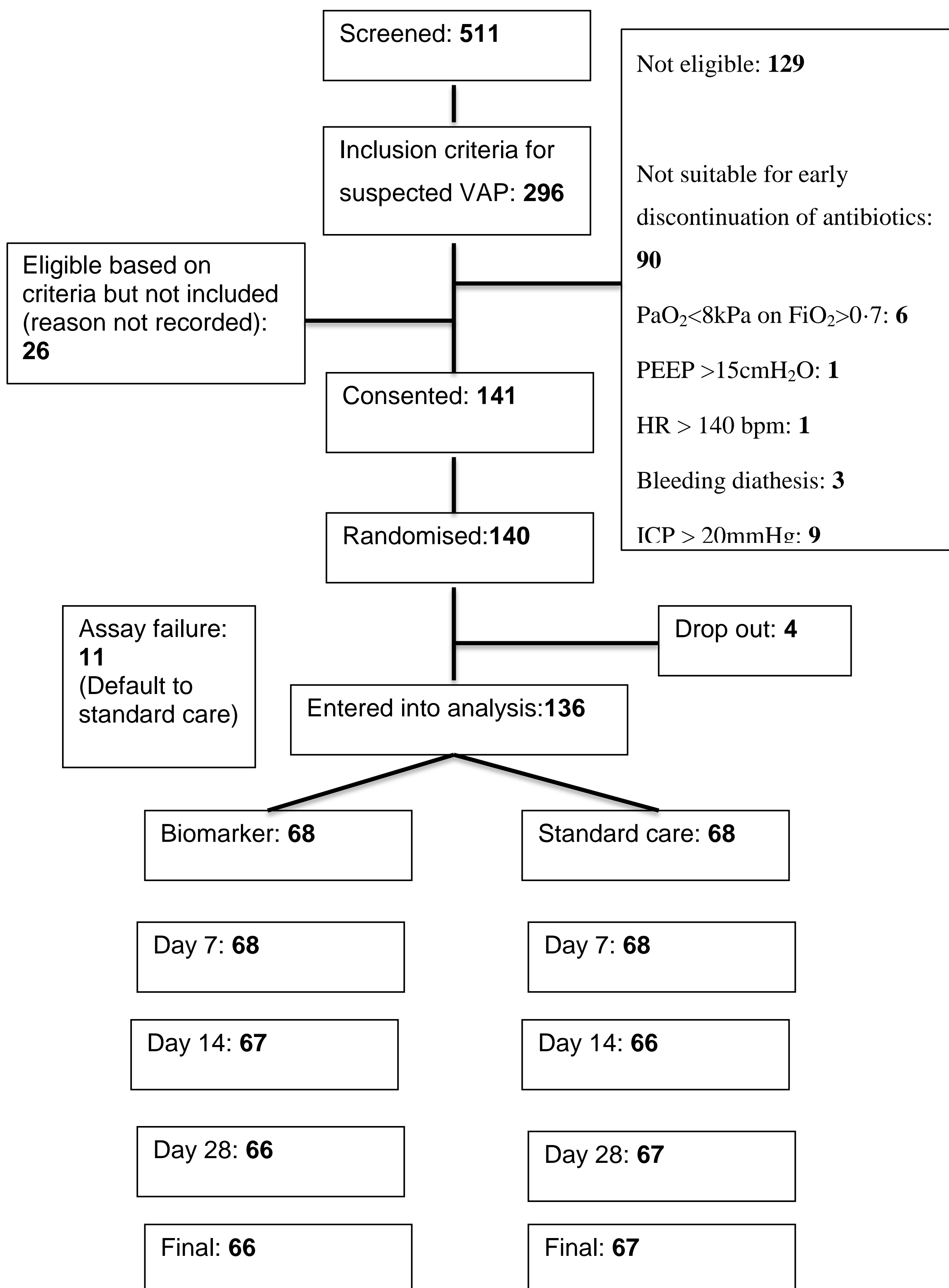

Figure 14: CONSORT diagram of patients enrolled by end of July 2015.

4.3 Process of trial conduct

The trial intervention was complex and so examining the detailed delivery of the trial is relevant. The majority of patients were consented, randomised and had BAL performed on the same day. Consent was obtained on the day before BAL in 8 patients. For these patients, the BAL was performed in the morning in 6 patients. Overall the median time that BAL was performed was at 13:30 with the earliest time of 8:35 and latest time of 17:00. The median time that BAL fluid was sent to the lab was 14:00, with the earliest time of 9:00 and latest of 17:06. The median time at which randomisation was performed was 13:25 (earliest 8:25 and latest 16:50).

The median time that the samples arrived in the laboratory was 15:00 (earliest 10:10 and latest 17:50). The technicians contacted the clinical teams once the biomarker results were available or, if the patient was in the standard care group, after approximately 6 hours to ensure approximate equality of the period in which clinicians were blinded to the test result. Overall the clinical teams were contact at a median time of 19:08, with the earliest at 6:24am and latest 1:30am. Whether results were communicated to the clinical team on the next working day, is not recorded. If the result was called back at 1:30am then that would be the next calendar day but a call at 6:24am was assumed to be the next working day too. A further 3 results were communicated to clinical teams before midday but these were in the standard care arm, and in this setting it appears that there was no period of blinding given to the clinical team (ie the result was presumably communicated as soon as the patient was randomised to standard care, without a 6 hour delay). Comparing the two groups, the teams were contacted at a similar median time, 20:44 for the biomarker group and 20:00 for the control group. As might be expected the latest times for communication of results were in the biomarker group with 9 results being called back after 23:00. In the standard care group the latest the teams were contacted was at 22:30.

The validation study demonstrated that patients generally underwent BAL in the afternoon. This was a cause for concern for the RCT because the biomarker assay takes approximately 6 hours to complete, and this would make the logistics of delivering a result to clinical services on the same working day a challenge. This proved to be the case with clinical teams being contacted in the evening and in the case of some biomarker results, the night.

4.4 Demographics

The demographics of the two trial arms are presented as summary statistics (**Table 30**). The two trial arms were overall similar in terms of age, sex, and severity of disease on admission (APACHE 2 and SOFA at baseline). The case mix of admission categories and degree of comorbidities as measured by the functional comorbidities index was also similar between the two groups. In terms of organ support, more than twice as many patients were receiving RRT in the biomarker group than in the standard care group. The rate of ARDS was slightly higher in the control group. The rate of VAP was also slightly greater in the biomarker group. A significant number of microbiology samples (17 patients) were handled incorrectly with the laboratory not reporting semi-quantitative results. This occurred disproportionally more frequently in the biomarker group (12 of the 17 patients), which could be the cause of this imbalance. The proportion of patients receiving antibiotics at baseline was similarly high in both groups.

Characteristic	Biomarker-guided recommendation on antibiotics N=68	Routine use of antibiotics N=68
Age in years, median (IQR)	61.5 (50.0-70.5)	57.5 (43.5-68.5)
Female, n (%)	24 (35.3)	26 (38.3)
APACHE 2, median (IQR)	18.0 (12.5-24.0)	17.0 (12.0-22.0)
SOFA Day 0, mean (SD)[1]	5.2 (2.5)	6.0 (2.8)
Medical admission (n=81), n (%)[2]	39 (59.1)	42 (64.6)
Surgical admission (n=50), n (%)[2]	27 (40.9)	23 (35.4)
Admission category, n (%)[3]		
Respiratory	10 (15.2)	16 (23.5)
Gastrointestinal/Liver	7 (10.6)	7 (10.3)
Cardiovascular	7 (10.6)	6 (8.8)
Trauma	13 (19.7)	17 (25.0)
Sepsis	2 (3.0)	0
Obstetric/Gynaecology	0	1 (1.5)
Neurology	16 (24.2)	11 (16.2)
Other	11 (16.7)	10 (14.7)
Function comorbidities index score, median (IQR)[4]	1.0 (0-2.0)	1.0 (0-2.0)
Renal replacement therapy, n (%)[3]	7 (10.6)	3 (4.4)
Vasopressors, n (%)[3]	25 (37.3)	22 (32.8)
ARDS, n (%)	10 (14.7)	16 (23.5)
ALI, n (%)	8 (11.8)	3 (4.4)
Corticosteriod use, n (%)	11 (16.2)	12 (17.6)
Antibiotics at randomisation, n (%)	53 (77.9)	57 (83.8)
Days from ICU admission to BAL, median (IQR)	7 (5-9.5)	8 (4-13)
Confirmed VAP, n (%)[5]	23 (41.1)	18 (28.6)
Clinician pre-test suspicion of VAP, n (%)[6]		
Low	7 (10.4)	4 (6.0)
Medium	19 (28.4)	34 (50.7)
High	41 (61.2)	29 (43.3)

Table 30: Baseline demographics of trial population.
Missing values, total (biomarker arm): [1] 9(6), [2] 5(2), [3] 2(2), [4] 3(2), [5] 17(12), [6] 2(1)

The clinical and laboratory characteristics of the two groups are presented in **Table 31**.

Clinical/laboratory parameters	**Biomarker-guided recommendation on antibiotics N=68**	**Routine use of antibiotics N=68**
Temperature ^{O}C, median (IQR)	37.4 (36.6-38.1)	37.7 (37.0-38.3)
WCC $x10^9/L$, median (IQR)	13.8 (10.7-16.4)	14.3 (11.8-16.7)
Neutrophils $x10^9/L$, median (IQR)	10.8 (8.1-14.1)	11.0 (9.2-13.8)
Platelets $x10^9/L$, mean (SD)	251 (146)	282 (195)
CRP *mg/L*, mean (SD)[1]	170 (87)	165 (112)
PEEP cmH_2O, median (IQR)[2]	8 (5-10)	7.5 (5-10)
PAP cmH_2O, median (IQR)	21 (13-23)	18 (12.5-25)
Heart rate *bpm*, mean (SD)[3]	89 (22)	92 (21)
MAP *mmHg*, median (IQR)[4]	75 (68-85)	78 (69-88)
ICP *mmHg,* median (IQR) *(monitored patients only, n=14, biomarker-guided=7)*	13 (9-15)	10 (0-11)
pH, median (IQR)[5]	7.43 (7.40-7.46)	7.44 (7.39-7.47)
$PaCO_2$ *kPa*, median (IQR)[6]	5.48 (4.90-6.33)	5.60 (4.50-6.30)
PaO_2:FiO_2 *kPa*, mean (SD)[6]	30.9 (12.2)	26.6 (12.7)
Tracheal secretion, n (%)[7]		
None	1 (1.5)	3 (4.5)
Non-purulent	9 (13.2)	9 (13.4)
Purulent	58 (85.3)	55 (82.1)
CXR, n (%)[8]		
Diffuse	7 (10.8)	9 (13.2)
Localised	37 (56.9)	35 (51.5)
Bilateral	21 (32.3)	24 (35.3)

Table 31: Clinical and laboratory characteristics for trial population. Missing values, total (biomarker-guided arm): [1]53(28), [2]5(3), [3]4(2), [4]10(4), [5]7(3), [6]5(2), [7]1(0), [8]3(3)

4.5 BAL and safety data

The majority of sampling occurred in the lower lobes with 64 (47%) being performed in the right lower lobe and 48 (36%) in the left lower lobe. Numbers performed in the right upper lobe, right middle lobe and left upper lobe were 10 (7%), 11 (8%) and 2 (2%), respectively. In keeping with the BAL SOP the median volume instilled was 120mls (IQR 120-120), and a minimum and maximum volume of 60mls and 160mls respectively.

Prior to BAL the median oxygen saturation was 97% (IQR 96-99) with a median FiO_2 0.41 (IQR 0.35-0.50). Prior to BAL, on a FiO_2 of 1.0, the lowest oxygen saturation was 94%. During BAL oxygen saturation fell below 5% of starting saturations in 32 patients. In these patients the saturation fell to a median of 88% (IQR 85-88) for a median time of 2 minutes (IQR 2-4). Amongst these, 23 patients had saturations that fell below 90% and this lasted for a median time of 2 minutes (IQR 0-3). One patient had saturations that fell to 59% for 60 seconds during BAL and saturations remained below 90% for 45 minutes but at the two hour follow up point, this patient was saturating at pre-BAL level with only an increase in FiO_2 of 0.05. Five patients met our predefined criteria to report an adverse event during BAL (drop of 5% or below 90% for greater than 5 minutes), although 4 were recorded as adverse events in the CRF (these did not require separate reporting to NCTU).

Two hours after BAL the median saturation was 97% (IQR 96-99) and the median FiO_2 was 0.50 (IQR 0.40-0.60). Fifty-three patients had an increase in FiO_2 of ≥0.1. Of these patients, 6 had ≥3% lower oxygen saturations than at baseline despite this increase in FiO_2 (range -3 - -5%). A further 9 patients had a reduction in saturations of between -1% and -2%. The remaining patients had either no change in baseline saturations (5 patients) or an increase in oxygen saturations with the increase in FiO_2. Nine further patients had falls in oxygen saturations at 2 hours post BAL of 3-9% without any increase in FiO_2. A total of 43 adverse events were recorded in the CRF with our predefined criteria for adverse events during BAL (these did not require separate reporting to NCTU).

Five serious adverse events (SAE) were recorded and all were judged to be in relation to the patient's critical illness rather than trial involvement. Three SAE were in relation to

one patient who was admitted with gastrointestinal bleeding and had two further episodes of GI bleeding. This patient died and the death was recorded as SAE. In the other two patients, one suffered a tension pneumothorax and the other a ventricular fibrillation cardiac arrest. Both of these incidents happened around the time of the BAL but in both cases the PI judged that there was no causal relationship. In both cases the Sponsor reviewed the incident and was in agreement with the PIs. In the case of the cardiac arrest, the DMEC also reviewed the case and felt there was no causal link.

4.6 Microbiology

The median time taken from BAL to return of microbiology results was 2 days (IQR 2-4). As described above, a total of 17 samples were not reported as a semi-quantitative result, and so these could not be defined as VAP or non-VAP. Therefore, based on our criteria for defining VAP as a culture growth of $\geq 10^4$ cfu/ml, VAP was confirmed in 41 of 119 patients (34.5%). Of those below the threshold and classed as non-VAP, 32 (26.9%) had a culture growth below the threshold and 46 (38.7%) had a sterile culture. Considering all organisms grown (VAP and sub-VAP), 18% were yeasts, 30% were Gram-positive and 52% were Gram-negative. Of organisms cultured above the threshold of $\geq 10^4$ cfu/ml, 5% were yeasts, 39% Gram-positive and 56% Gram-negative. Polymicrobial growth occurred in 37% of patients with VAP. The organisms cultured at $\geq 10^4$ cfu/ml are presented in **Table 32**.

Organism	**Frequency**	**Percent**
MSSA	16	27.6
Haemophilus spp.	10	17.2
Pseudomonas aeruginosa	5	8.6
Proteus mirabilis	4	6.9
Escherichia coli	3	5.2
Klebsiella pneumoniae	3	5.2
Candida spp.	2	3.4
Serratia marcescens	2	3.4
Staphylococcus spp.	2	3.4
Stenotrophomonas maltophilia	2	3.4
Acinetobacter baumannii	1	1.7
Corynebacterium spp.	1	1.7
Enterobacter spp.	1	1.7
Moraxella catarrhalis	1	1.7
Prevotella bivia	1	1.7
Beta haemolytic Streptococcus	1	1.7
Streptococcus pneumoniae	1	1.7
Normal upper respiratory tract flora	1	1.7
Yeasts	1	1.7
Total	58	100.0

Table 32: Organisms cultured above the threshold to define VAP. More than one pathogen could be cultured per BAL sample.

The proportion of patients already in receipt of antibiotics at the time of enrolment was high in both trial arms (**Table 26**). This was observed in the validation study too and in the validation study there was a greater proportion of non-VAP in patients who had received antibiotics (raising the theoretical possibility of false negative culture). Taking the trial population as a whole, there were more non-VAP patients who had received antibiotics (87.2%) in comparison to VAP patients (68.3%) (Chi-squared p=0.013).

At the time of recruitment, before BAL was performed, clinicians were asked to give their pre-test level of suspicion of VAP (low, medium or high). There was no relationship between clinician pre-test and VAP diagnosis (**Table 33**). Interestingly, for the majority of patients, the clinician's suspicion of VAP was rated as 'medium' (40%) or 'high' (51%), with only 9.1% of non-VAP and 7.5% of VAP rated as 'low'. Considering that VAP was confirmed in 34.5% of this RCT cohort and in 35% of the validation study cohort, clinician expectation of confirming VAP is incongruous with the observed VAP rate.

N (%)	**Pre BAL LOW**	**Pre BAL MEDIUM**	**Pre BAL HIGH**
Non-VAP	7 (9.1)	32 (41.6)	38 (49.4)
VAP	3 (7.5)	15 (37.5)	22 (55.0)

Table 33: Clinician pre-test suspicion of VAP compared to VAP status. Chi-squared: p=0.84. n=117, clinician pre-BAL suspicion of VAP not available for 2 patients.

4.7 Biomarker performance

One area of concern in the design of this trial was whether the biomarker assay would demonstrate similar diagnostic parameters as it did in the validation study. In other words, would the assay accurately identify patients with non-VAP in whom early discontinuation of antibiotics would be most appropriate?

Of the 68 patients who underwent the biomarker testing, there were 11 assay failures and 12 patients who did not have semi-quantitative culture (2 patients fell into both categories). A further 5 patients had missing data for biomarker results and so 42 patients were included in this analysis. Overall there were 15 patients in whom the biomarker assay fell below the threshold. Amongst the 42 patients in this analysis, there were 11 assays that fell below the threshold to exclude VAP (26%). There was one false negative (SUN005), which was below the threshold on the biomarker test but had a positive culture (*Enterococcus* spp. $\geq 10^4$ cfu/ml). Of the 23 patients classed as 'non-VAP', the biomarker correctly identified 10 as 'non-VAP' (43.5%)

As a further 'validation' of the biomarker assay, ROC curves were constructed for IL-1β, IL-8 and the linear predictor of the IL-1β/IL-8 combination. The median concentrations of IL-1β and IL-8 and the AUROC curves are presented in **Table 34**.

	VAP n=19	Non-VAP n=23	*p* value	AUROC
IL-1β pg/ml	246 (191-1994)	36 (3-325)	0.0021	0.77
IL-8 pg/ml	6382 (3175-18219)	1024 (350-6361)	0.0066	0.74

Table 34: Biomarker concentrations between VAP and non-VAP groups. Concentrations presented as median (IQR). ***p*** **value reported from Mann Whitney U test.**

The smaller numbers in this analysis limits drawing comparisons to the validation study. The concentrations of IL-1β and IL-8 in the non-VAP groups are similar to the validation study, as is the level of IL-8 in the VAP group (see Results: Validation study, 3.7). The IL-1β concentration in the VAP group is lower than in the validation study. However the IQR for these values closely reflect the validation study.

The AUROC for IL-8 closely replicates the validation study value but the AUROC for IL-1β is lower. When the coordinates of the ROC are examined to determine cut-points that are close to the validation cut-points for single biomarkers (17pg/ml for IL-1β and 382pg/ml for IL-8), a cut-point of 17.5pg/ml for IL-1β had a sensitivity of 95% and a specificity of 39% and a cut-point of 379pg/ml for IL-8 had a sensitivity of 100% and a specificity of 26%. The sensitivity and specificity at these cut-points are close to the validation study values (IL-1β sensitivity 96% and specificity 43%, IL-8 sensitivity 98% and specificity 25%).

The logistic regression output from the combination of IL-1β and IL-8 was also plotted on a ROC curve. The AUROC for the linear predictor was 0.76. At a coordinate close to the validation study cut-point (-1.7616) of -1.713, the sensitivity was 95% and the specificity was 43.5%. Again this is close to the validation study biomarker performance (IL-1β/IL-8 sensitivity 100% and specificity 44%).

This close replication of the validation study sensitivity and specificity is reflected by 43.5% of the non-VAP patients being correctly identified as non-VAP by the biomarker test at this stage in the trial.

4.8 Primary outcome analysis in the *ITT population*

The AFD in the 7 days following BAL was presented as an integer value ranging from 0-7 AFD. The frequency distribution was compared in a 2x8 contingency table (**Table 35**).

	AFD							
	0	1	2	3	4	5	6	7
Biomarker arm	33	9	6	5	4	0	2	6
Control	28	10	6	10	6	0	4	3

Table 35: Frequency of AFD in the 7 days following BAL. Figures represent the number of patients in each AFD category. Chi-squared *p*=0.65. Missing values, total (biomarker): 4(3).

There was no difference between to the two arms of the trial when compared by Chi-squared test, *p*=0.65. The frequency distribution of AFD is illustrated in **Figure 15**.

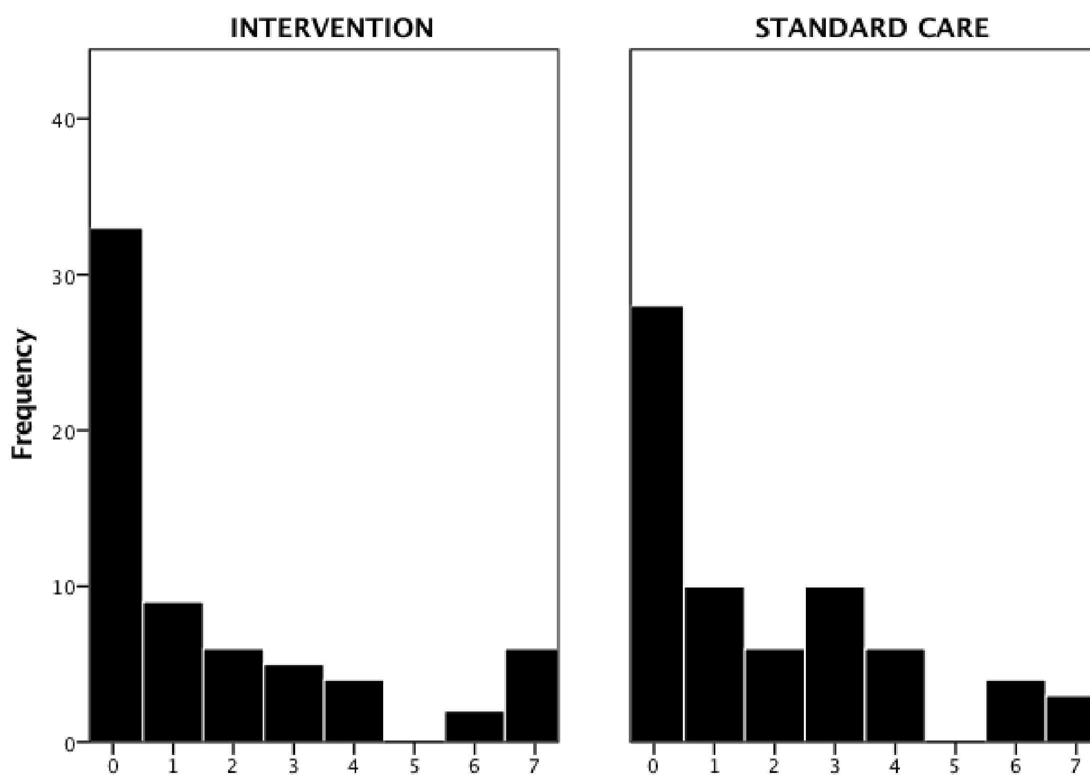

Figure 15: Frequency distribution of AFD in ITT population.

The distribution of AFD was similar to the observed distribution in the validation study with a skew to the left, reflecting the majority of patients having few AFD. The proportion of patients with 0 AFD was 50.8% in the biomarker arm and 41.8% in the control arm. The remaining categories have between 3.1% and 14.9% of patients. The distribution between the two groups is similar.

One limitation of this analysis is that it does not account for deaths within the first 7 days, ie a patient that dies on day 4 on antibiotics has the same number of AFD as the patient who has 7 days of antibiotics and does not die. To determine whether deaths

within the first 7 days had a significant impact on the primary outcome, the number of days of antibiotics, number of days alive without antibiotics and the number of days dead in the first 7 days were calculated (**Table 36**).

There is no imbalance between the two groups at day 7 with regards to number of antibiotic days, number of days alive without antibiotics and number of days dead, which suggests that deaths within the first 7 days has not had a significant influence on the primary outcome.

Outcome	Biomarker-guided recommendation on antibiotics N=68	Routine use of antibiotics N=68
Day 7 antibiotic days (mean, 95% CI)	5.17 (4.57-5.77)	4.93 (4.38-5.47)
Number of days alive without antibiotics at day 7 (mean, 95% CI)	1.65 (1.08-2.21)	1.81 (1.29-2.32)
Number of days dead at day 7 (mean, 95% CI)	0.18 (0-0.38)	0.28 (0.04-0.52)

Table 36: Days alive without antibiotics

4.9 Secondary outcome analysis in the *ITT population*

Secondary outcome measures were compared between the two groups with no significant difference between the two groups in any of these (**Table 37**). The antibiotic use at days 7, 14 and 28 was high in both groups. The median AFD at day 7 was 0 (IQR 0-3) in the biomarker arm and 1 (IQR 0-3) in the control arm. The AFD at day 14 and day 28 in the biomarker arm was 5.0 (SD 3.9) and 11.6 (SD 7.6) respectively and in the control arm was 5.2 (SD 3.8) and 12.5 (SD 8.2) respectively.

There were no significant differences between the biomarker arm and the control arm in terms of ICU-mortality (19.1% and 23.5%, respectively; p=0.627); 28-day mortality (25% and 23.5%, respectively; p=0.844); VFD at 28 days (13.0 [IQR 0-21.0] and 12.0 [IQR 0-19.0], respectively; p=0.723); duration of invasive ventilation (16.0 days [IQR 11.5-26.5] and 19.0 days [IQR 12.5-29.0], respectively; p=0.433); length of hospital stay (33 days [IQR 25-57] and 43 days [25.5-77.0], respectively; p=0.242); and length

of critical care stay (21.0 days [16.0-31.0] and 24.5 days [17.0-36.0], respectively; p=0.394).

The presence of antibiotic-resistant pathogens or *C. difficile* could be influenced by antibiotic use and so the occurrence of these were monitored up to final follow up at hospital discharge, death or 56 days. There were a total of 8 MRSA positive cultures with no difference between the biomarker and control arms. There were 2 *C. difficile* infections and these occurred equally in both trial arms. Multi-resistant organisms (defined as resistance to 2 or more antibiotics) were detected in 29.4% of the patients in the biomarker arm and 20.6% of the patients in the control arm (p= 0.235). The median number of multi-resistant cultures was 2 (IQR 1-3) in both the biomarker and control arms.

Outcome	Biomarker-guided recommendation on antibiotics N=68	Routine use of antibiotics N=68	*p* value
28-day mortality n (%, 95%CI)†[1]	16 (25, 14.5-35.8)	16 (23.5, 14.3-33.3)	0.844
ICU mortality n (%, 95% CI)†[2]	13 (19.1, 10.6-29.3)	14 (22.6, 11.4-33.3)	0.627
Day 3 SOFA score*[3]	3.0 (2.0-5.5)	4.0 (2.0-6.0)	0.419
Day 7 SOFA score*[4]	3.5 (2.0-4.5)	3.5 (2.0-5.0)	0.546
Day 14 SOFA score*[5]	3.0 (0.5-4.0)	3.0 (2.0-4.0)	0.711
Day 7 antibiotic days, IQR (95% CI)*[6]	6,4-7 (6-7)	6, 4-7 (4-7)	0.360
Day 7 AFD, IQR (95% CI)*[6]	0, 0-3 (0-1)	1, 0-3 (0-2)	0.380
Day 14 antibiotic days, SD (95% CI)**[7]	7.8, 4.0(6.8-8.8)	7.3, 3.9 (6.3-8.2)	0.464
Day 14 AFD, SD (95% CI)**[7]	5.0, 3.9 (4.0-6.0)	5.3, 3.8 (4.4-6.2)	0.625
Day 28 antibiotic days, SD (95% CI)**[8]	9.7, 6.0 (8.2-11.3)	9.3, 6.4 (7.7-10.9)	0.724
Day 28 AFD, SD (95% CI)**[8]	11.6, 7.6(9.6-13.6)	12.5, 8.2 (10.5-14.5)	0.552
VFD at 28 days, IQR (95% CI)*[9]	13, 0.0-21(3.5-18.0)	12, 0-19 (2-16)	0.723
Duration invasive ventilation, days, IQR (95% CI)*[10]	16.0, 11.5-26.5 (14-21.5)	19.0, 12.5-29.0 (16.5-21)	0.433
Length of hospital stay, days, IQR (95% CI)*[11]	33.0, 25.0-57.0 (27.5-41)	43.0, 25.5-77.0 (32-54)	0.242
Length of critical care stay, days, IQR (95% CI)*[12]	21.0, 16.0-31.0 (18-26)	24.5, 17.0-36.0 (20-27)	0.394
Length of Level 3 stay, days*[13]	16.0 (12.0-26.0)	20.0 (13.0-26.0)	0.282
Length of Level 2 stay, days*[14]	2.0 (0.0-6.0)	3.0 (0.0-6.0)	0.690
MRSA n (%)***[15]	3 (4.5)	5 (7.6)	0.718
Clostridium difficile n (%)***[15]	1 (1.5)	1 (1.5)	1.000
Presence of multi-resistant organisms (56 days) n (%)†	20 (29.4)	14 (20.6)	0.235
Number of multi-resistant cultures (56 days)*	2 (1-3)	2 (1-3)	1.000

Table 37: Secondary outcome measures in the ITT population. †Chi-squared, **parametric mean (SD) & T-test, *non-parametric median (IQR) & Mann-Whitney U, ***Fisher's exact. Missing values, total (biomarker-guided arm): [1]4(4), [2]6(0), [3]12(5), [4]42(20), [5]83(40), [6]4(3), [7]6(5), [8]11(9), [9]11(7), [10] 17(9), [11]14 (9), [12]8(6), [13]18(11), [14]19(11), [15]4(2).

4.10 Primary outcome analysis in the *per-protocol population*

When BAL fluid from patients who were randomised to the biomarker arm resulted in a failed biomarker-assay, the clinical teams were advised to default to 'standard care'. In the 11 instances where this occurred, all resulted from a failure of the assay to meet the internal control thresholds. This indicated that there was a problem in performing the assay. The other quality control used was the presence of IL-8. Given the usual abundance of IL-8 in BAL fluid, if no IL-8 was detectable in the sample, this called into question the quality of the BAL. No samples failed on this criterion. The 11 patients in whom the assay failed were excluded from the per-protocol analysis. All of the standard care group were included.

There were no differences in the frequency distribution of AFD in the 7 days following BAL between the biomarker and control arms (p=0.42) (**Table 38** and **Figure 16**).

	AFD							
	0	1	2	3	4	5	6	7
Biomarker	29	5	6	5	3	0	1	6
Control	28	10	6	10	6	0	4	3

Table 38: Frequency of AFD in the 7 days following BAL in the per-protocol population. Number of patients in each AFD category. Chi-squared *p*=0.42. n=55, 1 patient with missing AFD data at 7 days amongst the 11 patients excluded in the per-protocol analysis. Missing values, total (biomarker): 3(2).

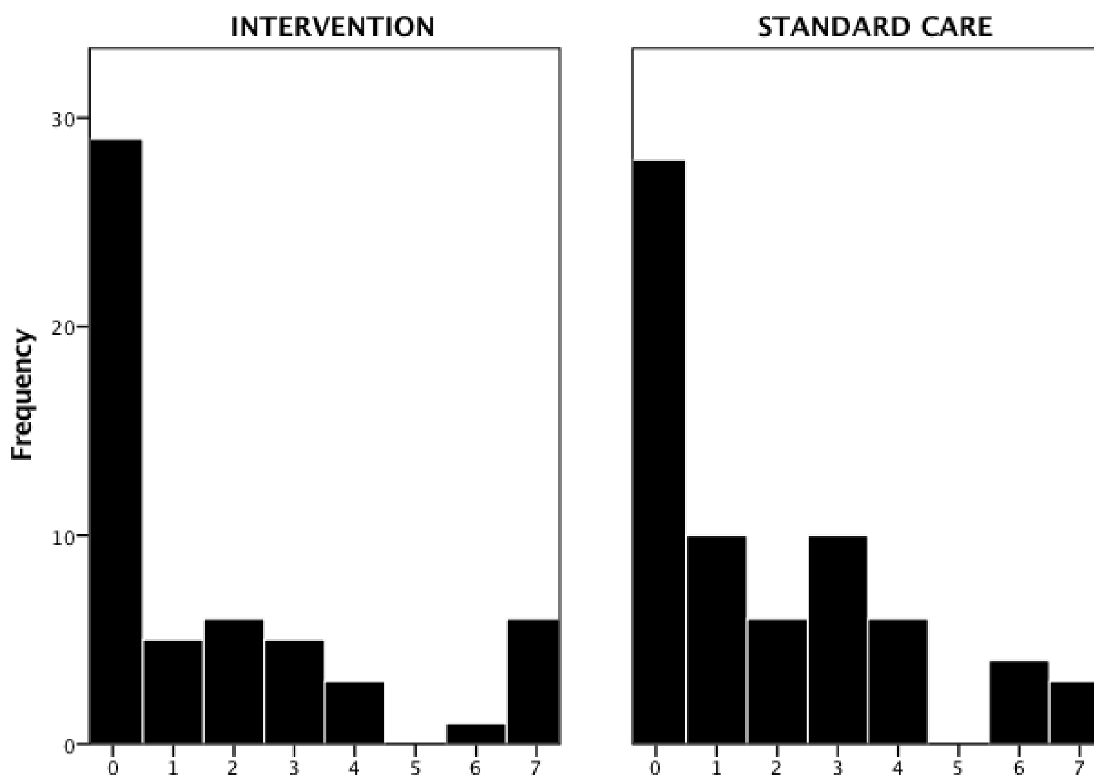

Figure 16: Frequency distribution of AFD in the 7 days following BAL for the per-protocol population.

4.11 Secondary outcome analysis in the *per-protocol population*

There were minimal differences observed in the biomarker arm with the removal of the 11 patients in whom there was an assay failure. There remained no differences between the biomarker and control arms of the trial (**Table 39**).

Outcome	Biomarker-guided recommendation on antibiotics N=57	Routine use of antibiotics N=68	*p* value
28 day mortality (%, 95% CI)†[1]	12 (22.6, 11.5-35.2)	16 (23.5, 13.8-33.3)	0.909
ICU mortality (%, 95% CI)†[2]	10 (17.5, 7.8-28.8)	14 (22.6, 12.3-33.9)	0.494
Day 3 SOFA score*[3]	4.0 (2.5-6.0)	4.0 (2.0-6.0)	0.641
Day 7 SOFA score*[4]	3.0 (2.0-4.0)	3.5 (2.0-5.0)	0.295
Day 14 SOFA score*[5]	3.0 (0.5-4.0)	3.0 (2.0-4.0)	0.570
Day 7 antibiotic days, IQR (95% CI)*[6]	6.0, 4.0-7.0 (5-7)	6.0, 4.0-7.0 (4-7)	0.433
Day 7 AFD, IQR (95% CI)*[6]	0.0, 0.0-3.0 (0-2)	1.0, 0.0-3.0 (0-2)	0.400
Day 14 antibiotic days, SD (95% CI)**[7]	7.7, 4.2 (6.6-8.9)	7.3, 3.9 (6.3-8.2)	0.530
Day 14 AFD, SD (95% CI)**[7]	5.2, 4.1 (4.0-6.3)	5.3, 3.8 (4.4-6.2)	0.804
Day 28 antibiotic days, SD (95% CI)**[8]	9.5, 5.8 (7.8-11.2)	9.3, 6.4 (7.7-10.9)	0.883
Day 28 AFD, SD (95% CI)**[8]	11.9, 7.5 (9.7-14.0)	12.5, 8.2 (10.5-14.5)	0.699
VFD at 28 days, IQR (95% CI)*[9]	17.0, 0.0-21.0 (7-19)	12.0, 0.0-19.0 (2-16)	0.331
Duration invasive ventilation, days, IQR (95% CI)*[10]	16.0, 11.0-26.0 (14-22)	19.0, 12.5-29.0 (16-21)	0.358
Length of hospital stay, days, IQR (95% CI)*[11]	32.5, 25.0-55.0 (28-41)	43.0, 25.5-77.0 (29-52)	0.228
Length of Critical care stay, days, IQR (95% CI)*[12]	20.5, 15.5-28.5 (18-26)	24.5, 17.0-36.0 (19-25)	0.318
Length of Level 3 stay, days*[13]	16.5 (12.0-26.5)	20.0 (13.0-26.0)	0.341
Length of Level 2 stay, days*[14]	2.0 (0.0-6.0)	3.0 (0.0-6.0)	0.694
MRSA n (%)***[15]	3 (5.5)	5 (7.6)	0.727
Clostridium difficile n (%)***[15]	1 (1.8)	1 (1.5)	1.000
Multi-resistant organisms (56 days) n (%)†	16 (28.1)	14 (20.6)	0.329
Number of multi-resistant cultures (56 days)*	2.0 (1.0-3.5)	2.0 (1.0-3.0)	0.552

Table 39: Secondary outcome measures for the per-protocol population. †Chi-squared, **parametric mean (SD) & T-test, *non-parametric median (IQR) & Mann-Whitney U, ***Fishers exact. Missing values, total (biomarker-guided arm): [1]4(4), [2]6(0), [3]12(5), [4]40(18), [5]76(33), [6]3(2), [7]5(4), [8]10(8), [9]11(7), [10]15(7), [11]12(7), [12]7(5), [13]16(9), [14]17(9), [15]4(2).

4.12 Antibiotic use overall

Since there were no differences between the trial arms in terms of antibiotic use, a description of antibiotic prescribing practices in the trial population as a whole was undertaken.

The median (IQR) antibiotic days at 7, 14 and 28 days are 6 (4-7), 7.5 (4-10) and 8 (6-13), respectively. The median AFD (IQR) at 7, 14 and 28 days are 1 (0-3), 5 (2-8) and 12 (5-19), respectively. The distribution of AFD at day 7, 14 and 28 are shown in **Figure 17, 18 and 19**.

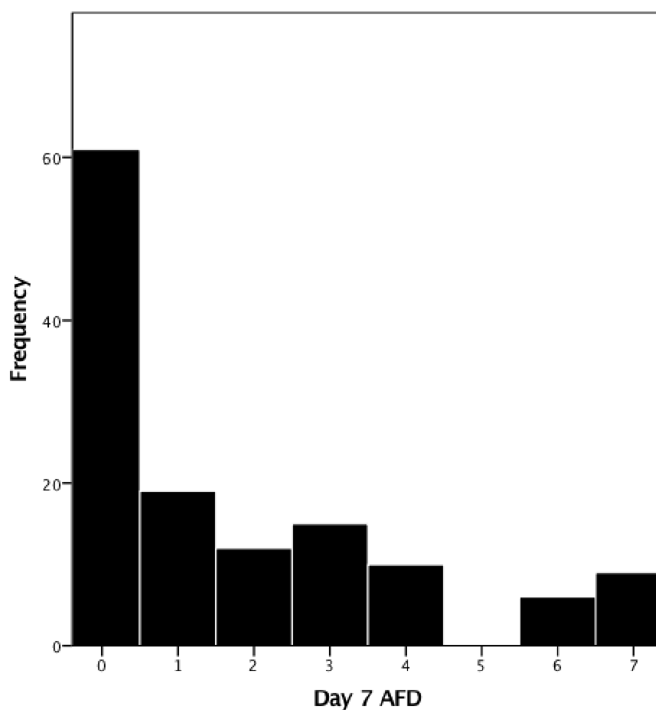

Figure 17: Distribution of AFD at 7 days n=132.

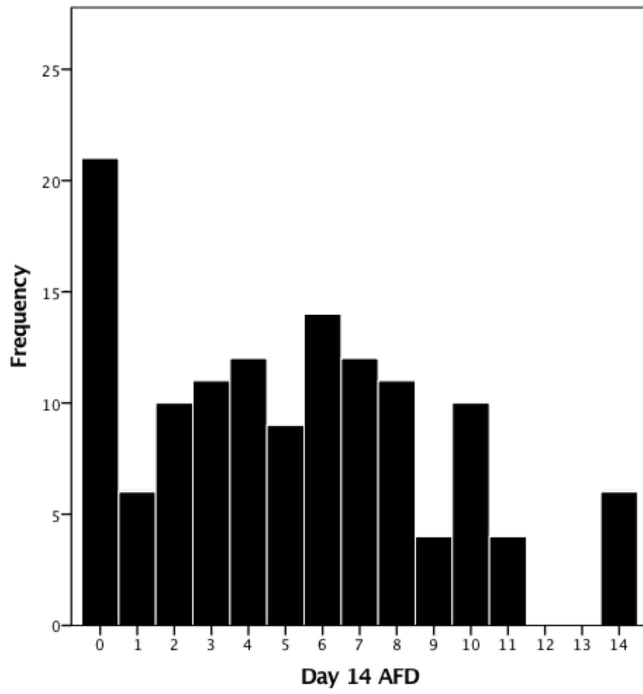

Figure 18: Distribution of AFD at 14 days n=130.

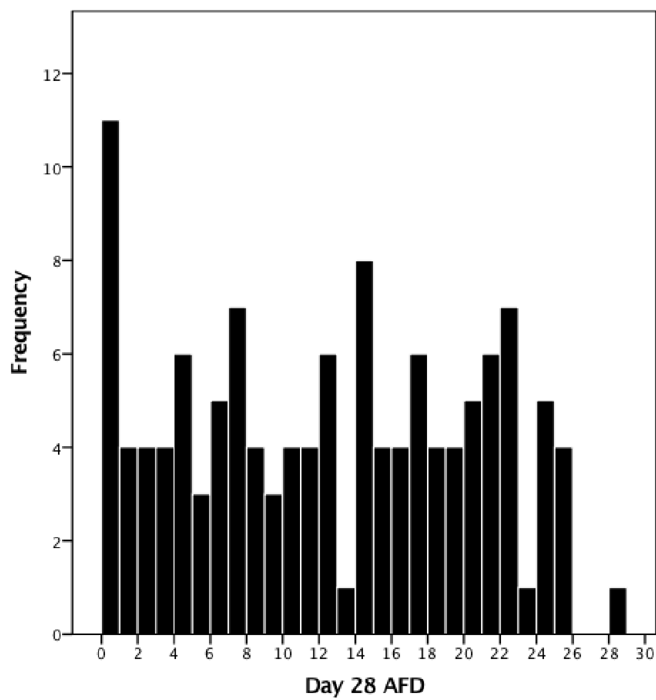

Figure 19: Distribution of AFD at 28 days n=125

Indications for antibiotic prescriptions were recorded for all antibiotics. Patients could have more than one indication for antibiotics recorded. At baseline the proportion of patients not receiving antibiotics was 18%. At baseline all indications for antibiotics were recorded, including antibiotics used for prophylaxis. Antibiotics for prophylaxis were subsequently not recorded at day 7, 14 or 28 as these did not contribute to AFD. At baseline the proportion of patients with 1, 2, 3 or 4 indications for antibiotics were 60%, 13%, 8% and 1% respectively. The proportion of patients with 1, 2, 3 or 4 antibiotics concurrently prescribed was 46%, 21%, 8% and 7% respectively. The indication for antibiotics at baseline is illustrated in **Figure 20**.

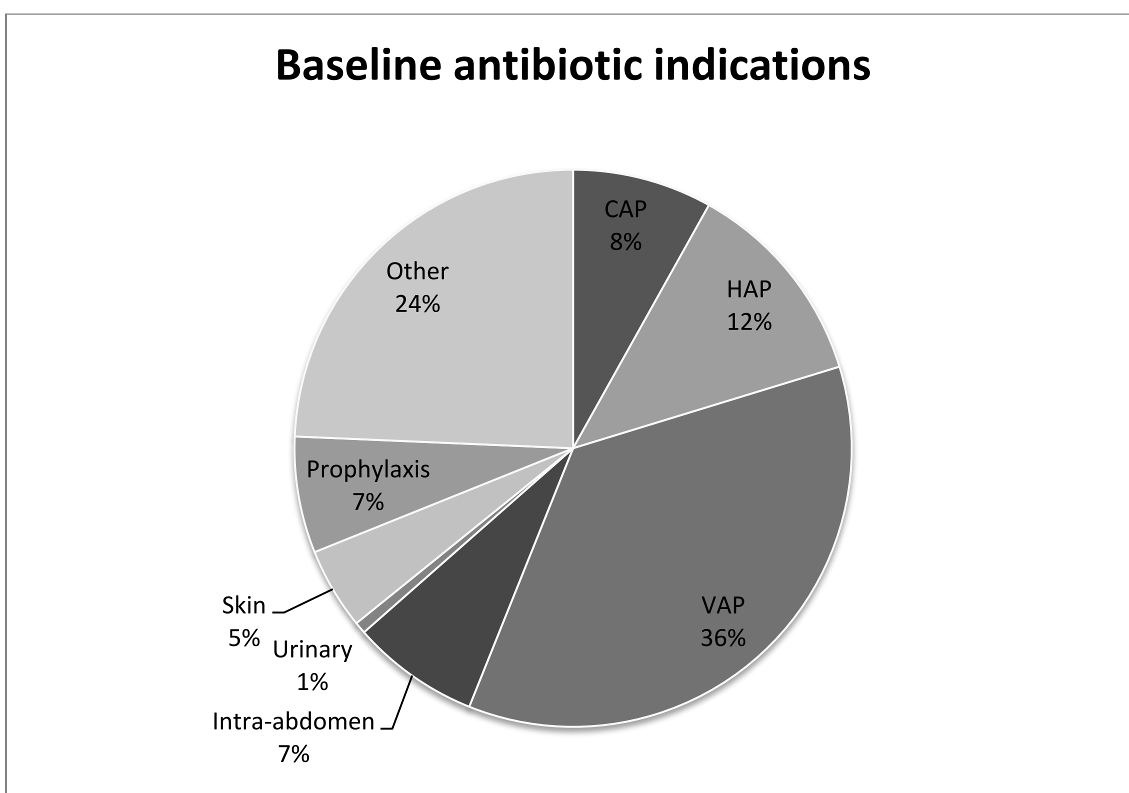

Figure 20: Indications for antibiotics at baseline. The figure presents the indication for antibiotics as a proportion of all recorded indications (rather than patients). More than one indication can be stated per patient.

At day 7, 58% of patients had 1 indication for antibiotics. A further 16% had 2 indications, 5% had 3 indications and 21% were not on antibiotics. The proportions of patients receiving 0, 1, 2, 3, 4, 5 and 6 antibiotics were 21%, 32%, 22%, 21%, 3% and 1%, respectively. Indications for antibiotics prescribed at day 7 are illustrated in **Figure 21**. VAP was an indication for antibiotics in 67 (49%) patients receiving antibiotics. VAP was the sole indication for antibiotics in 46 (34%) patients. Considering prescriptions classified as 'Other' but that were for positive respiratory cultures (either sputum or BAL), then VAP was a reason in addition to another indication in 29 (21%) patients. 32 (24%) patients had antibiotics for a non-VAP source.

Of those patients with confirmed VAP by semi-quantitative culture (41), 15 (37%) patients were on antibiotics solely for VAP, 13 (32%) were on antibiotics for VAP and another indication and 11 (27%) were on antibiotics for a reason other than VAP. Of those patients who did not have VAP confirmed (78), 24 (31%) were on antibiotics solely for VAP, 11 (14%) for VAP and another indication and 18 (23%) were on antibiotics for a non-VAP indication.

Considering the antibiotic indications at day 7 for 15 patients who had a BAL fluid biomarker assay result below the cut off for VAP (without making the exclusions made in section 4.7), 4 patients had treatment for VAP solely, 2 had treatment for VAP and another indication, 4 had treatment for a non-VAP indication and 5 had no antibiotics.

The indications for antibiotics at day 14 and day 28 show that a significant proportion of recorded indications where for pulmonary infection, either HAP or VAP (**Figure 22** and **23**). In addition to this 3 patients at day 14 and 5 at day 28 were recorded as 'Other' but were treated for 'chest', 'lower respiratory tract infection' or 'positive sputum culture'.

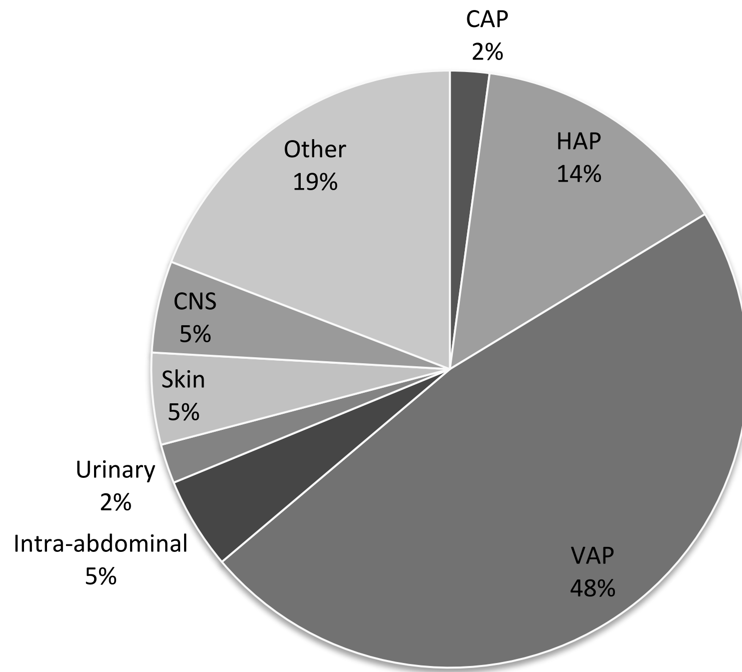

Figure 21: Indications for antibiotics at day 7. Indications presented as a proportion of all recorded indications.

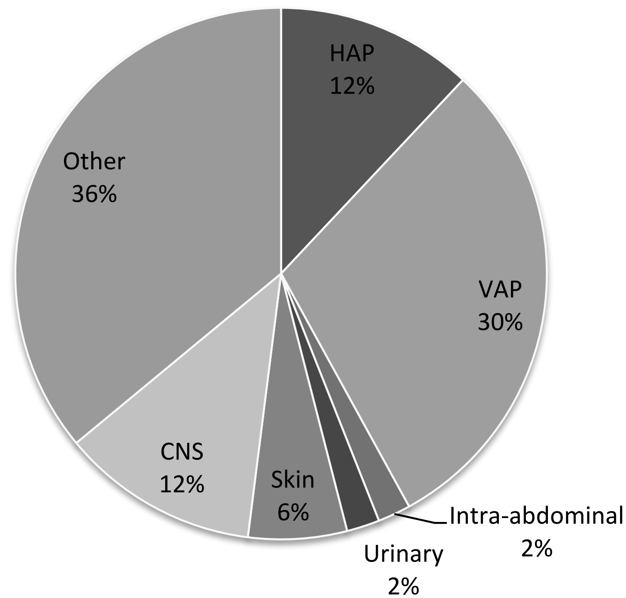

Figure 22: Indications for antibiotics at day 14. Indications presented as a proportion of all recorded indications.

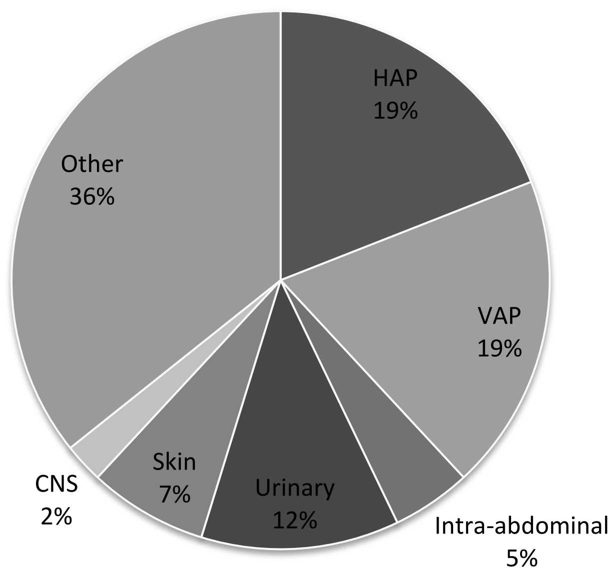

Figure 23: Indications for antibiotics at day 28. Indications presented as a proportion of all recorded indications.

4.13 Discussion

In this chapter I have reported the findings of an analysis of the first 140 patients (136 included in ITT) of an RCT that aims to recruit 210 patients. This strategy was pursed because the delays in trial set up and recruitment (**Figure 13**) meant that it was impractical to await the trial completion for inclusion in this thesis. This created a conflict between meeting the requirements of this thesis while not compromising the trial integrity. The trial was not blinded beyond the first 6 hours after randomisation and so the outcome measures were not blinded. Therefore accessing these outcomes before the close of the trial did not compromise the trial blinding. The time required to access the data, clean the dataset and analyse the data (approximately 7 months), was such that the analysis occurred after the last patient was randomised to the trial. The chief investigator was not aware of these results until after the trial had closed to recruitment and the trial statistician provided assistance with the analysis with only blinded data. Therefore this analysis could not bias the conduct and outcome of the trial as a whole. The trial sponsor and the DMEC approved this strategy.

Although this analysis does not have the statistical power to make firm conclusions on the trial overall, it does provide some valuable insights into the intervention, the trial design and the clinical context in which the intervention was tested.

This trial was designed to determine whether rapid, biomarker-based exclusion of VAP could improve antibiotic stewardship and reduce antibiotic use in patients with suspected VAP without compromising patient safety. VAP is a common reason for antibiotic prescriptions in ICU(Vincent *et al.*, 2009) and since infection is confirmed in 30-60% of patients with suspected VAP(Fagon *et al.*, 2000; Canadian Critical Care Trials Group et, 2006), the assumption is that many patients receive unnecessary antibiotics. Since microbiology results typically take 2-3 days to become available, rapid diagnostics, and in the case of this intervention, a rule-out test, could allow for antibiotics to be discontinued early if VAP can be excluded with high probability.

To determine whether this would be achieved, I conducted a multi-centre RCT in 23 ICUs in the UK. I measured whether antibiotic exposure in the 7 days following BAL, as measured by AFD, would be different between patients randomised to an antibiotic management strategy based on a rapid biomarker-based rule out of VAP and patients randomised to an antibiotic strategy based solely on microbiology results. The biomarker result was returned generally on the same day as the BAL (as compared to 2 days later for the microbiology results). There was no difference in the two trial arms in

the distribution of AFD in the 7 days following BAL. Furthermore there were no differences between the two groups in any secondary outcomes including antibiotic use at 14- or 28-days, duration of critical care stay, duration of hospital stay or mortality.

These were general ICUs and the patients included in this study were admitted with a broad range of admission categories. The cohort of patients enrolled was similar in characteristics to the cohort of patients enrolled in the validation study as reflected by median age, APACHE 2 score, and length of critical care and hospital stay. This is a sicker group of patients with a longer length of ICU and hospital stay than the average UK critical care population (Intensive Care National Audit and Research Centre, 2015) but with similar characteristics to previous trials in patients with suspected VAP(Fagon *et al.*, 2000; Canadian Critical Care Trials Group et, 2006). Overall VAP was confirmed in 34.5% of patients, consistent with the validation study. There was an imbalance in the VAP rate between the intervention and the control arm of the trial, although a greater proportion of microbiology specimens were not reported as semi-quantitative in the intervention arm, which could account for this difference. The median number of days between ICU admission and BAL was 7 (IQR 5-9.5) for the intervention group and 8 (IQR 4-13) for the control group, suggesting this is predominantly a group of patients with late-onset VAP. In keeping with this, 56% of organisms cultured were Gram-negative, although the single most common organism was methicillin-sensitive *S. aureus*, as it was in the validation study. Similar to the validation study there were more patients classed as non-VAP who had antibiotics at enrolment than VAP patients, raising the possibility of false negative cultures and misclassification as 'non-VAP'. Previous studies have shown that antibiotics do reduce the chances of obtaining a positive result for microbiology samples(Fabregas *et al.*, 1996), which is a limitation when using a microbiological definition for VAP.

The trial aimed to influence antibiotic use and this trial demonstrated that this is an environment and patient group in whom the antibiotic exposure is high. At baseline 77.9% of the intervention group and 83.8% of the control group were receiving antibiotics. Antibiotic use at 7 days was high in both arms of the trial, with a median of 0 AFD (IQR 0-3) in the intervention arm and 1 AFD (IQR 0-3) in the standard care arm, with no difference in the frequency distribution of AFD between the two groups. Since there was no difference in antibiotic use between the two trial arms, antibiotic use was also described using the trial population as a whole. The median number of antibiotic days at day 7, 14 and 28 was 6 (IQR 4-7), 7.5 (IQR 4-10) and 8 (6-13), respectively.

This suggests that the bulk of antibiotic use was in the 7 days following BAL, at the moment of suspicion of VAP. The median number of AFD at 28-days of 12 (IQR 5-19) was similar to other clinical trials. In a trial comparing 8 versus 15 days of antibiotic treatment for VAP, the mean AFD at 28-days in the 8-day arm was 13.1 (SD 7.4)(Chastre *et al.*, 2003). Considering that a 7-day course of antibiotics is considered standard in UK practice(Browne *et al.*, 2014), it seems reasonable that the number of AFD would match the 8-day arm of that trial. In an ICU population with suspected bacterial infections (in the context of a trial of PCT-guided antibiotic discontinuation), the 28-day AFD were 14.3 (SD 9.1) in the PCT group and 11.6 (8.2) in the control group(Bouadma *et al.*, 2010). In contrast to this AFD at 30-days has been reported to be much lower in other studies. A trial of early versus late tracheostomy found that the median AFD at 30-days was only 5 days (IQR 1-10)(Young *et al.*, 2013).

Biomarker-based antibiotic stewardship interventions have mainly utilised PCT, with mixed results. A number of high quality trials have utilised serum PCT to guide antibiotic use in suspected infection in ICU (ie. not VAP specific) and have clearly shown that it can influence antibiotic use. The PRORATA trial showed a significant reduction in antibiotics at 28-days but a non-significant trend to increased mortality at 60 days (30% vs 26%)(Bouadma *et al.*, 2010). A recent pragmatic trial aimed to determine the utility of PCT to reduce antibiotics while also assessing mortality as a safety endpoint(de Jong *et al.*, 2016). In this trial of 4507 patients, there was a reduction in antibiotic use in those randomised to PCT-guided antibiotic management, as measured by DDD, with a median of 7.5 DDD (IQR 4.0-12.7) in the PCT group and 9.3 DDD (5.0-16.6) in the control group. In this trial there was in fact a reduction in mortality in the PCT group at 28-days (20% in PCT versus 25% in control group, p=0.0122). These two trials used a PCT threshold of 0.5ng/ml to rule out infection. In contrast to these trials, a trial using a lower threshold of 0.1ng/ml was unable to find a reduction in antibiotic use with PCT-guided antibiotic management compared to the control group(Shehabi *et al.*, 2014). More specifically investigating the impact in respiratory infections, a trial conducted in the emergency department setting, using PCT to guide antibiotic use in community-acquired LRTI found that serum PCT use was both safe and reduced the median duration of antibiotic use (5.7 days versus 8.7 days for PCT and control groups respectively)(Schuetz *et al.*, 2009). Furthermore in a trial of patients with VAP (using a clinical definition), the use of serum PCT resulted in more AFD at 28-days with 13 (IQR 2-21) and 9.5 (IQR 1.5-17) for the PCT and control groups respectively(Stolz *et al.*, 2009). As a further demonstration of the ability to PCT

to influence antibiotic use, in a trial of a PCT intervention to increase early antibiotic use in suspected infections, an algorithm that encouraged antibiotic use, did in fact increase antibiotic use in the PCT group but there were also worse outcomes, with the length of ICU stay increasing by 1 day, an increased rate of mechanical ventilation and worsening renal function in the PCT group(Jensen *et al.*, 2011).

Studies of newer diagnostic technologies, such as PCR have generally included patients with confirmed blood culture and through rapid identification of the pathogen, have aimed to rapidly optimise treatment. In a non-randomised study of patients with *S. aureus* bacteraemia, the use of PCR allowed for more rapid de-escalation of empirical antibiotics (1.7 days earlier) and also resulted in a reduction in hospital costs(Bauer *et al.*, 2010). In a randomised trial of patients with a positive blood culture the use of rapid multiplex PCR resulted in more rapid detection of microorganism (1.3 hours versus 22.3 hours for PCR and control groups respectively) and less time on broad-spectrum antibiotics (44 hours versus 56 hours for PCR and control groups respectively)(Banerjee *et al.*, 2015). There were no differences in mortality, length of stay or cost between the two groups. A recent randomised trial utilising PNA FISH diagnostics, including patients with confirmed blood culture, did not find a reduction in empirical antibiotic use in the absence of an antibiotic stewardship programme to drive the change(Cosgrove *et al.*, 2016). These studies are valuable in terms of showing that these technologies can more rapidly refine antibiotic prescribing but fail to address the issue around avoiding antibiotic prescribing in the absence of infection.

Considering the lack of difference between the two trial arms of this trial, it is worth considering the 15 patients in whom the biomarker was below the threshold and in whom the clinicians were advised to discontinue antibiotics. Considering the antibiotic use in these patients could give an indication into clinician compliance with the biomarker antibiotic-discontinuation recommendation. Conclusions drawn from this group of 15 patients must be considered with considerable caution. The median AFD at 7 days amongst these 15 patients was 2.5 (IQR 0.0-6.0) in comparison to a median AFD in the control group of 1.0 (IQR 0.0-3.0). Four of these patients had antibiotics for VAP and so the recommendation on antibiotics was not followed. For another 6 patients there was an indication other than VAP for which antibiotics were given. Antibiotics were not given in 5 patients (33%).

The suggestion from these 15 patients is that clinicians complied with the antibiotic-discontinuation recommendation in some circumstances but not all. In fact in the

description of antibiotic prescribing in the whole trial population (at baseline and at days 7, 14 and 28), VAP was an indication for antibiotics in 19-48% of recorded indications. Therefore it is likely that alternative sources of infection were a barrier to biomarker-recommendation compliance.

In the PRORATA trial, PCT thresholds were used for both starting and stopping antibiotics. Although there was significantly lower antibiotic use in the PCT group, clinician compliance with the recommendations was surprisingly low with the recommendation not followed in 72% of episodes(Bouadma *et al.*, 2010). This included 57% of episodes in which antibiotics were given despite recommendation to not give antibiotics and 14% of episodes in which antibiotics were stopped despite recommendations to give antibiotics. Overwhelmingly the trend was for clinicians to give antibiotics based on their own judgement of the likelihood of infection rather than based on the PCT level. Interpreting this is difficult because the diagnostic accuracy of PCT in meta-analyses has been far from perfect. The most recent meta-analysis of PCT for bacterial infections concluded a positive PCT had a PTP to rule in sepsis of 48% and a negative PTP to exclude sepsis of only 7%(Wacker *et al.*, 2013)(see Introduction, 1.10.2). Since PCT is not a gold standard for diagnosis of bacterial infections, not complying with the recommendation may be considered entirely reasonable.

The BAL biomarker test used in this trial has a diagnostic threshold to achieve a clear diagnostic purpose, the exclusion of VAP. At the threshold, the validation study suggested that VAP could be excluded with a PTP of 0% and a 95% CI of 0.0-9.2%. The hope was that this very low PTP would give clinicians confidence that a rule-out biomarker would really mean a rule-out of VAP. Research teams were asked to complete a questionnaire providing information as to why antibiotic-discontinuation recommendations were not followed. Only two of these were completed by the time of my data collection and for both of those patients an alternative source of infection was being treated. Interestingly, if we consider the antibiotic prescribing for the non-VAP patients, 45% were receiving antibiotics for VAP, although 11% had an alternative indication. This suggests that the semi-quantitative culture was not used to guide antibiotic use either.

The validation AFD data had demonstrated that this was a high antibiotic clinical environment and there was a concern that detection of a signal of reduced antibiotic use would be difficult. This was taken into consideration when designing the trial. In addition to the inclusion criteria used in the validation study, the criterion that the

patient must be considered suitable for early discontinuation of antibiotics was added. The exact description of this criterion was difficult to determine. Alternatives that were considered were to ‘exclude patients on any antibiotics’ or on ‘antibiotics for a non-VAP source of infection’. These were considered inappropriate because they would have excluded so many patients that accrual would have been too difficult. The description of the criterion that was used was not as clearly defined as the alternatives, and depended on clinicians’ interpretation and judgement as to whether they thought the biomarker test would be appropriate for the patient and whether they would consider discontinuing antibiotics if VAP was ruled out. Changing the inclusion criteria risked recruiting a different trial population to the validation study. In fact the most common reason for a screen failure was the patient not being suitable for early antibiotic discontinuation. These were patients who may well have been suitable for inclusion in the validation study. If the population were different to that in the validation study, then a natural concern would be that the biomarker would not perform with the same profile of sensitivity and specificity. In fact the patient demographics and VAP rate were similar to those in the validation study. Furthermore, in analysing the limited number of biomarker results, the ROC coordinates at thresholds used in the validation study gave similar sensitivities and specificities. The specificity of the combination of IL-1β/IL-8 at a threshold of -1.713 (close to the -1.7616 of the validation study), was 43.5% and this was reflected in the number of non-VAP patients correctly identified as ‘non-VAP'. We can therefore conclude that the biomarker performed as expected, and that any failure to detect a difference in the trial arms was not due to failure of the biomarker performance.

Deciding on the primary outcome measure for a trial is of paramount importance. It must be clinically relevant and the intervention should be expected to produce a signal in this measure. Mortality is a commonly used outcome for many trials in the ICU (and other clinical environments) and is clearly an important patient-centred outcome, particularly in a clinical setting where mortality is high. Many ICU RCTs fail to show a significant difference in the primary outcome measure and this is particularly true of trials with mortality endpoints, with just 10% having a positive result(Harhay *et al.*, 2014).

A mortality endpoint has a number of benefits in that preventing death in critically ill patients is an important clinical goal, it is a clear endpoint with no confusion over definition and if ICU- or hospital-mortality is used, completed follow up is easy to attain. Mortality endpoints are limited by the fact that arbitrary time intervals are

selected for measurement. Although ICU- and hospital-mortality are easy to measure, they are of limited use if patients die shortly after discharge from hospital. In fact there is an increased risk of death post-hospital discharge for ICU patients that is highest at 3 months and continues for 3 years(Brinkman *et al.*, 2013). Other arbitrary time points to measure mortality (eg. 14-, 28-, 60- or 90-day mortality) are similarly limited by making assumptions that the intervention will have its effect within this timescale.

Amongst a patient group with high baseline mortality, the influence of a particular complication on overall mortality can be hard to define. This is highly relevant in VAP where the attributable mortality has been reported to range from 1.5-9%(Bekaert *et al.*, 2011; Melsen *et al.*, 2011). With such low attributable mortality rates, detecting a mortality benefit in a VAP trial would require an impractically large sample size.

Measurement of antibiotic therapy in terms of antibiotic-days or antibiotic-free days can be considered a 'process outcome' and measures healthcare delivery(McGregor & Furuno, 2014). Process outcomes are intermediate outcomes that should be associated with other favourable outcomes, eg clinical outcomes. This raises interesting questions when using antibiotic therapy as an outcome, as it has been in previous trials(Fagon *et al.*, 2000; Chastre *et al.*, 2003). The association between antibiotic use and antibiotic resistance is established and reducing antibiotic selective pressure reduces antibiotic resistance(Geissler *et al.*, 2003; Goossens *et al.*, 2005). This represents a benefit to society but whether there is a benefit to an individual patient is not established. There are data from quasi-experimental studies that challenge perceptions that antibiotics are a benign treatment and suggest firstly that over-treatment with antibiotics could be harmful and secondly that a more restrictive approach to antibiotics could result in better clinical outcomes(Kett *et al.*, 2011; Hranjec *et al.*, 2012).

The primary outcome measure used was the change in the frequency distribution of AFD in the 7 days following BAL. There were a number of statistical considerations with respect to the analysis of AFD at 7 days (outlined in Methods, 2.13.2). Due to the high baseline use of antibiotics, detecting a change in antibiotic use in terms of location-shift or change in proportions, may not have been sensitive enough to detect subtle reductions in antibiotic use. Analysing a change in frequency distribution of AFD at 7 days was expected to be able to detect more subtle changes. Despite this, at this stage of the trial, no difference was detected.

As outlined, both the primary outcome measure and inclusion criteria were influenced by the high baseline antibiotic use and this gave the trial elements of an efficacy trial

and an effectiveness trial(Singal, Higgins and Waljee, 2014). Selecting patients in whom antibiotic discontinuation was felt to be appropriate (a highly selected group of patients) is in keeping with an efficacy trial. This population does not represent all patients receiving antibiotics for suspected VAP, rather a subgroup thought likely to benefit from the biomarker assay. The complexity of the timing of the trial processes of enrolment, randomisation and BAL meant that the process of VAP investigation for these patients might not reflect real-world practice. In fact, the control arm was termed 'routine use of antibiotics' because the term 'standard care' was felt inappropriate, since many ICUs do not use BAL and semi-quantitative culture as standard(Browne *et al.*, 2014). On the other hand, there were elements of the trial that were more representative of a 'pragmatic' or 'effectiveness' trial. The trial was conducted in a large number of general ICUs and the intervention had to be worked into the usual workflow of the ICU. Clinicians in the ICUs, rather than a centralised group of researchers, performed BALs. How the biomarker results were utilised in clinical practice was not dictated in the trial protocol. The decision to give antibiotics was entirely left to the clinical team. An element of blinding was included in the trial, a feature of an efficacy trial, but in fact beyond the first 6 hours, once the results were made known to the clinical team, the trial arm was unblinded. An argument could have been made not to perform the blinding element of the trial, and instead to take a more pragmatic approach of allowing clinicians the opportunity to know that the biomarker assay was going to be performed, so that it could be incorporated into their decision-making process.

The trial intervention should be considered a 'complex intervention' for a number of reasons(Craig *et al.*, 2008). There were a number of behavioural components to the intervention, including: identification of suspected VAP based on pre-determined criteria versus pure clinician judgement; decisions regarding suitability for early antibiotic discontinuation; clinician judgement to discontinue antibiotics on the basis of biomarker assay results in the context of other clinical information such as previous positive microbiology; clinician pre-test level of suspicion of VAP; acceptability of BAL; and confidence in the biomarker test. There were logistical complexities to the intervention including the time in which the biomarker result was returned to clinicians (which was often late in the evening or even in the night). It is possible that the clinical teams may not have got these results until the next day, or that they were received at a time when they were unlikely to be incorporated into main decision points of the working day (ie. the ward rounds). The intervention involved a large number of individuals including research, clinical and laboratory teams, and of course the patients

and their representatives. This required communication between all of these individuals, often in different sites, to ensure delivery of the intervention. The complexity and difficulty of delivering the intervention could have been a barrier to patient accrual. To help explain the results of trials of complex interventions, it is advised that these trials are carried out in conjunction with a 'process evaluation'(Craig *et al.*, 2008). A process evaluation is a qualitative method of understanding the process of implementing the intervention, to shed light on why an intervention failed or, if there is a positive result, to guide further implementation of the intervention. A process evaluation has been nested in this trial although it does not form part of this thesis and is in fact part of another student's PhD thesis. The findings of that process evaluation will be of great importance in understanding the findings of this trial, particularly if the completed trial fails to show a difference.

There were a number of safety issues considered in the design of this study, but overall the trial has proven to be safe. Firstly, performing BAL in critically ill patients is not without risk. Patients had saturations monitored and recorded during BAL and 2 hours post-BAL. There were pre-defined criteria for AE to be recorded in relation to the BAL. These criteria were the same as those used in the validation study. A total of 43 AE based on these criteria were reported. Only 4 patients were recorded as having an AE during BAL (a drop in SaO_2 of >5% or an absolute SaO_2 level below 90% for >5mins). The remaining patients met the AE criteria for increased oxygen requirements 2 hours after BAL (an increase in FiO_2 of ≥0.1 to maintain saturations within 3% of initial saturations). In retrospect these criteria may not have been fit for purpose in that it resulted in AE being recorded although they may not be considered important clinically. The main weakness is that this criterion is not one that is used clinically. It is not usual practice to ensure that saturations remain within 3% of starting saturations, with the FiO_2 increased accordingly to achieve this. Similarly the FiO_2 is not always titrated down after 2 hours to starting levels. This makes interpreting these values difficult but overall there was not clear evidence of harm from the BAL. Five SAEs were reported but these were felt not be related to the trial intervention and rather were a consequence of the critical illness the patients were suffering from. Furthermore a DMEC had oversight of the trial and did not raise any concerns over trial safety.

There was also a concern during design in relation to asking clinicians to discontinue antibiotics early. There was a risk that if early antibiotic discontinuation were inappropriate, patients would come to harm from untreated infections. Measurements

that were taken to detect a signal of harm were the SOFA score at days 3, 7 and 14, in addition to mortality and length of stay end points. There was no difference in any of these outcomes between the trial arms, although since antibiotic use was the same, these findings are not unexpected. However previous trials suggest that encouraging earlier discontinuation of antibiotics is not only safe, it could also be beneficial(Fagon *et al.*, 2000; Singh *et al.*, 2000; Hranjec *et al.*, 2012; de Jong *et al.*, 2016). An additional design concern in asking clinicians to discontinue antibiotics related to the risk of false negative biomarker results. The upper limit of the 95% CI for the PTP of the biomarker, based on the validation study, was 9.2%. Therefore some false negative patients were expected and 1 false negative occurred. Antibiotics were not discontinued in this patient, as antibiotics were given for an intra-abdominal source of infection and the patient had 0 AFD at 7 days.

If the main trial analysis finds no difference between the trial arms, consideration will have to be given to why the trial was ‘negative’. An important distinction will have to be made between whether the intervention is not effective, whether there was a failure of implementation (the process evaluation will help with this), or whether the trial design was inadequate and not the correct design to answer the questions. It is possible that all of these reasons are factors. In terms of effectiveness, this study has shown that even when inclusion criteria aimed to select out a group of patients likely to benefit from the intervention, the number of antibiotic days at 7 days is still high and antibiotics were being given for a wide range of indications. Clearly a biomarker for VAP can only influence antibiotics for VAP and on the basis of these results we should not think of a clinical moment in which the suspicion of VAP is considered in isolation, but rather of a moment in which a complex decision must be made around suspected infection, where VAP is one possibility. It may be that the biomarker was not acceptable to the clinicians or that they did not have confidence in the test. Antibiotic prescribing in relation to confirmation of VAP by semi-quantitative culture would suggest that clinicians did not base antibiotic use on microbiology evidence, although we cannot be sure exactly what microbiology evidence the clinician was using in his/her decision making. If BAL is not routine in the ICU and the clinician is accustomed to using positive ETA culture results in the decision-making process, then he/she may be less trusting of BAL biomarker results. In this study clinicians’ pre-test suspicion of VAP had no relation to the microbiological diagnosis VAP. One possibility is that if the clinician’s pre-test probability is high, then clinical investigations (microbiology or biomarker results) may have little effect on the decision to prescribe antibiotics.

Antibiotic use was high at baseline and at day 7 and so to influence this is a significant challenge. If we consider other trials that have reported significant differences in antibiotic use, the PRORATA trial reported 3 days difference at 30 days(Bouadma *et al.*, 2010). Similarly a trial of PCT-guided early discontinuations of antibiotics in VAP found a difference of 3 days at 28 days(Stolz *et al.*, 2009). Using a 7-day time period and using days as the smallest unit of measurement required our trial to detect a greater proportional change. Although using frequency distribution of AFD in the 7 days aimed to make for a more sensitive analysis, it may have been more appropriate to measure individual antibiotic doses or DDD rather than days, as more sensitive instruments to detect change.

One might ask whether this was the right trial to answer the question and how might the trial have been changed in retrospect? At the time of planning this trial, the design was approached in the framework of a simple intervention that would be tested by a RCT. In the final stages of the trial set up and during the recruitment period the complexity of the intervention became much more apparent. Greater consideration should have been given early in the planning stage to the complexity of the intervention and how the results would be interpreted. The process evaluation will go some way to answer some of these questions, but it is unlikely to give insights from a statistical and trial design standpoint. There would have been a strong case for carrying out a feasibility study and/or a pilot study. I used AFD data from the validation study to inform the power calculation for the RCT. A pilot study would have allowed for more detailed AFD data to be collected and would have given the opportunity to consider whether this was the most appropriate primary outcome measure. A feasibility study would have given an opportunity to test the inclusion and exclusion criteria. The high rate of antibiotics used for non-VAP indications would suggest that our criteria were not able to select the population we intended to include (ie. those most likely to benefit from the biomarker). A feasibility study could have also given an indication of accrual rate and how the intervention would fit in with clinical practice. It took 9 months to randomise 60 patients, so pilot studies and/or feasibility studies would have required significant increases in time and resources.

In summary, in this subgroup analysis, no benefit in terms of antibiotic use could be detected in a trial of a biomarker-based VAP exclusion. It must be stressed that this subgroup study is underpowered to detect a difference and the completed trial dataset must be awaited to make firm conclusions. This analysis does however highlight the

considerable challenge and complexity of conducting the trial, of the intervention itself and, most importantly, of the clinical environment that it the trial was conducted in. If the final results fail to show a difference in antibiotic use between the trial arms, it will highlight the challenge that remains in altering antibiotic use in ICU. Calls have been made for novel, rapid biomarker-based diagnostics for infections(Davies, 2011). Through the validation study, this was achieved, but whether the test will be accepted and implemented into clinical use remains a far more difficult question.

Chapter 5. Conclusions

The importance of addressing AMR, and in particular through novel diagnostics, has been stressed in a number of government reports(Davies, 2011; O'Neill, 2016). It has been estimated that by 2050, 10 million people could die each year from the consequences of AMR as resistance continues to rise, with an estimated global cost of 100 trillion US dollars(O'Neill, 2014). It is currently estimated that AMR costs 55 billion US dollars to the USA, although this is thought to be a conservative estimate(Smith and Coast, 2013).

Patients admitted to the ICU are at considerable risk of a poor outcome in terms of morbidity and mortality, as dictated by their critical illness. The role of clinician is to strive for their recovery and to protect them from (and treat the consequences of) hospital-acquired complications including infections. For all of these reasons, critically ill patients commonly receive a treatment that is a cornerstone of our armamentarium against disease, namely antibiotics. As AMR increases on a global scale, the clinician is faced with the challenge of treating presumed infections while avoiding unnecessary antibiotic use.

Clinicians are justified to a significant degree in the rapid administration of antibiotics in the face of suspected infection. Data show that delay in the administration of antibiotics in patients with sepsis is associated with worse outcome(Kumar *et al.*, 2006; Ferrer *et al.*, 2014; Garnacho-Montero *et al.*, 2015). In fact one study found that there was an associated increase in mortality if the recognition of sepsis and administration of antibiotics were delayed(Puskarich *et al.*, 2011), providing further incentive to give antibiotics at the moment of suspected infection. Furthermore, outcomes for severe community-acquired pneumonia are shown to be better with early administration of antibiotics(Gattarello *et al.*, 2014). The other side to this argument is that administering antibiotics at the point of suspicion may result in the overuse of antibiotics in patients who do not have a disease that would respond to antibiotics. Data on the use of antibiotics in the ambulatory setting shows that many patients receive broad-spectrum antibiotics for conditions that are unlikely to benefit from antibiotic treatment(Shapiro *et al.*, 2014). Furthermore global antibiotic consumption is also increasing, including increasing use of antibiotics that should be reserved for the most serious and resistant infections(Van Boeckel *et al.*, 2014). International and national guidelines for the management of sepsis recommend the early initiation of antibiotics for suspected sepsis

and in particular for severe sepsis(Dellinger *et al.*, 2013; NICE, 2016). The unintended consequence of this could be the over-prescription of antibiotics and pressures to meet targets have already been associated with the overuse of antibiotics(Nicks, Manthey and Fitch, 2009). The challenge of balancing the need to treat infections versus the need to conserve antibiotic use is primarily a problem of diagnostics. We do not have tests that can confidently rule-in or rule-out infections rapidly.

It has been outlined in this thesis that VAP is a common infection and reason for antibiotic use in ICUs. It also typifies the challenge of antibiotic stewardship. Many non-infectious and inflammatory conditions can mimic VAP in mechanically ventilated patients causing fever and changes on a CXR. The ability to rule out infection based on culture of pathogens is significantly limited by the time taken to culture organisms from respiratory samples. This work has aimed to address these limitations through the multi-centre validation of a rapid biomarker-based diagnostic test and then through testing its clinical effectiveness to reduced antibiotic use in a RCT.

It was outlined in the Introduction (section 1.10.3) that many biomarkers have been investigated for the diagnosis of VAP. Only one of these, to my knowledge, has been externally validated(Laupland, Church and Gregson, 2005). In this thesis I have described the results of a multi-centre validation study, which have shown that IL-1β concentrations in BAL fluid in particular, if low, can exclude VAP with confidence. This diagnostic performance is made more robust with the addition of IL-8. As a further 'validation' of these results, the biomarker performance appears to have been consistent in the RCT. This has answered the call for a rapid biomarker-based diagnostic test. It has a high sensitivity, which means it can exclude VAP confidently, and the expectation of the RCT was that it would allow antibiotics to be withheld.

This biomarker test is based on BAL and a microbiology-based definition of VAP. The acceptability of this strategy is uncertain in routine clinical practice. Trials to determine whether an invasive or clinical diagnostic strategy is superior have given conflicting results (see Introduction, 1.6.3)(Fagon *et al.*, 2000; Canadian Critical Care Trials Group et, 2006). Furthermore a meta-analysis did not support the use of an invasive strategy over a clinical strategy(Berton, Kalil and Teixeira, 2012). Updated guidelines for the management of VAP do not recommend invasive sampling methods(Kalil *et al.*, 2016) and the use of BAL is not routine practice in UK ICUs(Browne *et al.*, 2014). This biomarker assay moves away from the longstanding debate over invasive sampling versus clinical diagnosis, in that it is using invasive sampling to obtain biomarkers at the

site of pulmonary inflammation. However for it to be accepted clinically, BAL must be accepted as a strategy for VAP diagnosis.

Following the successful validation of the biomarker test, I carried out a RCT to determine whether a biomarker-based exclusion of VAP would result in reduced antibiotic use in patients with suspected VAP. No reduction in antibiotic use could be detected in the analysis included in this thesis, although it does not include the complete RCT dataset. This raises interesting questions as to why no difference was found. The possibility of using a primary outcome measure that was not sensitive enough to detect a change has been discussed (Results: RCT, 4.14). However it is arguable that a clinically relevant change should be easily detectable. The RCT results would suggest that clinicians did not follow the recommendation to discontinue antibiotics but they also did not seem to discontinue antibiotics in the face of negative BAL microbiology.

This intervention was tested alongside normal clinical practice but an alternative approach would have been to embed it in an antibiotic stewardship programme (ASP). Recommendations and guidelines for the implementation of ASP have been produced(CDC, 2014; NICE, 2015). ASP have multiple elements including educational tools; surveillance of antibiotic prescribing and AMR; organisational approaches such as creating the human resources to dedicate to ASP; and appointment of a single leader to take accountability of driving the ASP and to implement specific interventions(CDC, 2014). Specific interventions include persuasive interventions such as audit and feedback, education and reminders; and restrictive interventions such as compulsory order forms, expert approval and antibiotic restriction(Davey *et al.*, 2013). Embedding a novel diagnostic test in an ASP could have a significant impact on the size of the effect of the intervention. In a study using MALDI-TOF MS to rapidly identify Gram-negative blood cultures, the technology was able to identify the organism much quicker than conventional culture(Perez *et al.*, 2014). Importantly, the results of the test were sent to infectious diseases-trained pharmacists as part of an antibiotic stewardship team. Changes to antibiotic prescriptions were then implemented via this individual, resulting in a more rapid optimal antibiotic therapy with improved clinical outcomes. In contrast, a trial using PNA FISH technology without embedding the intervention in an ASP did not find an improvement in time to optimal antibiotic treatment(Cosgrove *et al.*, 2016).

The biomarker assay used in my studies was based on CBA and was a manual assay that took the technician approximately 5 hours to complete. Most laboratory tests are automated and it is unusual for hospital biomedical scientists to perform such a hands-

on assay. The application of this assay for clinical purposes in this trial was of course experimental. Considering the manual nature of the assay, it is not surprising that there were a number of assays in which the internal control did not fall into the required range and so had to be rejected. It was impractical for the technician to simply repeat the assay given the time taken to perform the assay. The clinical acceptability of a new technology is not just based on the clinician's view. It would be hard to view the application of this assay, in its current form, in the context of the workflow of a busy hospital laboratory. Our industry partner, BD, will have to consider the further development of the assay. As a minimum it would need to be automated, but maybe its greatest impact would be to develop it into a point of care test.

Understanding clinical behaviours and attitudes to antibiotic stewardship, infections in ICU, and management of VAP, would provide valuable insights into how antibiotic use can be reduced. Behavioural elements have been identified as factors affecting decisions to prescribe antibiotics, such as the time of day that the decision is made and 'decision fatigue'(Linder, 2014). Exploring the clinical environment and context of decisions in the ICU through an ethnographic study has the potential to identify areas where antibiotic stewardship could be improved. It could also inform the design of future trials by ensuring that interventions are implemented in clinical practice in such a way as to have the greatest influence on behaviours.

The consumption of antibiotics in the ICU is known to be amongst the highest in hospitals(Dumartin *et al.*, 2010). However detailed information on antibiotic consumption in the ICU is limited. Empiric antibiotic prescription accounts for a considerable proportion of antibiotics used(Candeloro *et al.*, 2012) and the duration of antibiotic courses varies (for example in bacteraemia)(Havey *et al.*, 2013; Daneman *et al.*, 2016). Antibiotic-free days at 28-days as an outcome measure in trials also varies(Bouadma *et al.*, 2010; Young *et al.*, 2013). Having a deeper understanding of prescribing practices in the ICU in a more general sense would allow areas for antibiotic stewardship to be identified.

As I have alluded to above, embedding our biomarker test in an ASP may allow for a greater impact of the assay. A RCT may not necessarily be the best method to evaluate the impact of the intervention. A before-and-after study design may be more suitable for an intervention that aimed to have more wide reaching changes. This could include an educational programme on VAP management and antibiotic stewardship; reinforcement of the biomarker assay and encouragement to follow the recommendation; and

education and reinforcement of the use of semi-quantitative culture. An alternative outcome measure for antibiotic use could be the total antibiotic consumption over the time periods studied, rather than the duration of antibiotics for individual patients.

In summary, this work has responded to calls to deliver rapid novel diagnostic tests for infections. To develop a novel biomarker assay for patients with suspected VAP represents a significant step forward for a group of the most vulnerable and critically unwell patients in the hospital. Despite the potential of our test to improve antibiotic stewardship in an environment in which it is urgently needed, we were unable to demonstrate a benefit for biomarker-based antibiotic use. However striving to meet the goal of better antibiotic stewardship remains a priority and this biomarker should play an important role in meeting that goal.

Appendix A: Publications

Hellyer, T. P., Anderson, N. H., Parker, J., Dark, P., Van Den Broeck, T., Singh, S., McMullan, R., Agus, A. M., Emerson, L. M., Blackwood, B., Gossain, S., Walsh, T. S., Perkins, G. D., Conway Morris, A., McAuley, D. F. and Simpson, A. J. (2016) 'Effectiveness of biomarker-based exclusion of ventilator-acquired pneumonia to reduce antibiotic use (VAPrapid-2): study protocol for a randomised controlled trial', *Trials*. Trials, 17(1), p. 318.

Hellyer, T. P., Ewan, V., Wilson, P. and Simpson, A. J. (2016) 'The Intensive Care Society recommended bundle of interventions for the prevention of ventilator-associated pneumonia', *Journal of the Intensive Care Society*, 17(3), pp. 238–243.

Hellyer, T. P., Morris, A. C., McAuley, D. F., Walsh, T. S., Anderson, N. H., Singh, S., Dark, P., Roy, A. I., Baudouin, S. V, Wright, S. E., Perkins, G. D., Kefala, K., Jeffels, M., McMullan, R., O'Kane, C. M., Spencer, C., Laha, S., Robin, N., Gossain, S., Gould, K., Ruchaud-Sparagano, M.-H., Scott, J., Browne, E. M., MacFarlane, J. G., Wiscombe, S., Widdrington, J. D., Dimmick, I., Laurenson, I. F., Nauwelaers, F. and Simpson, A. J. (2015) 'Diagnostic accuracy of pulmonary host inflammatory mediators in the exclusion of ventilator-acquired pneumonia.', *Thorax*, 70(1), pp. 41–7.

References

Abul, H., Abul, A., Khan, I., Mathew, T. C., Ayed, A. and Al-Athary, E. (2001) 'Levels of IL-8 and myeloperoxidase in the lungs of pneumonia patients.', *Molecular and cellular biochemistry*, 217(1–2), pp. 107–12. doi: 10.1023/A:1007264411006.

Aggarwal, a, Baker, C. S., Evans, T. W. and Haslam, P. L. (2000) 'G-CSF and IL-8 but not GM-CSF correlate with severity of pulmonary neutrophilia in acute respiratory distress syndrome.', *The European respiratory journal*, 15(5), pp. 895–901. doi: 10.1034/j.1399-3003.2000.15e14.x.

Agrawal, A., Zhuo, H., Brady, S., Levitt, J., Steingrub, J., Siegel, M. D., Soto, G., Peterson, M. W., Chesnutt, M. S., Matthay, M. A., Liu, K. D., Bernard, G., Artigas, A., Brigham, K., Carlet, J., Falke, K., Hudson, L., Lamy, M., Legall, J., Morris, A., Spragg, R., Brower, R., Lanken, P., MacIntyre, N., Matthay, M., Morris, A., Ancukiewicz, M., Schoenfeld, D., Thompson, B., Calfee, C., Budev, M., Matthay, M., Church, G., Brady, S., Uchida, T., Ishizaka, A., Lara, A., Ranes, J., deCamp, M., Arroliga, A., Calfee, C., Eisner, M., Ware, L., Thompson, B., Parsons, P., Wheeler, A., Korpak, A., Matthay, M., Calfee, C., Ware, L., Eisner, M., Parsons, P., Thompson, B., Wickersham, N., Matthay, M., Clark, J., Milberg, J., Steinberg, K., Hudson, L., Eisner, M., Parsons, P., Matthay, M., Ware, L., Greene, K., Fremont, R., Koyama, T., Calfee, C., Wu, W., Dossett, L., Bossert, F., Mitchell, D., Wickersham, N., Bernard, G., Matthay, M., May, A., Ware, L., Liu, K., Levitt, J., Zhuo, H., Kallet, R., Brady, S., Steingrub, J., Tidswell, M., Siegel, M., Soto, G., Peterson, M., Chesnutt, M., Phillips, C., Weinacker, A., Thompson, B., Eisner, M., Matthay, M., Matthay, M., Matthay, M., Brower, R., Carson, S., Douglas, I., Eisner, M., Hite, D., Holets, S., Kallet, R., Liu, K., Macintyre, N., Moss, M., Schoenfeld, D., Steingrub, J., Thompson, B., McClintock, D., Zhuo, H., Wickersham, N., Matthay, M., Ware, L., Ortiz, R., Cilley, R., Bartlett, R., Parsons, P., Eisner, M., Thompson, B., Matthay, M., Ancukiewicz, M., Bernard, G., Wheeler, A., Pittet, J., Wiener-Kronish, J., Serikov, V., Matthay, M., Prabhakaran, P., Ware, L., White, K., Cross, M., Matthay, M., Olman, M., Rice, T., Wheeler, A., Thompson, B., deBoisblanc, B., Steingrub, J., Rock, P., Rice, T., Wheeler, A., Thompson, B., Steingrub, J., Hite, R., Moss, M., Morris, A., Dong, N., Rock, P., Rubenfeld, G., Caldwell, E., Peabody, E., Weaver, J., Martin, D., Neff, M., Stern, E., Hudson, L., Seeley, E., McAuley, D., Eisner, M., Miletin, M., Matthay, M., Kallet, R., Ware, L., Conner, E., Matthay, M., Ware, L., Eisner, M., Thompson, B., Parsons, P., Matthay, M., Ware, L., Fang, X.,

Matthay, M., Ware, L., Koyama, T., Billheimer, D., Wu, W., Bernard, G., Thompson, B., Brower, R., Standiford, T., Martin, T., Matthay, M., Ware, L., Matthay, M., Ware, L., Matthay, M., Parsons, P., Thompson, B., Januzzi, J., Eisner, M., Wheeler, A., Bernard, G., Thompson, B., Schoenfeld, D., Wiedemann, H., deBoisblanc, B., Connors, A., Hite, R., Harabin, A., Wiedemann, H., Wheeler, A., Bernard, G., Thompson, B., Hayden, D., deBoisblanc, B., Connors, A., Hite, R. and Harabin, A. (2012) 'Pathogenetic and predictive value of biomarkers in patients with ALI and lower severity of illness: results from two clinical trials.', *American journal of physiology. Lung cellular and molecular physiology*. American Physiological Society, 303(8), pp. L634-9. doi: 10.1152/ajplung.00195.2012.

Albert, M., Williamson, D., Muscedere, J., Lauzier, F., Rotstein, C., Kanji, S., Jiang, X., Hall, M. and Heyland, D. (2014) 'Candida in the respiratory tract secretions of critically ill patients and the impact of antifungal treatment: a randomized placebo controlled pilot trial (CANTREAT study)', *Intensive Care Medicine*. Springer Berlin Heidelberg, 40(9), pp. 1313–1322. doi: 10.1007/s00134-014-3352-2.

Altman, D. G. and Bland, J. M. (1994) 'Diagnostic Tests 1: Sensitivity and Specificity', BMJ, 308, pp. 1552–1553. doi: 10.1258/phleb.2012.012J05.

Altman, D. G. and Bland, J. M. (1994) 'Diagnostic tests 2: Predictive values.', BMJ, p. 102.

American Thoracic Society (2005) 'Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia.', *American journal of respiratory and critical care medicine*, 171(4), pp. 388–416. doi: 10.1164/rccm.200405-644ST.

Anderson, D. J., Kirkland, K. B., Kaye, K. S., Thacker, P. A., Kanafani, Z. A., Auten, G. and Sexton, D. J. (2007) 'Underresourced hospital infection control and prevention programs: penny wise, pound foolish?', *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*. The University of Chicago Press, 28(7), pp. 767–73. doi: 10.1086/518518.

Arias, C. A. and Murray, B. E. (2012) 'The rise of the Enterococcus: beyond vancomycin resistance.', *Nature reviews. Microbiology*, 10(4), pp. 266–78. doi: 10.1038/nrmicro2761.

Armand-Lefèvre, L., Angebault, C., Barbier, F., Hamelet, E., Defrance, G., Ruppé, E., Bronchard, R., Lepeule, R., Lucet, J.-C., El Mniai, A., Wolff, M., Montravers, P.,

Plésiat, P. and Andremont, A. (2013) 'Emergence of imipenem-resistant gram-negative bacilli in intestinal flora of intensive care patients.', *Antimicrobial agents and chemotherapy*, 57(3), pp. 1488–95. doi: 10.1128/AAC.01823-12.

Assicot, M., Gendrel, D., Carsin, H., Raymond, J., Guilbaud, J. and Bohuon, C. (1993) 'High serum procalcitonin concentrations in patients with sepsis and infection.', *Lancet (London, England)*, 341(8844), pp. 515–8.

Avila, M., Ojcius, D. M. and Yilmaz, O. (2009) 'The oral microbiota: living with a permanent guest.', *DNA and cell biology*. Mary Ann Liebert, Inc., 28(8), pp. 405–11. doi: 10.1089/dna.2009.0874.

Bahrani-Mougeot, F. K., Paster, B. J., Coleman, S., Barbuto, S., Brennan, M. T., Noll, J., Kennedy, T., Fox, P. C. and Lockhart, P. B. (2007) 'Molecular analysis of oral and respiratory bacterial species associated with ventilator-associated pneumonia.', *Journal of clinical microbiology*, 45(5), pp. 1588–93. doi: 10.1128/JCM.01963-06.

Baker, A. M., Meredith, J. W. and Haponik, E. F. (1996) 'Pneumonia in intubated trauma patients. Microbiology and outcomes.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 153(1), pp. 343–9.

Baldwin, F., Gray, R. and Chequers, A. (2014) 'Survey of the use and practicalities of subglottic suction drainage in the UK', *Critical Care*, 18(Suppl 1), p. P332. doi: 10.1186/cc13522.

Banerjee, R., Teng, C. B., Cunningham, S. A., Ihde, S. M., Steckelberg, J. M., Moriarty, J. P., Shah, N. D., Mandrekar, J. N. and Patel, R. (2015) 'Randomized Trial of Rapid Multiplex Polymerase Chain Reaction-Based Blood Culture Identification and Susceptibility Testing', *Clinical Infectious Diseases*, 61(7), pp. 1071–1080. doi: 10.1093/cid/civ447.

Bartels, E. M. and Ribel-Madsen, S. (2013) 'Cytokine measurements and possible interference from heterophilic antibodies--problems and solutions experienced with rheumatoid factor.', *Methods (San Diego, Calif.)*, 61(1), pp. 18–22. doi: 10.1016/j.ymeth.2012.12.015.

Bauer, K. A., West, J. E., Balada- Llasat, J., Pancholi, P., Stevenson, K. B. and Goff, D. A. (2010) 'An Antimicrobial Stewardship Program's Impact with Rapid Polymerase Chain Reaction Methicillin- Resistant *Staphylococcus aureus* / *S. aureus* Blood Culture Test in Patients with *S. aureus* Bacteremia', *Clinical Infectious Diseases*, 51(9), pp. 1074–1080. doi: 10.1086/656623.

Beal, S. G., Ciurca, J., Smith, G., John, J., Lee, F., Doern, C. D. and Gander, R. M. (2013) 'Evaluation of the nanosphere verigene gram-positive blood culture assay with the VersaTREK blood culture system and assessment of possible impact on selected patients.', *Journal of clinical microbiology*. American Society for Microbiology (ASM), 51(12), pp. 3988–92. doi: 10.1128/JCM.01889-13.

Bekaert, M., Timsit, J.-F., Vansteelandt, S., Depuydt, P., Vésin, A., Garrouste-Orgeas, M., Decruyenaere, J., Clec'h, C., Azoulay, E. and Benoit, D. (2011) 'Attributable mortality of ventilator-associated pneumonia: a reappraisal using causal analysis.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 184(10), pp. 1133–9. doi: 10.1164/rccm.201105-0867OC.

Bercault, N. and Boulain, T. (2001) 'Mortality rate attributable to ventilator-associated nosocomial pneumonia in an adult intensive care unit: a prospective case-control study.', *Critical care medicine*, 29(12), pp. 2303–9.

Berenholtz, S. M., Pham, J. C., Thompson, D. A., Needham, D. M., Lubomski, L. H., Hyzy, R. C., Welsh, R., Cosgrove, S. E., Sexton, J. B., Colantuoni, E., Watson, S. R., Goeschel, C. A. and Pronovost, P. J. (2011) 'Collaborative cohort study of an intervention to reduce ventilator-associated pneumonia in the intensive care unit.', *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 32(4), pp. 305–14. doi: 10.1086/658938.

Bergmans, D. C., Bonten, M. J., Gaillard, C. A., Paling, J. C., van der Geest, S., van Tiel, F. H., Beysens, A. J., de Leeuw, P. W. and Stobberingh, E. E. (2001) 'Prevention of ventilator-associated pneumonia by oral decontamination: a prospective, randomized, double-blind, placebo-controlled study.', *American journal of respiratory and critical care medicine*, 164(3), pp. 382–8. doi: 10.1164/ajrccm.164.3.2005003.

Berry, A. M., Davidson, P. M., Masters, J., Rolls, K. and Ollerton, R. (2011) 'Effects of three approaches to standardized oral hygiene to reduce bacterial colonization and ventilator associated pneumonia in mechanically ventilated patients: a randomised control trial.', *International journal of nursing studies*, 48(6), pp. 681–8. doi: 10.1016/j.ijnurstu.2010.11.004.

Berton, D. C., Kalil, A. C. and Teixeira, P. J. (2012) 'Quantitative versus qualitative cultures of respiratory secretions for clinical outcomes in patients with ventilator-associated pneumonia', *Cochrane Database Syst Rev*. 2012/01/20, 1, p. CD006482. doi: 10.1002/14651858.CD006482.pub3.

Betrosian, A. P., Frantzeskaki, F., Xanthaki, A. and Douzinas, E. E. (2008) 'Efficacy and safety of high-dose ampicillin/sulbactam vs. colistin as monotherapy for the treatment of multidrug resistant Acinetobacter baumannii ventilator-associated pneumonia.', *The Journal of infection*, 56(6), pp. 432–6. doi: 10.1016/j.jinf.2008.04.002.

Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., Perez-Perez, G., Blaser, M. J. and Relman, D. A. (2006) 'Molecular analysis of the bacterial microbiota in the human stomach.', *Proceedings of the National Academy of Sciences of the United States of America*, 103(3), pp. 732–7. doi: 10.1073/pnas.0506655103.

Blackwood, B., Alderdice, F., Burns, K., Cardwell, C., Lavery, G. and O'Halloran, P. (2011) 'Use of weaning protocols for reducing duration of mechanical ventilation in critically ill adult patients: Cochrane systematic review and meta-analysis.', *BMJ (Clinical research ed.)*, 342(jan13_2), p. c7237. doi: 10.1136/bmj.c7237.

Blot, S., Koulenti, D., Dimopoulos, G., Martin, C., Komnos, A., Krueger, W. A., Spina, G., Armaganidis, A., Rello, J. and EU-VAP Study Investigators (2014) 'Prevalence, risk factors, and mortality for ventilator-associated pneumonia in middle-aged, old, and very old critically ill patients.', *Critical care medicine*, 42(3), pp. 601–9. doi: 10.1097/01.ccm.0000435665.07446.50.

Boeck, L., Graf, R., Eggimann, P., Pargger, H., Raptis, D. A., Smyrnios, N., Thakkar, N., Siegemund, M., Rakic, J., Tamm, M. and Stolz, D. (2011) 'Pancreatic stone protein: a marker of organ failure and outcome in ventilator-associated pneumonia.', *Chest*, 140(4), pp. 925–32. doi: 10.1378/chest.11-0018.

Van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A. and Laxminarayan, R. (2014) 'Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data', *The Lancet Infectious Diseases*. Elsevier Ltd, 14(8), pp. 742–750. doi: 10.1016/S1473-3099(14)70780-7.

Bonten, M. J., Gaillard, C. A., van der Geest, S., van Tiel, F. H., Beysens, A. J., Smeets, H. G. and Stobberingh, E. E. (1995) 'The role of intragastric acidity and stress ulcus prophylaxis on colonization and infection in mechanically ventilated ICU patients. A stratified, randomized, double-blind study of sucralfate versus antacids.', *American journal of respiratory and critical care medicine*, 152(6 Pt 1), pp. 1825–34. doi: 10.1164/ajrccm.152.6.8520743.

Boots, R. J., Howe, S., George, N., Harris, F. M. and Faoagali, J. (1997) 'Clinical utility of hygroscopic heat and moisture exchangers in intensive care patients.', *Critical care medicine*, 25(10), pp. 1707–12.

Boraschi, D. and Tagliabue, A. (2013) 'The interleukin-1 receptor family', *Seminars in Immunology*. Elsevier Ltd, 25(6), pp. 394–407. doi: 10.1016/j.smim.2013.10.023.

Borregaard, N. (2010) 'Neutrophils, from marrow to microbes.', *Immunity*, 33(5), pp. 657–70. doi: 10.1016/j.immuni.2010.11.011.

Bos, L. D. J., Martin-Loeches, I., Kastelijn, J. B., Gili, G., Espasa, M., Povoa, P., Kolk, A. H. J., Janssen, H.-G., Sterk, P. J., Artigas, A. and Schultz, M. J. (2014) 'The volatile metabolic fingerprint of ventilator-associated pneumonia', *Intensive Care Medicine*, 40(5), pp. 761–762. doi: 10.1007/s00134-014-3260-5.

Bos, L. D. J., Sterk, P. J. and Schultz, M. J. (2013) 'Volatile Metabolites of Pathogens: A Systematic Review', *PLoS Pathogens*, 9(5), pp. 1–8. doi: 10.1371/journal.ppat.1003311.

Bouadma, L., Luyt, C.-E., Tubach, F., Cracco, C., Alvarez, A., Schwebel, C., Schortgen, F., Lasocki, S., Veber, B., Dehoux, M., Bernard, M., Pasquet, B., Régnier, B., Brun-Buisson, C., Chastre, J. and Wolff, M. (2010) 'Use of procalcitonin to reduce patients' exposure to antibiotics in intensive care units (PRORATA trial): a multicentre randomised controlled trial.', *Lancet*, 375(9713), pp. 463–74. doi: 10.1016/S0140-6736(09)61879-1.

Boucher, H. W., Talbot, G. H., Benjamin, D. K., Bradley, J., Guidos, R. J., Jones, R. N., Murray, B. E., Bonomo, R. A. and Gilbert, D. (2013) '10 x '20 Progress--Development of New Drugs Active Against Gram-Negative Bacilli: An Update From the Infectious Diseases Society of America', *Clinical Infectious Diseases*, 56(12), pp. 1685–1694. doi: 10.1093/cid/cit152.

Boutten, A., Dehoux, M. S., Seta, N., Ostinelli, J., Venembre, P., Crestani, B., Dombret, M. C., Durand, G. and Aubier, M. (1996) 'Compartmentalized IL-8 and elastase release within the human lung in unilateral pneumonia.', *American journal of respiratory and critical care medicine*, 153(1), pp. 336–42.

Brinkman, S., de Jonge, E., Abu-Hanna, A., Arbous, M. S., de Lange, D. W. and de Keizer, N. F. (2013) 'Mortality after hospital discharge in ICU patients.', *Critical care medicine*, 41(5), pp. 1229–36. doi: 10.1097/CCM.0b013e31827ca4e1.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. and Zychlinsky, A. (2004) ‘Neutrophil extracellular traps kill bacteria.’, *Science*, 303(5663), pp. 1532–5. doi: 10.1126/science.1092385.

Brinkmann, V. and Zychlinsky, A. (2012) ‘Neutrophil extracellular traps: Is immunity the second function of chromatin?’, *The Journal of Cell Biology*. Rockefeller University Press, 198(5), pp. 773–783. doi: 10.1083/jcb.201203170.

Browne, E., Hellyer, T. P., Baudouin, S. V, Conway Morris, A., Linnett, V., McAuley, D. F., Perkins, G. D. and Simpson, A. J. (2014) ‘A national survey of the diagnosis and management of suspected ventilator-associated pneumonia.’, *BMJ open respiratory research*. British Thoracic Society, 1(1), p. e000066. doi: 10.1136/bmjresp-2014-000066.

Brun-Buisson, C., Sollet, J. P., Schweich, H., Brière, S. and Petit, C. (1998) ‘Treatment of ventilator-associated pneumonia with piperacillin-tazobactam/amikacin versus ceftazidime/amikacin: a multicenter, randomized controlled trial. VAP Study Group.’, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 26(2), pp. 346–54.

Burns, K. E. A., Adhikari, N. K. J., Keenan, S. P. and Meade, M. (2009) ‘Use of non-invasive ventilation to wean critically ill adults off invasive ventilation: meta-analysis and systematic review.’, *BMJ (Clinical research ed.)*, 338, p. b1574.

Burns, K. E. A., Adhikari, N. K. J. and Meade, M. O. (2006) ‘A meta-analysis of noninvasive weaning to facilitate liberation from mechanical ventilation.’, *Canadian journal of anaesthesia = Journal canadien d’anesthésie*, 53(3), pp. 305–15.

Canadian Critical Care Trials Group et (2006) ‘A randomized trial of diagnostic techniques for ventilator-associated pneumonia’, *N Engl J Med*. 2006/12/22, 355(25), pp. 2619–2630. doi: 10.1056/NEJMoa052904.

Candeloro, C. L., Kelly, L. M., Bohdanowicz, E., Martin, C. M. and Bombassaro, A. M. (2012) ‘Antimicrobial use in a critical care unit: a prospective observational study.’, *The International journal of pharmacy practice*, 20(3), pp. 164–71. doi: 10.1111/j.2042-7174.2011.00176.x.

Capellier, G., Mockly, H., Charpentier, C., Annane, D., Blasco, G., Desmettre, T., Roch, A., Faisy, C., Cousson, J., Limat, S., Mercier, M. and Papazian, L. (2012) ‘Early-onset ventilator-associated pneumonia in adults randomized clinical trial: comparison of 8 versus 15 days of antibiotic treatment.’, *PloS one*. Edited by B. Spellberg. Public

Library of Science, 7(8), p. e41290. doi: 10.1371/journal.pone.0041290.

Carsin, H., Assicot, M., Feger, F., Roy, O., Pennacino, I., Le Bever, H., Ainaud, P. and Bohuon, C. (1997) 'Evolution and significance of circulating procalcitonin levels compared with IL-6, TNF alpha and endotoxin levels early after thermal injury.', *Burns : journal of the International Society for Burn Injuries*, 23(3), pp. 218–24.

CDC (2014) 'Core Elements of Hospital Antibiotic Stewardship Programs', *Centers for Disease Control and Prevention*, pp. 1–25.

Center for Disease Control (2004) 'National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004', *American Journal of Infection Control*, 32(8), pp. 470–485. doi: 10.1016/j.ajic.2004.10.001.

Centers for Disease Control and Prevention (2013) *Antibiotic resistance threats in the USA, 2013*.

Chai, W., Zhang, J., Duan, Y., Pan, D., Liu, W., Li, Y., Yan, X. and Chen, B. (2014) 'Pseudomonas pyocyanin stimulates IL-8 expression through MAPK and NF-κB pathways in differentiated U937 cells.', *BMC microbiology*. BioMed Central, 14, p. 26. doi: 10.1186/1471-2180-14-26.

Chan, E. Y., Ruest, A., Meade, M. O. and Cook, D. J. (2007) 'Oral decontamination for prevention of pneumonia in mechanically ventilated adults: systematic review and meta-analysis.', *BMJ (Clinical research ed.)*, 334(7599), p. 889.

Chastre, J. and Fagon, J. Y. (2002) 'Ventilator-associated pneumonia', *Am J Respir Crit Care Med*. 2002/04/06, 165(7), pp. 867–903.

Chastre, J., Fagon, J. Y., Bornet-Lecso, M., Calvat, S., Dombret, M. C., al Khani, R., Basset, F. and Gibert, C. (1995) 'Evaluation of bronchoscopic techniques for the diagnosis of nosocomial pneumonia.', *American journal of respiratory and critical care medicine*, 152(1), pp. 231–40.

Chastre, J., Viau, F., Brun, P., Pierre, J., Dauge, M. C., Bouchama, A., Akesbi, A. and Gibert, C. (1984) 'Prospective evaluation of the protected specimen brush for the diagnosis of pulmonary infections in ventilated patients.', *The American review of respiratory disease*, 130(5), pp. 924–9.

Chastre, J., Wolff, M., Fagon, J.-Y., Chevret, S., Thomas, F., Wermert, D., Clementi, E., Gonzalez, J., Jusserand, D., Asfar, P., Perrin, D., Fieux, F. and Aubas, S. (2003)

‘Comparison of 8 vs 15 days of antibiotic therapy for ventilator-associated pneumonia in adults: a randomized trial.’, *JAMA : the journal of the American Medical Association*, 290(19), pp. 2588–98. doi: 10.1001/jama.290.19.2588.

Chastre, J., Wunderink, R., Prokocimer, P., Lee, M., Kaniga, K. and Friedland, I. (2008) ‘Efficacy and safety of intravenous infusion of doripenem versus imipenem in ventilator-associated pneumonia: a multicenter, randomized study.’, *Critical care medicine*, 36(4), pp. 1089–96. doi: 10.1097/CCM.0b013e3181691b99.

Chiu, S. W., Wang, J. H., Chang, K. H., Chang, T. H., Wang, C. M., Chang, C. L., Tang, C. T., Chen, C. F., Shih, C. H., Kuo, H. W., Wang, L. C., Chen, H., Hsieh, C. C., Chang, M. F., Liu, Y. W., Chen, T. J., Yang, C. H., Chiueh, H., Shyu, J. M. and Tang, K. T. (2014) ‘A fully integrated nose-on-a-chip for rapid diagnosis of ventilator-associated pneumonia’, *IEEE Transactions on Biomedical Circuits and Systems*, 8(6), pp. 765–778. doi: 10.1109/TBCAS.2014.2377754.

Chlebicki, M. P. and Safdar, N. (2007) ‘Topical chlorhexidine for prevention of ventilator-associated pneumonia: a meta-analysis.’, *Critical care medicine*, 35(2), pp. 595–602. doi: 10.1097/01.CCM.0000253395.70708.AC.

Chung, D. R., Song, J.-H., Kim, S. H., Thamlikitkul, V., Huang, S.-G., Wang, H., So, T. M.-K., Yasin, R. M. D., Hsueh, P.-R., Carlos, C. C., Hsu, L. Y., Buntaran, L., Lalitha, M. K., Kim, M. J., Choi, J. Y., Kim, S. Il, Ko, K. S., Kang, C.-I. and Peck, K. R. (2011) ‘High prevalence of multidrug-resistant nonfermenters in hospital-acquired pneumonia in Asia.’, *American journal of respiratory and critical care medicine*. American Thoracic Society, 184(12), pp. 1409–17. doi: 10.1164/rccm.201102-0349OC.

Cobb, J. P., Moore, E. E., Hayden, D. L., Minei, J. P., Cuschieri, J., Yang, J., Li, Q., Lin, N., Brownstein, B. H., Hennessy, L., Mason, P. H., Schierding, W. S., Dixon, D. J., Tompkins, R. G., Warren, H. S., Schoenfeld, D. A. and Maier, R. V (2009) ‘Validation of the riboleukogram to detect ventilator-associated pneumonia after severe injury.’, *Annals of surgery*, 250(4), pp. 531–9. doi: 10.1097/SLA.0b013e3181b8fbd5.

Conway Morris, A., Anderson, N., Brittan, M., Wilkinson, T. S., McAuley, D. F., Antonelli, J., McCulloch, C., Barr, L. C., Dhaliwal, K., Jones, R. O., Haslett, C., Hay, A. W., Swann, D. G., Laurenson, I. F., Davidson, D. J., Rossi, A. G., Walsh, T. S. and Simpson, A. J. (2013) ‘Combined dysfunctions of immune cells predict nosocomial infection in critically ill patients.’, *British journal of anaesthesia*, 111(5), pp. 778–87. doi: 10.1093/bja/aet205.

Conway Morris, A., Hay, a W., Swann, D. G., Everingham, K., McCulloch, C., McNulty, J., Brooks, O., Laurenson, I. F., Cook, B. and Walsh, T. S. (2011) 'Reducing ventilator-associated pneumonia in intensive care: Impact of implementing a care bundle.', *Critical care medicine*, 39(10), pp. 2218–2224. doi: 10.1097/CCM.0b013e3182227d52.

Conway Morris, A., Kefala, K., Simpson, A. J., Wilkinson, T. S., Everingham, K., Kerslake, D., Raby, S., Laurenson, I. F., Swann, D. G. and Walsh, T. S. (2009) 'Evaluation of the effect of diagnostic methodology on the reported incidence of ventilator-associated pneumonia', *Thorax*. 2009/02/14, 64(6), pp. 516–522. doi: 10.1136/thx.2008.110239.

Conway Morris, A., Kefala, K., Wilkinson, T. S., Moncayo-Nieto, O. L., Dhaliwal, K., Farrell, L., Walsh, T. S., Mackenzie, S. J., Swann, D. G., Andrews, P. J., Anderson, N., Govan, J. R., Laurenson, I. F., Reid, H., Davidson, D. J., Haslett, C., Sallenave, J. M. and Simpson, A. J. (2010) 'Diagnostic importance of pulmonary interleukin-1beta and interleukin-8 in ventilator-associated pneumonia', *Thorax*. 2009/10/15, 65(3), pp. 201–207. doi: 10.1136/thx.2009.122291.

Cook, A., Norwood, S. and Berne, J. (2010) 'Ventilator-associated pneumonia is more common and of less consequence in trauma patients compared with other critically ill patients.', *The Journal of trauma*, 69(5), pp. 1083–91. doi: 10.1097/TA.0b013e3181f9fb51.

Cook, D., Guyatt, G., Marshall, J., Leasa, D., Fuller, H., Hall, R., Peters, S., Rutledge, F., Griffith, L., McLellan, A., Wood, G. and Kirby, A. (1998) 'A comparison of sucralfate and ranitidine for the prevention of upper gastrointestinal bleeding in patients requiring mechanical ventilation. Canadian Critical Care Trials Group.', *The New England journal of medicine*, 338(12), pp. 791–7. doi: 10.1056/NEJM199803193381203.

Cook, D. J., Fuller, H. D., Guyatt, G. H., Marshall, J. C., Leasa, D., Hall, R., Winton, T. L., Rutledge, F., Todd, T. J. and Roy, P. (1994) 'Risk factors for gastrointestinal bleeding in critically ill patients. Canadian Critical Care Trials Group.', *The New England journal of medicine*, 330(6), pp. 377–81. doi: 10.1056/NEJM199402103300601.

Cook, D. J., Reeve, B. K., Guyatt, G. H., Heyland, D. K., Griffith, L. E., Buckingham, L. and Tryba, M. (1996) 'Stress ulcer prophylaxis in critically ill patients. Resolving

discordant meta-analyses.', *JAMA : the journal of the American Medical Association*, 275(4), pp. 308–14.

Cook, D. J., Walter, S. D., Cook, R. J., Griffith, L. E., Guyatt, G. H., Leasa, D., Jaeschke, R. Z. and Brun-Buisson, C. (1998) 'Incidence of and risk factors for ventilator-associated pneumonia in critically ill patients.', *Annals of internal medicine*, 129(6), pp. 433–40.

Cook, P. P., Catrou, P., Gooch, M. and Holbert, D. (2006) 'Effect of reduction in ciprofloxacin use on prevalence of meticillin-resistant Staphylococcus aureus rates within individual units of a tertiary care hospital.', *The Journal of hospital infection*, 64(4), pp. 348–51. doi: 10.1016/j.jhin.2006.06.033.

Corley, D. E., Kirtland, S. H., Winterbauer, R. H., Hammar, S. P., Dail, D. H., Bauermeister, D. E. and Bolen, J. W. (1997) 'Reproducibility of the histologic diagnosis of pneumonia among a panel of four pathologists: analysis of a gold standard.', *Chest*, 112(2), pp. 458–65.

Cosgrove, S. E., Li, D. X., Tamma, P. D., Avdic, E., Hadhazy, E., Wakefield, T., Gherna, M. and Carroll, K. C. (2016) 'Use of PNA FISH for blood cultures growing Gram-positive cocci in chains without a concomitant antibiotic stewardship intervention does not improve time to appropriate antibiotic therapy.', *Diagnostic microbiology and infectious disease*, 86(1), pp. 86–92. doi: 10.1016/j.diagmicrobio.2016.06.016.

Craig, A., Mai, J., Cai, S. and Jeyaseelan, S. (2009) 'Neutrophil recruitment to the lungs during bacterial pneumonia.', *Infection and immunity*, 77(2), pp. 568–75. doi: 10.1128/IAI.00832-08.

Craig, P., Dieppe, P., Macintyre, S., Michie, S., Nazareth, I. and Petticrew, M. (2008) 'Developing and evaluating complex interventions: the new Medical Research Council guidance', *Bmj Clinical Research Ed.*, 337(September), p. a1655. doi: 10.1136/bmj.a1655.

Craven, D. E., Kunches, L. M., Kilinsky, V., Lichtenberg, D. A., Make, B. J. and McCabe, W. R. (1986) 'Risk factors for pneumonia and fatality in patients receiving continuous mechanical ventilation.', *The American review of respiratory disease*, 133(5), pp. 792–6.

Croce, M. A., Brasel, K. J., Coimbra, R., Adams, C. A., Miller, P. R., Pasquale, M. D., McDonald, C. S., Vuthipadadon, S., Fabian, T. C. and Tolley, E. A. (2013) 'National Trauma Institute prospective evaluation of the ventilator bundle in trauma patients:

Does it really work?', *The journal of trauma and acute care surgery*, 74(2), pp. 354–362. doi: 10.1097/TA.0b013e31827a0c65.

Cuthbertson, B. H., Campbell, M. K., Maclennan, G., Duncan, E. M., Marshall, A. P., Wells, E. C., Prior, M. E., Todd, L., Rose, L., Seppelt, I. M., Bellingan, G. and Francis, J. J. (2013) 'Clinical stakeholders' opinions on the use of selective decontamination of the digestive tract in critically ill patients in intensive care units: an international Delphi study.', *Critical care (London, England)*, 17(6), p. R266. doi: 10.1186/cc13096.

D'Amico, R., Pifferi, S., Torri, V., Brazzi, L., Parmelli, E. and Liberati, A. (2009) 'Antibiotic prophylaxis to reduce respiratory tract infections and mortality in adults receiving intensive care.', *The Cochrane database of systematic reviews*, (4), p. CD000022. doi: 10.1002/14651858.CD000022.pub3.

Dalboni, T. M., Abe, A. E., de Oliveira, C. E., Lara, V. S., Campanelli, A. P., Gasparoto, C. T. and Gasparoto, T. H. (2013) *Activation profile of CXCL8-stimulated neutrophils and aging*, *Cytokine*. doi: 10.1016/j.cyto.2013.01.016.

Daley, R. J., Rebuck, J. A., Welage, L. S. and Rogers, F. B. (2004) 'Prevention of stress ulceration: current trends in critical care.', *Critical care medicine*, 32(10), pp. 2008–13.

Damas, P., Garweg, C., Monchi, M., Nys, M., Canivet, J.-L., Ledoux, D. and Preiser, J.-C. (2006) 'Combination therapy versus monotherapy: a randomised pilot study on the evolution of inflammatory parameters after ventilator associated pneumonia [ISRCTN31976779].', *Critical care (London, England)*, 10(2), p. R52. doi: 10.1186/cc4879.

Dandona, P., Nix, D., Wilson, M. F., Aljada, A., Love, J., Assicot, M. and Bohuon, C. (1994) 'Procalcitonin increase after endotoxin injection in normal subjects.', *The Journal of clinical endocrinology and metabolism*, 79(6), pp. 1605–8. doi: 10.1210/jcem.79.6.7989463.

Daneman, N., Rishu, A. H., Xiong, W., Bagshaw, S. M., Dodek, P., Hall, R., Kumar, A., Lamontagne, F., Lauzier, F., Marshall, J., Martin, C. M., McIntyre, L., Muscedere, J., Reynolds, S., Stelfox, H. T., Cook, D. J., Fowler, R. A. and Canadian Critical Care Trials Group (2016) 'Duration of Antimicrobial Treatment for Bacteremia in Canadian Critically Ill Patients.', *Critical care medicine*, 44(2), pp. 256–64. doi: 10.1097/CCM.0000000000001393.

Daneman, N., Sarwar, S., Fowler, R. A. and Cuthbertson, B. H. (2013) 'Effect of selective decontamination on antimicrobial resistance in intensive care units: a

systematic review and meta-analysis.', *The Lancet infectious diseases*, 13(4), pp. 328–41. doi: 10.1016/S1473-3099(12)70322-5.

Davey, P., Brown, E., Charani, E., Fenelon, L., Gould, I. M., Holmes, A., Ramsay, C. R., Wiffen, P. J. and Wilcox, M. (2013) 'Interventions to improve antibiotic prescribing practices for hospital inpatients.', *The Cochrane database of systematic reviews*, 4(4), p. CD003543. doi: 10.1002/14651858.CD003543.pub3.

Davies, J. and Davies, D. (2010) 'Origins and evolution of antibiotic resistance.', *Microbiology and molecular biology reviews : MMBR*, 74(3), pp. 417–33. doi: 10.1128/MMBR.00016-10.

Davies, J. and Ryan, K. S. (2012) 'Introducing the parvome: bioactive compounds in the microbial world.', *ACS chemical biology*, 7(2), pp. 252–9. doi: 10.1021/cb200337h.

Davies, S. (2011) 'Annual Report of the Chief Medical Officer - Infections and the rise of antimicrobial resistance'.

Delisle, M. S., Williamson, D. R., Perreault, M. M., Albert, M., Jiang, X. and Heyland, D. K. (2008) 'The clinical significance of Candida colonization of respiratory tract secretions in critically ill patients', *Journal of Critical Care*, 23(1), pp. 11–17. doi: 10.1016/j.jcrc.2008.01.005.

Dellinger, R. P., Levy, M. M., Rhodes, A., Annane, D., Gerlach, H., Opal, S. M., Sevransky, J. E., Sprung, C. L., Douglas, I. S., Jaeschke, R., Osborn, T. M., Nunnally, M. E., Townsend, S. R., Reinhart, K., Kleinpell, R. M., Angus, D. C., Deutschman, C. S., MacHado, F. R., Rubenfeld, G. D., Webb, S., Beale, R. J., Vincent, J. L. and Moreno, R. (2013) 'Surviving sepsis campaign: International guidelines for management of severe sepsis and septic shock, 2012', *Intensive Care Medicine*, 39(2), pp. 165–228. doi: 10.1007/s00134-012-2769-8.

Dennesen, P. J., van der Ven, A. J., Kessels, A. G., Ramsay, G. and Bonten, M. J. (2001) 'Resolution of infectious parameters after antimicrobial therapy in patients with ventilator-associated pneumonia.', *American journal of respiratory and critical care medicine*, 163(6), pp. 1371–5. doi: 10.1164/ajrccm.163.6.2007020.

Depuydt, P. O., Vandijck, D. M., Bekaert, M. A., Decruyenaere, J. M., Blot, S. I., Vogelaers, D. P. and Benoit, D. D. (2008) 'Determinants and impact of multidrug antibiotic resistance in pathogens causing ventilator-associated-pneumonia.', *Critical care (London, England)*, 12(6), p. R142. doi: 10.1186/cc7119.

DeRiso, A. J., Ladowski, J. S., Dillon, T. A., Justice, J. W. and Peterson, A. C. (1996) ‘Chlorhexidine gluconate 0.12% oral rinse reduces the incidence of total nosocomial respiratory infection and nonprophylactic systemic antibiotic use in patients undergoing heart surgery.’, *Chest*. American College of Chest Physicians, 109(6), pp. 1556–61. doi: 10.1378/chest.109.6.1556.

Determann, R. M., Millo, J. L., Gibot, S., Korevaar, J. C., Vroom, M. B., van der Poll, T., Garrard, C. S. and Schultz, M. J. (2005) ‘Serial changes in soluble triggering receptor expressed on myeloid cells in the lung during development of ventilator-associated pneumonia.’, *Intensive care medicine*, 31(11), pp. 1495–500. doi: 10.1007/s00134-005-2818-7.

Dezfulian, C., Shojania, K., Collard, H. R., Kim, H. M., Matthay, M. A. and Saint, S. (2005) ‘Subglottic secretion drainage for preventing ventilator-associated pneumonia: a meta-analysis.’, *The American journal of medicine*, 118(1), pp. 11–8. doi: 10.1016/j.amjmed.2004.07.051.

Dickson, R. P., Erb-Downward, J. R., Prescott, H. C., Martinez, F. J., Curtis, J. L., Lama, V. N. and Huffnagle, G. B. (2014) ‘Analysis of Culture-Dependent versus Culture-Independent Techniques for Identification of Bacteria in Clinically Obtained Bronchoalveolar Lavage Fluid’, *Journal of Clinical Microbiology*. American Society for Microbiology, 52(10), pp. 3605–3613. doi: 10.1128/JCM.01028-14.

DiMango, E., Zar, H. J., Bryan, R. and Prince, A. (1995) ‘Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8.’, *Journal of Clinical Investigation*. American Society for Clinical Investigation, 96(5), pp. 2204–2210. doi: 10.1172/JCI118275.

Dinarello, C. (2011) ‘Interleukin-1 in the pathogenesis and treatment of inflammatory diseases’, *Blood*, 117(14), pp. 3720–3733. doi: 10.1182/blood-2010-07-273417.The.

Dinarello, C. A. (1996) ‘Biologic basis for interleukin-1 in disease.’, *Blood*, 87(6), pp. 2095–147.

Dinarello, C. A., Simon, A. and van der Meer, J. W. M. (2012) ‘Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases.’, *Nature reviews. Drug discovery*. Nature Publishing Group, 11(8), pp. 633–52. doi: 10.1038/nrd3800.

Dodemont, M., De Mendonca, R., Nonhoff, C., Roisin, S. and Denis, O. (2014) ‘Performance of the Verigene Gram-Negative Blood Culture Assay for Rapid Detection of Bacteria and Resistance Determinants’, *Journal of Clinical Microbiology*. American

Society for Microbiology, 52(8), pp. 3085–3087. doi: 10.1128/JCM.01099-14.

Drago, L., De Vecchi, E., Nicola, L., Tocalli, L. and Gismondo, M. R. (2005) 'In vitro selection of resistance in Pseudomonas aeruginosa and Acinetobacter spp. by levofloxacin and ciprofloxacin alone and in combination with beta-lactams and amikacin.', *The Journal of antimicrobial chemotherapy*, 56(2), pp. 353–9. doi: 10.1093/jac/dki204.

Drakulovic, M. B., Torres, A., Bauer, T. T., Nicolas, J. M., Nogué, S. and Ferrer, M. (1999) 'Supine body position as a risk factor for nosocomial pneumonia in mechanically ventilated patients: a randomised trial.', *Lancet*, 354(9193), pp. 1851–8. doi: 10.1016/S0140-6736(98)12251-1.

Dreyfuss, D., Djedaïni, K., Gros, I., Mier, L., Le Bourdellés, G., Cohen, Y., Estagnasié, P., Coste, F. and Boussougant, Y. (1995) 'Mechanical ventilation with heated humidifiers or heat and moisture exchangers: effects on patient colonization and incidence of nosocomial pneumonia.', *American journal of respiratory and critical care medicine*, 151(4), pp. 986–92. doi: 10.1164/ajrccm/151.4.986.

Dudeck, M., Horan, T. C., Peterson, K. D., Allen-Bridson, K., Morrell, G., Pollock, D. a and Edwards, J. R. (2011) 'National Healthcare Safety Network (NHSN) Report, data summary for 2010, device-associated module.', *American journal of infection control*, 39(10), pp. 798–816. doi: 10.1016/j.ajic.2011.10.001.

Duflo, F., Debon, R., Monneret, G., Bienvenu, J., Chassard, D. and Allaouchiche, B. (2002) 'Alveolar and serum procalcitonin: diagnostic and prognostic value in ventilator-associated pneumonia', *Anesthesiology*. 2001/12/26, 96(1), pp. 74–79.

Dumartin, C., L'Hériteau, F., Péfau, M., Bertrand, X., Jarno, P., Boussat, S., Angora, P., Lacavé, L., Saby, K., Savey, A., Nguyen, F., Carbonne, A. and Rogues, A.-M. (2010) 'Antibiotic use in 530 French hospitals: results from a surveillance network at hospital and ward levels in 2007.', *The Journal of antimicrobial chemotherapy*, 65(9), pp. 2028–36. doi: 10.1093/jac/dkq228.

Dziedzic, T., Slowik, A. and Szczudlik, A. (2004) 'Nosocomial infections and immunity: lesson from brain-injured patients.', *Critical care (London, England)*, 8(4), pp. 266–70. doi: 10.1186/cc2828.

Edwards, J. R., Peterson, K. D., Andrus, M. L., Dudeck, M. a, Pollock, D. a and Horan, T. C. (2008) 'National Healthcare Safety Network (NHSN) Report, data summary for 2006 through 2007, issued November 2008.', *American journal of infection control*.

Elsevier Ltd, 36(9), pp. 609–26. doi: 10.1016/j.ajic.2008.08.001.

Edwards, J. R., Peterson, K. D., Andrus, M. L., Tolson, J. S., Goulding, J. S., Dudeck, M. a, Mincey, R. B., Pollock, D. a and Horan, T. C. (2007) 'National Healthcare Safety Network (NHSN) Report, data summary for 2006, issued June 2007.', *American journal of infection control*, 35(5), pp. 290–301. doi: 10.1016/j.ajic.2007.04.001.

Edwards, J. R., Peterson, K. D., Mu, Y., Banerjee, S., Allen-Bridson, K., Morrell, G., Dudeck, M. a, Pollock, D. a and Horan, T. C. (2009) 'National Healthcare Safety Network (NHSN) report: data summary for 2006 through 2008, issued December 2009.', *American journal of infection control*. Association for Professionals in Infection Control and Epidemiology, Inc., 37(10), pp. 783–805. doi: 10.1016/j.ajic.2009.10.001.

el-Ebiary, M., Torres, A., González, J., Martos, A., Puig de la Bellacasa, J., Ferrer, M. and Rodriguez-Roisin, R. (1995) 'Use of elastin fibre detection in the diagnosis of ventilator associated pneumonia.', *Thorax*, 50(1), pp. 14–7.

Elshal, M. F. and McCoy, J. P. (2006) 'Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA.', *Methods (San Diego, Calif.)*, 38(4), pp. 317–23. doi: 10.1016/j.ymeth.2005.11.010.

Estes, R. J. and Meduri, G. U. (1995) 'The pathogenesis of ventilator-associated pneumonia: I. Mechanisms of bacterial transcolonization and airway inoculation', *Intensive Care Medicine*, 21(4), pp. 365–383. doi: 10.1007/BF01705418.

European Centre for Disease Control (2010) 'EUROPEAN SURVEILLANCE OF HEALTHCARE-ASSOCIATED INFECTIONS IN INTENSIVE CARE UNITS', (December).

European Centre for Disease Prevention and Control (2012a) *Antimicrobial resistance surveillance in Europe: Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2012*.

European Centre for Disease Prevention and Control (2012b) *ECDC surveillance report: Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals*.

European Centre for Disease Prevention and Control (2014) *Antimicrobial resistance surveillance in Europe 2014*. doi: 10.2900/93403.

European Centre for Disease Prevention and Control (no date) *Antimicrobial consumption interactive database (ESAC-Net)*. Available at:

http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/esac-net-database/Pages/database.aspx (Accessed: 22 December 2014).

Fabregas, N., Ewig, S., Torres, A., El-Ebiary, M., Ramirez, J., de La Bellacasa, J. P., Bauer, T. and Cabello, H. (1999) 'Clinical diagnosis of ventilator associated pneumonia revisited: comparative validation using immediate post-mortem lung biopsies', *Thorax*. 1999/09/24, 54(10), pp. 867–873.

Fabregas, N., Torres, A., El-Ebiary, M., Ramírez, J., Hernández, C., González, J., de la Bellacasa, J. P., de Anta, J. and Rodriguez-Roisin, R. (1996) 'Histopathologic and microbiologic aspects of ventilator-associated pneumonia.', *Anesthesiology*, 84(4), pp. 760–71.

Fagon, J.-Y., Chastre, J., Domart, Y., Trouillet, J.-L. and Gibert, C. (1996) 'Mortality Due to Ventilator-Associated Pneumonia or Colonization with Pseudomonas or Acinetobacter Species: Assessment by Quantitative Culture of Samples Obtained by a Protected Specimen Brush', *Clinical Infectious Diseases*. Oxford University Press, 23(3), pp. 538–542. doi: 10.1093/clinids/23.3.538.

Fagon, J.-Y., Chastre, J., Hance, A. J., Guiguet, M., Trouillet, J.-L., Domart, Y., Pierre, J. and Gibert, C. (1988) 'Detection of Nosocomial Lung Infection in Ventilated Patients: Use of a Protected Specimen Brush and Quantitative Culture Techniques in 147 Patients', *American Journal of Respiratory and Critical Care Medicine*. American Thoracic Society, 138(1), pp. 110–116.

Fagon, J.-Y., Chastre, J., Hance, A. J., Montravers, P., Novara, A. and Gibert, C. (1993) 'Nosocomial pneumonia in ventilated patients: A cohort study evaluating attributable mortality and hospital stay', *The American Journal of Medicine*, 94(3), pp. 281–288. doi: 10.1016/0002-9343(93)90060-3.

Fagon, J. Y., Chastre, J., Wolff, M., Gervais, C., Parer-Aubas, S., Stephan, F., Similowski, T., Mercat, A., Diehl, J. L., Sollet, J. P. and Tenaillon, A. (2000) 'Invasive and noninvasive strategies for management of suspected ventilator-associated pneumonia. A randomized trial', *Ann Intern Med*. 2000/04/15, 132(8), pp. 621–630.

Faisy, C., Guerot, E., Diehl, J.-L., Iftimovici, E. and Fagon, J.-Y. (2003) 'Clinically significant gastrointestinal bleeding in critically ill patients with and without stress-ulcer prophylaxis.', *Intensive care medicine*, 29(8), pp. 1306–13. doi: 10.1007/s00134-003-1863-3.

Farrell, J. J., Sampath, R., Ecker, D. J., Bonomo, R. A., Bhatia, N., Farrell, J., Sampath,

R., Ranken, R., Rounds, M., Puskarich, M., Trzeciak, S., Shapiro, N., Arnold, R., Horton, J., Houck, P., Bratzler, D., Niederman, M., Bartlett, J., McGarvey, R., Harper, J., Fitch, M., Beek, D. van de, Aronin, S., Peduzzi, P., Quagliarello, V., Proulx, N., Frechette, D., Toye, B., Chan, J., Kravcik, S., Sampath, R., Hall, T., Massire, C., Li, F., Blyn, L., Ecker, D., Sampath, R., Li, H., Massire, C., Matthews, H., Caliendo, A., Kaleta, E., Clark, A., Johnson, D., Gamage, D. and Wysocki, V. (2013) '"Salvage Microbiology": Detection of Bacteria Directly from Clinical Specimens following Initiation of Antimicrobial Treatment', *PLoS ONE*. Edited by R. C. Willson. Public Library of Science, 8(6), p. e66349. doi: 10.1371/journal.pone.0066349.

Fartoukh, M., Maitre, B., Honoré, S., Cerf, C., Zahar, J.-R. and Brun-Buisson, C. (2003) 'Diagnosing pneumonia during mechanical ventilation: the clinical pulmonary infection score revisited.', *American journal of respiratory and critical care medicine*, 168(2), pp. 173–9. doi: 10.1164/rccm.200212-1449OC.

Faurschou, M. and Borregaard, N. (2003) 'Neutrophil granules and secretory vesicles in inflammation', *Microbes and Infection*, 5(14), pp. 1317–1327. doi: 10.1016/j.micinf.2003.09.008.

Fennerty, M. B. (2002) 'Pathophysiology of the upper gastrointestinal tract in the critically ill patient: Rationale for the therapeutic benefits of acid suppression', *Critical Care Medicine*, 30(Suppl), pp. S351–S355.

Ferrer, M., Liapikou, A., Valencia, M., Esperatti, M., Theessen, A., Antonio Martinez, J., Mensa, J. and Torres, A. (2010) 'Validation of the American Thoracic Society-Infectious Diseases Society of America guidelines for hospital-acquired pneumonia in the intensive care unit.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. Oxford University Press, 50(7), pp. 945–52. doi: 10.1086/651075.

Ferrer, R., Martin-Loeches, I., Phillips, G., Osborn, T. M., Townsend, S., Dellinger, R. P., Artigas, A., Schorr, C. and Levy, M. M. (2014) 'Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program.', *Critical care medicine*, 42(8), pp. 1749–55. doi: 10.1097/CCM.0000000000000330.

Filipiak, W., Beer, R., Sponring, A., Filipiak, A., Ager, C., Schiefecker, A., Lanthaler, S., Helbok, R., Nagl, M., Troppmair, J. and Amann, A. (2015) 'Breath analysis for *in vivo* detection of pathogens related to ventilator-associated pneumonia in intensive care

patients: a prospective pilot study', *Journal of Breath Research*. IOP Publishing, 9(1), p. 16004. doi: 10.1088/1752-7155/9/1/016004.

Filius, P. M. G., Gyssens, I. C., Kershof, I. M., Roovers, P. J. E., Ott, A., Vulto, A. G., Verbrugh, H. A. and Endtz, H. P. (2005) 'Colonization and resistance dynamics of gram-negative bacteria in patients during and after hospitalization.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 49(7), pp. 2879–86. doi: 10.1128/AAC.49.7.2879-2886.2005.

Fink, J. B., Krause, S. A., Barrett, L., Schaaff, D. and Alex, C. G. (1998) 'Extending ventilator circuit change interval beyond 2 days reduces the likelihood of ventilator-associated pneumonia.', *Chest*, 113(2), pp. 405–11.

Fisher, C. J., Dhainaut, J.-F. A., Opal, S. M., Pribble, J. P., Balk, R. A., Slotman, G. J., Iberti, T. J., Rackow, E. C., Shapiro, M. J., Greenman, R. L., Reines, H. D., Shelly, M. P., Thompson, B. W., LaBrecque, J. F., Catalano, M. A., Knaus, W. A., Sadoff, J. C., Astiz, M., Carpati, C., Bone, R. C., Friedman, B., Mure, A. J., Brathwaite, C., Shapiro, E., Melhorn, L., Taylor, R., Keegan, M., O'Brien, J., Schein, R., Pena, M., Wasserlouf, M., Oropello, J., Benjamin, E., DelGuidice, R., Emmanuel, G., Lie, T., Anderson, L., Marshall, J., Majo, W. De, Rotstein, O., Foster, D., Abraham, E., Middleton, H., Perry, C., Levy, H., Fry, D. E., Simpson, S. Q., Crowell, R. E., Neidhart, M., Stevens, D., Coffman, T., Narasimhan, N., Merrick, D. K., Bergquist, W., Matzel, K. E., Huebler, M., Foulke, G. E., Albertson, T. E., Walby, W. F., Allen, R. P., Baughman, R., Hasselgren, P.-O., Fink, M. P., Favorito, F., Thompson, B. T., Corbin, R., Shellhorse, G. Y., Frazier, A., White, S., Garrard, C., A'Court, C., Storer, S., Gervich, D. H., Foshe, D., Brase, R., Bagdahn, A., Cooney, R., Smith, J. S., Martin, L. F., Vincent, J.-L., Friedman, G., Berlot, G., Fletcher, J. R., Williams, M. D., Wright, T. F., Johnson, S., Feild, C., Wolf, K., MacIntyre, N., Dubin, H. G., Durkin, M. R., Dubin, P. K., Staubach, K. H., Fein, A. M., Schulman, D. B., Niederman, M. S., Chalfin, D. B., Van, P. A. M., Leeuwen, Boermeester, M. A., Schneider, A. J., Bander, J., Imm, A., Bernard, G., Nelson, L., Stroud, M., Safcsak, K., Cerra, F., Rindal, J., Mann, H., Halpern, N., Silverstein, J., Alicea, M., Sibbald, W. J., Martin, C. M., Rutledge, F. S., Petti, K., Russell, J. A., Kruger, R., Drummond, A., Lange, P., Seifert, T., Durocher, A., Tenaillon, A., Boiteau, R., Lherm, T., Lowry, S. F., Coyle, S. M., Barie, P. S., DeMaria, E., Snydman, D. R., Schwaitzberg, S. D., Nasraway, S. A., Grindlinger, J., Summer, W., Boisblanc, B. de, Wahl, M., Alestig, K., Grossman, J., Maki, D., Paz, H. L., Weiner, M., Bihari, D., Campbell, D., Bleichner, G., Dahn, M. S., Lange, M. P., Hall, J., Pohlman,

A., Wenzel, R. P., Grosserode, M., Costigan, M., Mileski, W., Weigelt, J., Yeston, N., Irizarry, C., Ross, J., Robbins, J., Nightingale, P., Owen, K., Sandstedt, S., Berg, S., Simon, G. L., Seneff, M. G., Conry, K. M., Zimmerman, J. L., Dellinger, R. P., Johnston, R., Allee, P., Grande, P.-O., Myhre, E., Dhainaut, J.-F., Hamy, I., Mira, J.-P., Harmon, J., White, J., McKie, L., Silverman, H., Tuma, P., Bennett, D., Porter, J. C., Laurell, M. H., Jacobs, S., Ash, S., Stiles, D. M., Prior, M. J., Knatterud, G., Terrin, M., Kufera, J., Wilkens, P., Ra, K., Monroe, L., Sprung, C., Hamilton, C. M., Matthay, R., McCabe, W., Tonascia, J., Wiedeman, H., Wittes, J., Knatterud, G., Campion, G. V., Croft, C. R., Lustick, R., Lookabaugh, J., Gordon, G. S., Noe, L., Bloedow, D., Christopher, S. G., Brannon, D., Kush, R., Ng, D., Moore, E., Bazemore, K., Galvan, M., Wagner, D., Harrell, F., Stablein, D., RP, W., Bone RC, Fisher CJ Jr, Clemmer TP, Slotman GJ, Metz CA, B. R., Control, C. for D., Greenman RL, Schein RMH, Martin MA, et al, Ziegler EJ, Fisher CJ Jr, Sprung CL, et al, Fisher CJ Jr, B. G., Parrillo JE, Parker MM, Natanson C, et al, RC, B., JE, P., Hesse DG, Tracey KJ, Fong Y, et al, Tracey KJ, Vlassara H, C. A., Waage A, Brandtzaeg P, Halstensen A, Kierulf P, E. T., Marks JD, Marks CB, Luce JM, et al, Cannon JG, Tompkins RG, Gelfand JA, et al, Calandra T, Baumgartner JD, Grau EG, et al, Michie HR, Manogue KR, Spriggs DR, et al, Waage A, E. T., Tracey KJ, Beutler B, Lowry SF, et al, Okusawa S, Gefand JA, Ikejima T, Connolly RJ, D. C., Eisenberg SP, Evans RJ, Arend WP, et al, Hannum CH, Wilcox CJ, Arend WP, et al, Arend WP, Welgus HG, Thompson RC, E. S., Dripps DJ, Brandhuber BJ, Thompson RC, E. S., Dripps DJ, Verderber EV, Ng RK, Thompson RC, E. S., Granowitz EV, Santos AA, Poutsiaka DD, et al, Fischer E, Marano MA, VanZee KJ, et al, Fischer E, Marano MA, Barber AE, et al, Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, T. R., Wakabayashi G, Gelfand JA, Burke JF, Thompson RC, D. C., Aiura K, Gelfand JA, Burke JF, Thompson RC, D. C., Fisher CJ Jr, Slotman GJ, Opal SM, et al, Knaus WA, Wagner DP, Draper EA, et al, Knaus WA, Harrell FE, Fisher CJ Jr, et al, Prentice RL, M. P., Dinarello CA, Gelfand JA, W. S., BP, G. and Fisher CJ Jr, Opal SM, Dhainaut JF, et al (1994) 'Recombinant Human Interleukin 1 Receptor Antagonist in the Treatment of Patients With Sepsis Syndrome', *JAMA*. American Medical Association, 271(23), pp. 1836–1843. doi: 10.1001/jama.1994.03510470040032.

Fonkalsrud, E. W., Sanchez, M., Higashijima, I. and Arima, E. (1975) 'A comparative study of the effects of dry vs. humidified ventilation on canine lungs.', *Surgery*, 78(3), pp. 373–80.

Forel, J.-M., Voillet, F. F., Pulina, D., Gacouin, A., Perrin, G., Barrau, K., Jaber, S., Arnal, J.-M., Fathallah, M., Auquier, P., Roch, A., Azoulay, E. and Papazian, L. (2012) ‘Ventilator-associated pneumonia and ICU mortality in severe ARDS patients ventilated according to a lung-protective strategy’, *Critical Care*. BioMed Central, 16(2), p. R65. doi: 10.1186/cc11312.

Fourrier, F., Cau-Pottier, E., Boutigny, H., Roussel-Delvallez, M., Jourdain, M. and Chopin, C. (2000) ‘Effects of dental plaque antiseptic decontamination on bacterial colonization and nosocomial infections in critically ill patients’, *Intensive Care Medicine*, 26(9), pp. 1239–1247. doi: 10.1007/s001340000585.

Fourrier, F., Dubois, D., Pronnier, P., Herbecq, P., Leroy, O., Desmettre, T., Pottier-Cau, E., Boutigny, H., Di Pompéo, C., Durocher, A. and Roussel-Delvallez, M. (2005) ‘Effect of gingival and dental plaque antiseptic decontamination on nosocomial infections acquired in the intensive care unit: a double-blind placebo-controlled multicenter study.’, *Critical care medicine*, 33(8), pp. 1728–35.

Fowler, S. J., Basanta-Sanchez, M., Xu, Y., Goodacre, R. and Dark, P. M. (2015) ‘Surveillance for lower airway pathogens in mechanically ventilated patients by metabolomic analysis of exhaled breath: a case-control study.’, *Thorax*. doi: 10.1136/thoraxjnl-2014-206273.

Franke-Ullmann, G., Pförtner, C., Walter, P., Steinmüller, C., Lohmann-Matthes, M. L. and Kobzik, L. (1996) ‘Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages in vitro.’, *Journal of immunology (Baltimore, Md. : 1950)*, 157(7), pp. 3097–104.

Garnacho-Montero, J., Gutiérrez-Pizarraya, A., Escoresca-Ortega, A., Fernández-Delgado, E. and López-Sánchez, J. M. (2015) ‘Adequate antibiotic therapy prior to ICU admission in patients with severe sepsis and septic shock reduces hospital mortality.’, *Critical care (London, England)*. Critical Care, 19(1), p. 302. doi: 10.1186/s13054-015-1000-z.

Garnacho-Montero, J., Sa-Borges, M., Sole-Violan, J., Barcenilla, F., Escoresca-Ortega, A., Ochoa, M., Cayuela, A. and Rello, J. (2007) ‘Optimal management therapy for Pseudomonas aeruginosa ventilator-associated pneumonia: an observational, multicenter study comparing monotherapy with combination antibiotic therapy.’, *Critical care medicine*, 35(8), pp. 1888–95. doi: 10.1097/01.CCM.0000275389.31974.22.

Gastinne, H., Wolff, M., Delatour, F., Faurisson, F. and Chevret, S. (1992) 'A controlled trial in intensive care units of selective decontamination of the digestive tract with nonabsorbable antibiotics. The French Study Group on Selective Decontamination of the Digestive Tract.', *The New England journal of medicine*, 326(9), pp. 594–9. doi: 10.1056/NEJM199202273260903.

Gattarello, S., Borgatta, B., Solé-Violán, J., Vallés, J., Vidaur, L., Zaragoza, R., Torres, A. and Rello, J. (2014) 'Decrease in Mortality in severe community-acquired pneumococcal pneumonia: Impact of improving antibiotic strategies (2000-2013)', *Chest*, 146(1), pp. 22–31. doi: 10.1378/chest.13-1531.

Geissler, A., Gerbeaux, P., Granier, I., Blanc, P., Facon, K. and Durand-Gasselin, J. (2003) 'Rational use of antibiotics in the intensive care unit: impact on microbial resistance and costs.', *Intensive care medicine*, 29(1), pp. 49–54. doi: 10.1007/s00134-002-1565-2.

Gibot, S., Cravoisy, A., Levy, B., Bene, M. C., Faure, G. and Bollaert, P. E. (2004) 'Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia', *N Engl J Med*. 2004/01/30, 350(5), pp. 451–458. doi: 10.1056/NEJMoa031544.

Girard, T. D., Kress, J. P., Fuchs, B. D., Thomason, J. W. W., Schweickert, W. D., Pun, B. T., Taichman, D. B., Dunn, J. G., Pohlman, A. S., Kinniry, P. A., Jackson, J. C., Canonico, A. E., Light, R. W., Shintani, A. K., Thompson, J. L., Gordon, S. M., Hall, J. B., Dittus, R. S., Bernard, G. R. and Ely, E. W. (2008) 'Efficacy and safety of a paired sedation and ventilator weaning protocol for mechanically ventilated patients in intensive care (Awakening and Breathing Controlled trial): a randomised controlled trial.', *Lancet*, 371(9607), pp. 126–34. doi: 10.1016/S0140-6736(08)60105-1.

Gleeson, K., Maxwell, S. L. and Eggli, D. F. (1997) 'Quantitative Aspiration During Sleep in Normal Subjects', *Chest*, 111(5), pp. 1266–1272. doi: 10.1378/chest.111.5.1266.

Goossens, H., Ferech, M., Vander Stichele, R. and Elseviers, M. (2005) 'Outpatient antibiotic use in Europe and association with resistance: a cross-national database study.', *Lancet*, 365(9459), pp. 579–87. doi: 10.1016/S0140-6736(05)17907-0.

Grap, M. J., Munro, C. L., Elswick, R. K., Sessler, C. N. and Ward, K. R. (2004) 'Duration of action of a single, early oral application of chlorhexidine on oral microbial flora in mechanically ventilated patients: a pilot study.', *Heart & lung : the journal of*

critical care, 33(2), pp. 83–91. doi: 10.1016/j.hrtlng.2003.12.004.

Groll, D. L., To, T., Bombardier, C. and Wright, J. G. (2005) ‘The development of a comorbidity index with physical function as the outcome.’, *Journal of clinical epidemiology*, 58(6), pp. 595–602. doi: 10.1016/j.jclinepi.2004.10.018.

Hallander, H. O., Dornbusch, K., Gezelius, L., Jacobson, K. and Karlsson, I. (1982) ‘Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: interaction index and killing curve method.’, *Antimicrobial agents and chemotherapy*, 22(5), pp. 743–52.

Halpern, N. A., Hale, K. E., Sepkowitz, K. A. and Pastores, S. M. (2012) ‘A world without ventilator-associated pneumonia: time to abandon surveillance and deconstruct the bundle.’, *Critical care medicine*, 40(1), pp. 267–70. doi: 10.1097/CCM.0b013e318232e3ec.

Hammond, J. (1992) ‘Double-blind study of selective decontamination of the digestive tract in intensive care’, *The Lancet*, 340(8810), pp. 5–9. doi: 10.1016/0140-6736(92)92422-C.

Harada, a, Sekido, N., Akahoshi, T., Wada, T., Mukaida, N. and Matsushima, K. (1994) ‘Essential involvement of interleukin-8 (IL-8) in acute inflammation.’, *Journal of leukocyte biology*, 56(5), pp. 559–64.

Harhay, M. O., Wagner, J., Ratcliffe, S. J., Bronheim, R. S., Gopal, A., Green, S., Cooney, E., Mikkelsen, M. E., Kerlin, M. P., Small, D. S. and Halpern, S. D. (2014) ‘Outcomes and statistical power in adult critical care randomized trials.’, *American journal of respiratory and critical care medicine*. American Thoracic Society, 189(12), pp. 1469–78. doi: 10.1164/rccm.201401-0056CP.

Hartog, C. M., Wermelt, J. A., Sommerfeld, C. O., Eichler, W., Dalhoff, K. and Braun, J. (2003) ‘Pulmonary matrix metalloproteinase excess in hospital-acquired pneumonia.’, *American journal of respiratory and critical care medicine*. American Thoracic Society, 167(4), pp. 593–8. doi: 10.1164/rccm.200203-258OC.

Haslam, P. L. and Baughman, R. P. (1999) ‘Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL.’, *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 14(2), pp. 245–8.

Havey, T. C., Fowler, R. A., Pinto, R., Elligsen, M. and Daneman, N. (2013) ‘Duration

of antibiotic therapy for critically ill patients with bloodstream infections: A retrospective cohort study.', *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie médicale / AMMI Canada*, 24(3), pp. 129–37.

Health Protection Agency (2011) *English National Point Prevalence Survey on Healthcare-associated Infections and Antimicrobial Use, 2011*. Available at: http://webarchive.nationalarchives.gov.uk/20140714084352/http:/www.hpa.org.uk/Publications/InfectiousDiseases/AntimicrobialAndHealthcareAssociatedInfections/1205HCAIEnglishPPSforhcaiandamu2011prelim/ (Accessed: 16 April 2013).

Health Protection Agency (2012) *UK Standards for Microbiology Investigations. Investigations of Bronchoalveolar Lavage, Sputum and Associated Specimens*.

Health Protection Scotland (2011) 'Surveillance of Healthcare Associated Infections in Scottish Intensive Care Units'.

Heidelbaugh, J. J. and Inadomi, J. M. (2006) 'Magnitude and economic impact of inappropriate use of stress ulcer prophylaxis in non-ICU hospitalized patients.', *The American journal of gastroenterology*, 101(10), pp. 2200–5. doi: 10.1111/j.1572-0241.2006.00839.x.

Heininger, A., Meyer, E., Schwab, F., Marschal, M., Unertl, K. and Krueger, W. A. (2006) 'Effects of long-term routine use of selective digestive decontamination on antimicrobial resistance.', *Intensive care medicine*, 32(10), pp. 1569–76. doi: 10.1007/s00134-006-0304-5.

Hellyer, T. P., Morris, A. C., McAuley, D. F., Walsh, T. S., Anderson, N. H., Singh, S., Dark, P., Roy, A. I., Baudouin, S. V, Wright, S. E., Perkins, G. D., Kefala, K., Jeffels, M., McMullan, R., O'Kane, C. M., Spencer, C., Laha, S., Robin, N., Gossain, S., Gould, K., Ruchaud-Sparagano, M.-H., Scott, J., Browne, E. M., MacFarlane, J. G., Wiscombe, S., Widdrington, J. D., Dimmick, I., Laurenson, I. F., Nauwelaers, F. and Simpson, A. J. (2015) 'Diagnostic accuracy of pulmonary host inflammatory mediators in the exclusion of ventilator-acquired pneumonia.', *Thorax*, 70(1), pp. 41–7. doi: 10.1136/thoraxjnl-2014-205766.

Herzig, S. J., Howell, M. D., Ngo, L. H. and Marcantonio, E. R. (2009) 'Acid-suppressive medication use and the risk for hospital-acquired pneumonia.', *JAMA : the journal of the American Medical Association*, 301(20), pp. 2120–8. doi: 10.1001/jama.2009.722.

Hess, D. R., Kallstrom, T. J., Mottram, C. D., Myers, T. R., Sorenson, H. M. and Vines, D. L. (2003) 'Care of the ventilator circuit and its relation to ventilator-associated pneumonia.', *Respiratory care*, 48(9), pp. 869–79.

Heyland, D., Jiang, X., Day, A. G. and Laverdiere, M. (2011) 'Serum β-d-glucan of critically ill patients with suspected ventilator-associated pneumonia: preliminary observations.', *Journal of critical care*, 26(5), p. 536.e1-9. doi: 10.1016/j.jcrc.2011.01.002.

Heyland, D. K., Cook, D. J., Griffith, L., Keenan, S. P. and Brun-Buisson, C. (1999) 'The attributable morbidity and mortality of ventilator-associated pneumonia in the critically ill patient. The Canadian Critical Trials Group.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 159(4 Pt 1), pp. 1249–56.

Heyland, D. K., Dodek, P., Muscedere, J., Day, A. and Cook, D. (2008) 'Randomized trial of combination versus monotherapy for the empiric treatment of suspected ventilator-associated pneumonia.', *Critical care medicine*, 36(3), pp. 737–44. doi: 10.1097/01.CCM.0B013E31816203D6.

Holzapfel, L., Chevret, S., Madinier, G., Ohen, F., Demingeon, G., Coupry, A. and Chaudet, M. (1993) 'Influence of long-term oro- or nasotracheal intubation on nosocomial maxillary sinusitis and pneumonia: results of a prospective, randomized, clinical trial.', *Critical care medicine*, 21(8), pp. 1132–8.

Horan, T. C., Andrus, M. and Dudeck, M. A. (2008) 'CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting.', *American journal of infection control*, 36(5), pp. 309–32. doi: 10.1016/j.ajic.2008.03.002.

Horonenko, G., Hoyt, J. C., Robbins, R. A., Singarajah, C. U., Umar, A., Pattengill, J. and Hayden, J. M. (2007) 'Soluble triggering receptor expressed on myeloid cell-1 is increased in patients with ventilator-associated pneumonia: a preliminary report.', *Chest*, 132(1), pp. 58–63. doi: 10.1378/chest.06-2731.

Houston, S., Hougland, P., Anderson, J. J., LaRocco, M., Kennedy, V. and Gentry, L. O. (2002) 'Effectiveness of 0.12% chlorhexidine gluconate oral rinse in reducing prevalence of nosocomial pneumonia in patients undergoing heart surgery.', *American journal of critical care : an official publication, American Association of Critical-Care Nurses*, 11(6), pp. 567–70.

Hranjec, T., Rosenberger, L. H., Swenson, B., Metzger, R., Flohr, T. R., Politano, A. D., Riccio, L. M., Popovsky, K. A. and Sawyer, R. G. (2012) 'Aggressive versus conservative initiation of antimicrobial treatment in critically ill surgical patients with suspected intensive-care-unit-acquired infection: a quasi-experimental, before and after observational cohort study.', *The Lancet infectious diseases*, 12(10), pp. 774–80. doi: 10.1016/S1473-3099(12)70151-2.

Hsueh, P.-R., Badal, R. E., Hawser, S. P., Hoban, D. J., Bouchillon, S. K., Ni, Y. and Paterson, D. L. (2010) 'Epidemiology and antimicrobial susceptibility profiles of aerobic and facultative Gram-negative bacilli isolated from patients with intra-abdominal infections in the Asia-Pacific region: 2008 results from SMART (Study for Monitoring Antimicrobial Resistan', *International journal of antimicrobial agents*, 36(5), pp. 408–14. doi: 10.1016/j.ijantimicag.2010.07.002.

Huang, J., Cao, Y., Liao, C., Wu, L. and Gao, F. (2010) 'Effect of histamine-2-receptor antagonists versus sucralfate on stress ulcer prophylaxis in mechanically ventilated patients: a meta-analysis of 10 randomized controlled trials.', *Critical care (London, England)*, 14(5), p. R194. doi: 10.1186/cc9312.

Hugonnet, S., Eggimann, P., Borst, F., Maricot, P., Chevrolet, J.-C. and Pittet, D. (2004) 'Impact of ventilator-associated pneumonia on resource utilization and patient outcome.', *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 25(12), pp. 1090–6. doi: 10.1086/502349.

Huh, J. W., Lim, C.-M., Koh, Y., Oh, Y. M., Shim, T. S., Lee, S. Do, Kim, W. S., Kim, D. S., Kim, W. D. and Hong, S.-B. (2008) 'Diagnostic utility of the soluble triggering receptor expressed on myeloid cells-1 in bronchoalveolar lavage fluid from patients with bilateral lung infiltrates.', *Critical care (London, England)*, 12(1), p. R6. doi: 10.1186/cc6770.

Hurni, J. M., Feihl, F., Lazor, R., Leuenberger, P. and Perret, C. (1997) 'Safety of combined heat and moisture exchanger filters in long-term mechanical ventilation.', *Chest*, 111(3), pp. 686–91.

Ibrahim, E. H., Tracy, L., Hill, C., Fraser, V. J. and Kollef, M. H. (2001) 'The occurrence of ventilator-associated pneumonia in a community hospital: risk factors and clinical outcomes.', *Chest*, 120(2), pp. 555–61.

Infectious Diseases Society of America (2004) *Bad bugs, no drugs. As Antibiotic*

Discovery Stagnates ... A Public Health Crisis Brews. Available at: http://www.idsociety.org/uploadedFiles/IDSA/Policy_and_Advocacy/Current_Topics_and_Issues/Antimicrobial_Resistance/10x20/Images/Bad Bugs no Drugs.pdf (Accessed: 16 April 2013).

Institute for Healthcare Improvement (2012) *How-to Guide: Prevent Ventilator-Associated Pneumonia*. Available at: http://www.ihi.org/knowledge/Pages/Tools/HowtoGuidePreventVAP.aspx (Accessed: 4 October 2012).

Intensive care national audit and research centre (2015) *ICNARC: Summary Statistics 2014-2015*. Available at: https://www.icnarc.org/Our-Audit/Audits/Cmp/Reports/Summary-Statistics.

Iregui, M., Ward, S., Sherman, G., Fraser, V. J. and Kollef, M. H. (2002) 'Clinical importance of delays in the initiation of appropriate antibiotic treatment for ventilator-associated pneumonia', *Chest*. 2002/07/13, 122(1), pp. 262–268.

Jensen, J. U., Hein, L., Lundgren, B., Bestle, M. H., Mohr, T. T., Andersen, M. H., Thornberg, K. J., Løken, J., Steensen, M., Fox, Z., Tousi, H., Søe-Jensen, P., Lauritsen, A. Ø., Strange, D., Petersen, P. L., Reiter, N., Hestad, S., Thormar, K., Fjeldborg, P., Larsen, K. M., Drenck, N. E., Østergaard, C., Kjær, J., Grarup, J. and Lundgren, J. D. (2011) 'Procalcitonin-guided interventions against infections to increase early appropriate antibiotics and improve survival in the intensive care unit: A randomized trial*', *Critical Care Medicine*, 39(9), pp. 2048–2058. doi: 10.1097/CCM.0b013e31821e8791.

Jin, T., Xu, X. and Hereld, D. (2008) 'Chemotaxis, chemokine receptors and human disease.', *Cytokine*, 44(1), pp. 1–8. doi: 10.1016/j.cyto.2008.06.017.

Jo, E.-K., Kim, J. K., Shin, D.-M. and Sasakawa, C. (2016) 'Molecular mechanisms regulating NLRP3 inflammasome activation.', *Cellular & molecular immunology*. Nature Publishing Group, 13(2), pp. 148–59. doi: 10.1038/cmi.2015.95.

Johanson, W. G., Seidenfeld, J. J., Gomez, P., de los Santos, R. and Coalson, J. J. (1988) 'Bacteriologic diagnosis of nosocomial pneumonia following prolonged mechanical ventilation.', *The American review of respiratory disease*, 137(2), pp. 259–64.

Jones, D. S., McGovern, J. G., Woolfson, A. D. and Gorman, S. P. (1997) 'Role of physiological conditions in the oropharynx on the adherence of respiratory bacterial

isolates to endotracheal tube poly(vinyl chloride).', *Biomaterials*, 18(6), pp. 503–10.

de Jong, E., van Oers, J. A., Beishuizen, A., Vos, P., Vermeijden, W. J., Haas, L. E., Loef, B. G., Dormans, T., van Melsen, G. C., Kluiters, Y. C., Kemperman, H., van den Elsen, M. J., Schouten, J. A., Streefkerk, J. O., Krabbe, H. G., Kieft, H., Kluge, G. H., van Dam, V. C., van Pelt, J., Bormans, L., Otten, M. B., Reidinga, A. C., Endeman, H., Twisk, J. W., van de Garde, E. M. W., de Smet, A. M. G. A., Kesecioglu, J., Girbes, A. R., Nijsten, M. W. and de Lange, D. W. (2016) 'Efficacy and safety of procalcitonin guidance in reducing the duration of antibiotic treatment in critically ill patients: A randomised, controlled, open-label trial', *The Lancet Infectious Diseases*. Elsevier Ltd, 16(7), pp. 819–827. doi: 10.1016/S1473-3099(16)00053-0.

de Jonge, E., Schultz, M. J., Spanjaard, L., Bossuyt, P. M. M., Vroom, M. B., Dankert, J. and Kesecioglu, J. (2003) 'Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial.', *Lancet*, 362(9389), pp. 1011–6. doi: 10.1016/S0140-6736(03)14409-1.

Jung, B., Embriaco, N., Roux, F., Forel, J.-M., Demory, D., Allardet-Servent, J., Jaber, S., La Scola, B. and Papazian, L. (2010) 'Microbiogical data, but not procalcitonin improve the accuracy of the clinical pulmonary infection score.', *Intensive care medicine*, 36(5), pp. 790–8. doi: 10.1007/s00134-010-1833-5.

Kalil, A. C., Metersky, M. L., Klompas, M., Muscedere, J., Sweeney, D. A., Palmer, L. B., Napolitano, L. M., O'Grady, N. P., Bartlett, J. G., Carratalà, J., El Solh, A. A., Ewig, S., Fey, P. D., File, T. M., Restrepo, M. I., Roberts, J. A., Waterer, G. W., Cruse, P., Knight, S. L. and Brozek, J. L. (2016) 'Management of Adults With Hospital-acquired and Ventilator-associated Pneumonia: 2016 Clinical Practice Guidelines by the Infectious Diseases Society of America and the American Thoracic Society', *Clinical Infectious Diseases*, 63, pp. e61–e111. doi: 10.1093/cid/ciw353.

Karmakar, M., Sun, Y., Hise, A. G., Rietsch, A. and Pearlman, E. (2012) 'Cutting edge: IL-1β processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 189(9), pp. 4231–5. doi: 10.4049/jimmunol.1201447.

Keglowich, L., Baraket, M., Tamm, M. and Borger, P. (2014) 'Hypoxia exerts dualistic effects on inflammatory and proliferative responses of healthy and asthmatic primary human bronchial smooth muscle cells.', *PloS one*. Public Library of Science, 9(2), p.

e89875. doi: 10.1371/journal.pone.0089875.

Kelly, C. A., Kotre, C. J., Ward, C., Hendrick, D. J. and Walters, E. H. (1987) 'Anatomical distribution of bronchoalveolar lavage fluid as assessed by digital subtraction radiography.', *Thorax*, 42(8), pp. 624–628. doi: 10.1136/thx.42.8.624.

Kelly, M., Gillies, D., Todd, D. A. and Lockwood, C. (2010) 'Heated humidification versus heat and moisture exchangers for ventilated adults and children.', *The Cochrane database of systematic reviews*, (4), p. CD004711. doi: 10.1002/14651858.CD004711.pub2.

Kett, D. H., Cano, E., Quartin, A. A., Mangino, J. E., Zervos, M. J., Peyrani, P., Cely, C. M., Ford, K. D., Scerpella, E. G. and Ramirez, J. A. (2011) 'Implementation of guidelines for management of possible multidrug-resistant pneumonia in intensive care: an observational, multicentre cohort study', *Lancet Infect Dis*. 2011/01/25, 11(3), pp. 181–189. doi: 10.1016/S1473-3099(10)70314-5.

Khokha, R., Murthy, A. and Weiss, A. (2013) 'Metalloproteinases and their natural inhibitors in inflammation and immunity.', *Nature reviews. Immunology*. Nature Publishing Group, 13(9), pp. 649–65. doi: 10.1038/nri3499.

Kim, Y.-J., Paek, S.-H., Jin, S., Park, B. S. and Ha, U.-H. (2014) 'A novel Pseudomonas aeruginosa-derived effector cooperates with flagella to mediate the upregulation of interleukin 8 in human epithelial cells.', *Microbial pathogenesis*, 66, pp. 24–8. doi: 10.1016/j.micpath.2013.12.001.

Kirtland, S. H., Corley, D. E., Winterbauer, R. H., Springmeyer, S. C., Casey, K. R., Hampson, N. B. and Dreis, D. F. (1997) 'The diagnosis of ventilator-associated pneumonia: a comparison of histologic, microbiologic, and clinical criteria.', *Chest*, 112(2), pp. 445–57.

Kirton, O. C., DeHaven, B., Morgan, J., Morejon, O. and Civetta, J. (1997) 'A prospective, randomized comparison of an in-line heat moisture exchange filter and heated wire humidifiers: rates of ventilator-associated early-onset (community-acquired) or late-onset (hospital-acquired) pneumonia and incidence of endotracheal tube oc', *Chest*, 112(4), pp. 1055–9.

Klein Klouwenberg, P. M. C., van Mourik, M. S. M., Ong, D. S. Y., Horn, J., Schultz, M. J., Cremer, O. L. and Bonten, M. J. M. (2014) 'Electronic implementation of a novel surveillance paradigm for ventilator-associated events. Feasibility and validation.', *American journal of respiratory and critical care medicine*, 189(8), pp. 947–55. doi:

10.1164/rccm.201307-1376OC.

Klompas, M. (2010) 'Ventilator-associated pneumonia: is zero possible?', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 51(10), pp. 1123–6. doi: 10.1086/656738.

Klompas, M. (2013) 'Complications of Mechanical Ventilation — The CDC's New Surveillance Paradigm', *New England Journal of Medicine*, 368(16), pp. 1472–1475. doi: 10.1056/NEJMp1300633.

Klompas, M., Anderson, D., Trick, W., Babcock, H., Kerlin, M. P., Li, L., Sinkowitz-Cochran, R., Ely, E. W., Jernigan, J., Magill, S., Lyles, R., O'Neil, C., Kitch, B. T., Arrington, E., Balas, M. C., Kleinman, K., Bruce, C., Lankiewicz, J., Murphy, M. V, E Cox, C., Lautenbach, E., Sexton, D., Fraser, V., Weinstein, R. a and Platt, R. (2015) 'The preventability of ventilator-associated events. The CDC Prevention Epicenters Wake Up and Breathe Collaborative.', *American journal of respiratory and critical care medicine*, 191(3), pp. 292–301. doi: 10.1164/rccm.201407-1394OC.

Klompas, M., Speck, K., Howell, M. D., Greene, L. R. and Berenholtz, S. M. (2014) 'Reappraisal of routine oral care with chlorhexidine gluconate for patients receiving mechanical ventilation: systematic review and meta-analysis.', *JAMA internal medicine*, 174(5), pp. 751–61. doi: 10.1001/jamainternmed.2014.359.

Koeman, M., van der Ven, A. J. A. M., Hak, E., Joore, H. C. A., Kaasjager, K., de Smet, A. G. A., Ramsay, G., Dormans, T. P. J., Aarts, L. P. H. J., de Bel, E. E., Hustinx, W. N. M., van der Tweel, I., Hoepelman, A. M. and Bonten, M. J. M. (2006) 'Oral decontamination with chlorhexidine reduces the incidence of ventilator-associated pneumonia.', *American journal of respiratory and critical care medicine*, 173(12), pp. 1348–55. doi: 10.1164/rccm.200505-820OC.

Kohlenberg, A., Schwab, F., Behnke, M., Geffers, C. and Gastmeier, P. (2010) 'Pneumonia associated with invasive and noninvasive ventilation: an analysis of the German nosocomial infection surveillance system database.', *Intensive care medicine*, 36(6), pp. 971–8. doi: 10.1007/s00134-010-1863-z.

Kola, A., Eckmanns, T. and Gastmeier, P. (2005) 'Efficacy of heat and moisture exchangers in preventing ventilator-associated pneumonia: meta-analysis of randomized controlled trials.', *Intensive care medicine*, 31(1), pp. 5–11. doi: 10.1007/s00134-004-2431-1.

Kollef, M. H. (1994) 'The role of selective digestive tract decontamination on mortality

and respiratory tract infections. A meta-analysis.', *Chest*, 105(4), pp. 1101–8.

Kollef, M. H., Chastre, J., Fagon, J.-Y., François, B., Niederman, M. S., Rello, J., Torres, A., Vincent, J.-L., Wunderink, R. G., Go, K. W. and Rehm, C. (2014) 'Global Prospective Epidemiologic and Surveillance Study of Ventilator-Associated Pneumonia due to Pseudomonas aeruginosa*', *Critical care medicine*, 42(10), pp. 2178–2187. doi: 10.1097/CCM.0000000000000510.

Kollef, M. H., Hamilton, C. W. and Ernst, F. R. (2012) 'Economic impact of ventilator-associated pneumonia in a large matched cohort.', *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 33(3), pp. 250–6. doi: 10.1086/664049.

Kollef, M. H., Shapiro, S. D., Boyd, V., Silver, P., Von Harz, B., Trovillion, E. and Prentice, D. (1998) 'A randomized clinical trial comparing an extended-use hygroscopic condenser humidifier with heated-water humidification in mechanically ventilated patients.', *Chest*, 113(3), pp. 759–67.

Kollef, M. H., Shapiro, S. D., Fraser, V. J., Silver, P., Murphy, D. M., Trovillion, E., Hearns, M. L., Richards, R. D., Cracchilo, L. and Hossin, L. (1995) 'Mechanical ventilation with or without 7-day circuit changes. A randomized controlled trial.', *Annals of internal medicine*, 123(3), pp. 168–74.

Koncan, R., Parisato, M., Sakarikou, C., Stringari, G., Fontana, C., Favuzzi, V., Ligozzi, M. and Lo Cascio, G. (2015) 'Direct identification of major Gram-negative pathogens in respiratory specimens by respiFISH HAP Gram (-) Panel, a beacon-based FISH methodology', *European Journal of Clinical Microbiology and Infectious Diseases*, 34(10), pp. 2097–2102. doi: 10.1007/s10096-015-2458-y.

Kooguchi, K., Hashimoto, S., Kobayashi, A., Kitamura, Y., Kudoh, I., Wiener-Kronish, J. and Sawa, T. (1998) 'Role of alveolar macrophages in initiation and regulation of inflammation in Pseudomonas aeruginosa pneumonia.', *Infection and immunity*, 66(7), pp. 3164–9.

Kotani, M., Kotani, T., Ishizaka, A., Fujishima, S., Koh, H., Tasaka, S., Sawafuji, M., Ikeda, E., Moriyama, K., Kotake, Y., Morisaki, H., Aikawa, N., Ohashi, A., Matsushima, K., Huang, Y.-C. T. and Takeda, J. (2004) 'Neutrophil depletion attenuates interleukin-8 production in mild-overstretch ventilated normal rabbit lung.', *Critical care medicine*, 32(2), pp. 514–9. doi: 10.1097/01.CCM.0000110677.16968.E4.

Koulenti, D., Lisboa, T., Brun-Buisson, C., Krueger, W., Macor, A., Sole-Violan, J.,

Diaz, E., Topeli, A., DeWaele, J., Carneiro, A., Martin-Loeches, I., Armaganidis, A. and Rello, J. (2009) 'Spectrum of practice in the diagnosis of nosocomial pneumonia in patients requiring mechanical ventilation in European intensive care units.', *Critical care medicine*, 37(8), pp. 2360–8. doi: 10.1097/CCM.0b013e3181a037ac.

Kress, J. P., Pohlman, A. S., O'Connor, M. F. and Hall, J. B. (2000) 'Daily interruption of sedative infusions in critically ill patients undergoing mechanical ventilation.', *The New England journal of medicine*, 342(20), pp. 1471–7. doi: 10.1056/NEJM200005183422002.

Krige, A., Pattison, N., Booth, M., Walsh, T., Walsh, T., Fletcher, S., Krige, A., Perkins, G., Pattison, N., Reay, H., Finney, S., Harvey, S., Rowan, K. and Booth, M. (2013) 'Co-Enrolment to Intensive Care Studies - A UK Perspective', *Journal of the Intensive Care Society*, 14(2), pp. 103–106. doi: 10.1177/175114371301400203.

Kufareva, I., Salanga, C. L. and Handel, T. M. (2015) 'Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies.', *Immunology and cell biology*. Nature Publishing Group, 93(4), pp. 372–83. doi: 10.1038/icb.2015.15.

Kumar, A., Roberts, D., Wood, K. E., Light, B., Parrillo, J. E., Sharma, S., Suppes, R., Feinstein, D., Zanotti, S., Taiberg, L., Gurka, D., Kumar, A. and Cheang, M. (2006) 'Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock.', *Critical care medicine*, 34(6), pp. 1589–96. doi: 10.1097/01.CCM.0000217961.75225.E9.

de La Cal, M. A., Cerdá, E., García-Hierro, P., van Saene, H. K. F., Gómez-Santos, D., Negro, E. and Lorente, J. A. (2005) 'Survival benefit in critically ill burned patients receiving selective decontamination of the digestive tract: a randomized, placebo-controlled, double-blind trial.', *Annals of surgery*, 241(3), pp. 424–30.

Labeau, S. O., Van de Vyver, K., Brusselaers, N., Vogelaers, D. and Blot, S. I. (2011) 'Prevention of ventilator-associated pneumonia with oral antiseptics: a systematic review and meta-analysis.', *The Lancet infectious diseases*, 11(11), pp. 845–54. doi: 10.1016/S1473-3099(11)70127-X.

Lacherade, J.-C., Auburtin, M., Cerf, C., Van de Louw, A., Soufir, L., Rebufat, Y., Rezaiguia, S., Ricard, J.-D., Lellouche, F., Brun-Buisson, C. and Brochard, L. (2005) 'Impact of humidification systems on ventilator-associated pneumonia: a randomized multicenter trial.', *American journal of respiratory and critical care medicine*, 172(10),

pp. 1276–82. doi: 10.1164/rccm.200408-1028OC.

Lacroix, M., Barraud, O., Clavel, M., Filiputti, D., Prudent, S., François, B., Ploy, M. C., Jestin, M.-A., Rodrigue, M., Pachot, A., Yugueros-Marcos, J. and Moucadel, V. (2015) 'Rapid quantification of Staphylococcus aureus from endotracheal aspirates of ventilated patients: a proof-of-concept study.', *Diagnostic microbiology and infectious disease*. doi: 10.1016/j.diagmicrobio.2015.06.014.

Lacy, P. (2006) 'Mechanisms of degranulation in neutrophils.', *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology*. BioMed Central, 2(3), pp. 98–108. doi: 10.1186/1710-1492-2-3-98.

Landis, J. R. and Koch, G. G. (1977) 'The Measurement of Observer Agreement for Categorical Data', *Biometrics*, 33(1), p. 159. doi: 10.2307/2529310.

de Latorre, F. J., Pont, T., Ferrer, A., Rosselló, J., Palomar, M. and Planas, M. (1995) 'Pattern of tracheal colonization during mechanical ventilation.', *American journal of respiratory and critical care medicine*, 152(3), pp. 1028–33. doi: 10.1164/ajrccm.152.3.7663779.

Laupland, K. B., Church, D. L. and Gregson, D. B. (2005) 'Validation of a rapid diagnostic strategy for determination of significant bacterial counts in bronchoalveolar lavage samples.', *Archives of pathology & laboratory medicine*, 129(1), pp. 78–81. doi: 10.1043/1543-2165(2005)129<78:VOARDS>2.0.CO;2.

Lauzier, F., Ruest, A., Cook, D., Dodek, P., Albert, M., Shorr, A. F., Day, A., Jiang, X. and Heyland, D. (2008) 'The value of pretest probability and modified clinical pulmonary infection score to diagnose ventilator-associated pneumonia.', *Journal of critical care*, 23(1), pp. 50–7. doi: 10.1016/j.jcrc.2008.01.006.

Lemon, K. (2010) 'Comparative Analyses of the Bacterial Microbiota of the Human Nostril and Oropharynx', *mBio*, 1(3), p. e00129-10-. doi: 10.1128/mBio.00129-10.

Leng, S. X., McElhaney, J. E., Walston, J. D., Xie, D., Fedarko, N. S. and Kuchel, G. A. (2008) 'ELISA and multiplex technologies for cytokine measurement in inflammation and aging research.', *The journals of gerontology. Series A, Biological sciences and medical sciences*, 63(8), pp. 879–84.

Lesaffre, E., Scheys, I., Fröhlich, J. and Bluhmki, E. (1993) 'Calculation of power and sample size with bounded outcome scores', *Statistics in Medicine*, 12(11), pp. 1063–1078. doi: 10.1002/sim.4780121106.

Li, X.-Z., Zhang, L. and Poole, K. (2000) ‘Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of Pseudomonas aeruginosa’, *Journal of Antimicrobial Chemotherapy*, 45(4), pp. 433–436. doi: 10.1093/jac/45.4.433.

Lin, P.-C., Chang, C.-H., Hsu, P.-I., Tseng, P.-L. and Huang, Y.-B. (2010) ‘The efficacy and safety of proton pump inhibitors vs histamine-2 receptor antagonists for stress ulcer bleeding prophylaxis among critical care patients: a meta-analysis.’, *Critical care medicine*, 38(4), pp. 1197–205. doi: 10.1097/CCM.0b013e3181d69ccf.

Lin, Q., Fu, F., Shen, L. and Zhu, B. (2013) ‘Pentraxin 3 in the assessment of ventilator-associated pneumonia: an early marker of severity.’, *Heart & lung : the journal of critical care*, 42(2), pp. 139–45. doi: 10.1016/j.hrtlng.2012.11.005.

Linder, J. a. (2014) ‘Letter: Time of Day and the Decision to Prescribe Antibiotics’, *JAMA Internal Medicine*, 174(12), pp. 2029–2031. doi: 10.1001/jamainternmed.2014.5225.Author.

Linssen, C. F. M., Bekers, O., Drent, M. and Jacobs, J. A. (2008) ‘C-reactive protein and procalcitonin concentrations in bronchoalveolar lavage fluid as a predictor of ventilator-associated pneumonia.’, *Annals of clinical biochemistry*, 45(Pt 3), pp. 293–8. doi: 10.1258/acb.2007.007133.

Livermore, D. M. (2012) ‘Fourteen years in resistance.’, *International journal of antimicrobial agents*, 39(4), pp. 283–94. doi: 10.1016/j.ijantimicag.2011.12.012.

Lopez-Ranton, M. and Rodriguez-Alvarez, M. (2013) *Package ‘OptimalCutpoints’*. Available at: http://cran.r-project.org/web/packages/OptimalCutpoints/OptimalCutpoints.pdf (Accessed: 9 June 2013).

Lorente, L., Blot, S. and Rello, J. (2007) ‘Evidence on measures for the prevention of ventilator-associated pneumonia.’, *The European respiratory journal*, 30(6), pp. 1193–207. doi: 10.1183/09031936.00048507.

Lorente, L., Lecuona, M., Galván, R., Ramos, M. J., Mora, M. L. and Sierra, A. (2004) ‘Periodically changing ventilator circuits is not necessary to prevent ventilator-associated pneumonia when a heat and moisture exchanger is used.’, *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 25(12), pp. 1077–82. doi: 10.1086/502347.

Lorente, L., Lecuona, M., Jiménez, A., Mora, M. L. and Sierra, A. (2006) 'Ventilator-associated pneumonia using a heated humidifier or a heat and moisture exchanger: a randomized controlled trial [ISRCTN88724583].', *Critical care (London, England)*, 10(4), p. R116. doi: 10.1186/cc5009.

Luna, C. M., Aruj, P., Niederman, M. S., Garzón, J., Violi, D., Prignoni, a, Ríos, F., Baquero, S. and Gando, S. (2006) 'Appropriateness and delay to initiate therapy in ventilator-associated pneumonia.', *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 27(1), pp. 158–64. doi: 10.1183/09031936.06.00049105.

Luyt, C.-E., Chastre, J. and Fagon, J.-Y. (2004) 'Value of the clinical pulmonary infection score for the identification and management of ventilator-associated pneumonia.', *Intensive care medicine*, 30(5), pp. 844–52. doi: 10.1007/s00134-003-2125-0.

Luyt, C.-E., Combes, A., Reynaud, C., Hekimian, G., Nieszkowska, A., Tonnellier, M., Aubry, A., Trouillet, J.-L., Bernard, M. and Chastre, J. (2008) 'Usefulness of procalcitonin for the diagnosis of ventilator-associated pneumonia.', *Intensive care medicine*, 34(8), pp. 1434–40. doi: 10.1007/s00134-008-1112-x.

Luyt, C.-E., Guérin, V., Combes, A., Trouillet, J.-L., Ayed, S. Ben, Bernard, M., Gibert, C. and Chastre, J. (2005) 'Procalcitonin kinetics as a prognostic marker of ventilator-associated pneumonia.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 171(1), pp. 48–53. doi: 10.1164/rccm.200406-746OC.

MacLaren, R., Reynolds, P. M. and Allen, R. R. (2014) 'Histamine-2 receptor antagonists vs proton pump inhibitors on gastrointestinal tract hemorrhage and infectious complications in the intensive care unit.', *JAMA internal medicine*. American Medical Association, 174(4), pp. 564–74. doi: 10.1001/jamainternmed.2013.14673.

Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., Lynfield, R., Maloney, M., McAllister-Hollod, L., Nadle, J., Ray, S. M., Thompson, D. L., Wilson, L. E. and Fridkin, S. K. (2014) 'Multistate Point-Prevalence Survey of Health Care–Associated Infections', *New England Journal of Medicine*. Massachusetts Medical Society , 370(13), pp. 1198–1208. doi: 10.1056/NEJMoa1306801.

Maniar-Hew, K., Clay, C. C., Postlethwait, E. M., Evans, M. J., Fontaine, J. H. and Miller, L. A. (2013) 'Innate immune response to LPS in airway epithelium is dependent on chronological age and antecedent exposures.', *American journal of respiratory cell*

and molecular biology. American Thoracic Society, 49(5), pp. 710–20. doi: 10.1165/rcmb.2012-0321OC.

Marik, P. E., Vasu, T., Hirani, A. and Pachinburavan, M. (2010) 'Stress ulcer prophylaxis in the new millennium: a systematic review and meta-analysis.', *Critical care medicine*, 38(11), pp. 2222–8. doi: 10.1097/CCM.0b013e3181f17adf.

Markowicz, P., Wolff, M., Djedaïni, K., Cohen, Y., Chastre, J., Delclaux, C., Merrer, J., Herman, B., Veber, B., Fontaine, A. and Dreyfuss, D. (2000) 'Multicenter prospective study of ventilator-associated pneumonia during acute respiratory distress syndrome. Incidence, prognosis, and risk factors. ARDS Study Group.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 161(6), pp. 1942–8.

Marquette, C. H., Copin, M. C., Wallet, F., Neviere, R., Saulnier, F., Mathieu, D., Durocher, A., Ramon, P. and Tonnel, A. B. (1995) 'Diagnostic tests for pneumonia in ventilated patients: prospective evaluation of diagnostic accuracy using histology as a diagnostic gold standard.', *American journal of respiratory and critical care medicine*, 151(6), pp. 1878–88.

Martin-Loeches, I., Povoa, P., Rodríguez, A., Curcio, D., Suarez, D., Mira, J.-P., Cordero, M. L., Lepecq, R., Girault, C., Candeias, C., Seguin, P., Paulino, C., Messika, J., Castro, A. G., Valles, J., Coelho, L., Rabello, L., Lisboa, T., Collins, D., Torres, A., Salluh, J. and Nseir, S. (2015) 'Incidence and prognosis of ventilator-associated tracheobronchitis (TAVeM): a multicentre, prospective, observational study.', *The Lancet. Respiratory medicine*, 3(11), pp. 859–68. doi: 10.1016/S2213-2600(15)00326-4.

Martin, C., Perrin, G., Gevaudan, M. J., Saux, P. and Gouin, F. (1990) 'Heat and moisture exchangers and vaporizing humidifiers in the intensive care unit.', *Chest*, 97(1), pp. 144–9.

Masterton, R. G., Galloway, A., French, G., Street, M., Armstrong, J., Brown, E., Cleverley, J., Dilworth, P., Fry, C., Gascoigne, A. D., Knox, A., Nathwani, D., Spencer, R. and Wilcox, M. (2008) 'Guidelines for the management of hospital-acquired pneumonia in the UK: report of the working party on hospital-acquired pneumonia of the British Society for Antimicrobial Chemotherapy', *J Antimicrob Chemother*. 2008/05/01, 62(1), pp. 5–34. doi: 10.1093/jac/dkn162.

Mathy-Hartert, M., Damas, P., Nys, M., Deby-Dupont, G., Canivet, J., Ledoux, D. and Lamy, M. (2000) 'Nitrated proteins in bronchoalveolar lavage fluid of patients at risk of

ventilator-associated bronchopneumonia', *Eur. Respir. J.*, 16(2), pp. 296–301.

McGregor, J. C. and Furuno, J. P. (2014) 'Optimizing research methods used for the evaluation of antimicrobial stewardship programs.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 59 Suppl 3(suppl 3), pp. S185-92. doi: 10.1093/cid/ciu540.

McHugh, G. S., Butcher, I., Steyerberg, E. W., Marmarou, A., Lu, J., Lingsma, H. F., Weir, J., Maas, A. I. R. and Murray, G. D. (2010) 'A simulation study evaluating approaches to the analysis of ordinal outcome data in randomized controlled trials in traumatic brain injury: results from the IMPACT Project.', *Clinical trials (London, England)*, 7(1), pp. 44–57. doi: 10.1177/1740774509356580.

Meduri, G. U. and Chastre, J. (1992) 'The standardization of bronchoscopic techniques for ventilator-associated pneumonia', *Chest*. 1992/11/01, 102(5 Suppl 1), p. 557S–564S.

Meduri, G. U., Mauldin, G. L., Wunderink, R. G., Leeper, K. V, Jones, C. B., Tolley, E. and Mayhall, G. (1994) 'Causes of fever and pulmonary densities in patients with clinical manifestations of ventilator-associated pneumonia.', *Chest*, 106(1), pp. 221–35.

Melsen, W. G., Rovers, M. M. and Bonten, M. J. M. (2009) 'Ventilator-associated pneumonia and mortality: a systematic review of observational studies.', *Critical care medicine*, 37(10), pp. 2709–18.

Melsen, W. G., Rovers, M. M., Koeman, M. and Bonten, M. J. M. (2011) 'Estimating the attributable mortality of ventilator-associated pneumonia from randomized prevention studies.', *Critical care medicine*, 39(12), pp. 2736–42. doi: 10.1097/CCM.0b013e3182281f33.

Memish, Z. A., Oni, G. A., Djazmati, W., Cunningham, G. and Mah, M. W. (2001) 'A randomized clinical trial to compare the effects of a heat and moisture exchanger with a heated humidifying system on the occurrence rate of ventilator-associated pneumonia.', *American journal of infection control*, 29(5), pp. 301–5.

Messori, A., Trippoli, S., Vaiani, M., Gorini, M. and Corrado, A. (2000) 'Bleeding and pneumonia in intensive care patients given ranitidine and sucralfate for prevention of stress ulcer: meta-analysis of randomised controlled trials.', *BMJ (Clinical research ed.)*, 321(7269), pp. 1103–6.

Meyer, K. C., Raghu, G., Baughman, R. P., Brown, K. K., Costabel, U., du Bois, R. M., Drent, M., Haslam, P. L., Kim, D. S., Nagai, S., Rottoli, P., Saltini, C., Selman, M.,

Strange, C. and Wood, B. (2012) 'An official American Thoracic Society clinical practice guideline: the clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 185(9), pp. 1004–14. doi: 10.1164/rccm.201202-0320ST.

Miao, E. A., Alpuche-Aranda, C. M., Dors, M., Clark, A. E., Bader, M. W., Miller, S. I. and Aderem, A. A. (2006) 'Cytoplasmic flagellin activates caspase-1 and secretion of interleukin-1β via Ipaf', *Nat. Immunol.*, 7(6), pp. 569–575. doi: ni1344 [pii]\r10.1038/ni1344.

Michetti, C. P., Fakhry, S. M., Ferguson, P. L., Cook, A., Moore, F. O. and Gross, R. (2012) 'Ventilator-associated pneumonia rates at major trauma centers compared with a national benchmark: a multi-institutional study of the AAST.', *The journal of trauma and acute care surgery*, 72(5), pp. 1165–73. doi: 10.1097/TA.0b013e31824d10fa.

Millo, J. L., Schultz, M. J., Williams, C., Weverling, G. J., Ringrose, T., Mackinlay, C. I., van der Poll, T. and Garrard, C. S. (2004) 'Compartmentalisation of cytokines and cytokine inhibitors in ventilator-associated pneumonia.', *Intensive care medicine*, 30(1), pp. 68–74. doi: 10.1007/s00134-003-2060-0.

Montravers, P., Fagon, J. Y., Chastre, J., Lecso, M., Dombret, M. C., Trouillet, J. L. and Gibert, C. (1993) 'Follow-up protected specimen brushes to assess treatment in nosocomial pneumonia.', *The American review of respiratory disease*, 147(1), pp. 38–44. doi: 10.1164/ajrccm/147.1.38.

Morgan, E., Varro, R., Sepulveda, H., Ember, J. A., Apgar, J., Wilson, J., Lowe, L., Chen, R., Shivraj, L., Agadir, A., Campos, R., Ernst, D. and Gaur, A. (2004) 'Cytometric bead array: a multiplexed assay platform with applications in various areas of biology.', *Clinical immunology (Orlando, Fla.)*, 110(3), pp. 252–66. doi: 10.1016/j.clim.2003.11.017.

Mukaida, N. (2003) 'Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases.', *American journal of physiology. Lung cellular and molecular physiology*, 284(4), pp. L566-77. doi: 10.1152/ajplung.00233.2002.

Muscedere, J. G., Day, A. and Heyland, D. K. (2010) 'Mortality, attributable mortality, and clinical events as end points for clinical trials of ventilator-associated pneumonia and hospital-acquired pneumonia.', *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 51 Suppl 1, pp. S120-5. doi: 10.1086/653060.

Muscedere, J., Rewa, O., McKechnie, K., Jiang, X., Laporta, D. and Heyland, D. K. (2011) 'Subglottic secretion drainage for the prevention of ventilator-associated pneumonia: a systematic review and meta-analysis.', *Critical care medicine*, 39(8), pp. 1985–91. doi: 10.1097/CCM.0b013e318218a4d9.

Nardone, G. and Compare, D. (2015) 'The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases?', *United European gastroenterology journal*, 3(3), pp. 255–60. doi: 10.1177/2050640614566846.

Nathan, C. (2006) 'Neutrophils and immunity: challenges and opportunities.', *Nature reviews. Immunology*, 6(3), pp. 173–82. doi: 10.1038/nri1785.

National Audit Office (NAO) (2009) *Reducing Healthcare Associated Infections in Hospitals in England*. Available at: https://www.nao.org.uk/report/reducing-healthcare-associated-infections-in-hospitals-in-england/ (Accessed: 29 March 2016).

National Audit Office and Office, N. A. (2000) *The Management and Control of Hospital Acquired Infection in Acute NHS Trusts in England*. Available at: http://www.nao.org.uk/wp-content/uploads/2000/02/9900230.pdf (Accessed: 25 April 2013).

National Institute for Health and Care Excellence (2014) *Infection prevention and control*. NICE.

Netea, M. G., Simon, A., van de Veerdonk, F., Kullberg, B.-J., Van der Meer, J. W. M. and Joosten, L. A. B. (2010) 'IL-1beta processing in host defense: beyond the inflammasomes.', *PLoS pathogens*. Edited by M. Manchester. Public Library of Science, 6(2), p. e1000661. doi: 10.1371/journal.ppat.1000661.

Neuhauser, M. M. (2003) 'Antibiotic Resistance Among Gram-Negative Bacilli in US Intensive Care Units: Implications for Fluoroquinolone Use', *JAMA: The Journal of the American Medical Association*, 289(7), pp. 885–888. doi: 10.1001/jama.289.7.885.

NICE (2008) 'Technical patient safety solutions for ventilator-associated pneumonia in adults'. NICE.

NICE (2015) 'Antimicrobial stewardship : systems and processes for effectiv effective e antimicrobial medicine use', *National Institute for Health and Care Excellence*, (August). doi: 10.1017/CBO9781107415324.004.

NICE (2016) 'Sepsis: recognition, diagnosis and early management management. NG 51', (July), p. 50.

Nicks, B. A., Manthey, D. E. and Fitch, M. T. (2009) 'The centers for medicare and medicaid services (CMS) community-acquired pneumonia core measures lead to unnecessary antibiotic administration by emergency physicians', *Academic Emergency Medicine*, 16(2), pp. 184–187. doi: 10.1111/j.1553-2712.2008.00320.x.

van Nieuwenhoven, C. A., Vandenbroucke-Grauls, C., van Tiel, F. H., Joore, H. C. A., van Schijndel, R. J. M. S., van der Tweel, I., Ramsay, G. and Bonten, M. J. M. (2006) 'Feasibility and effects of the semirecumbent position to prevent ventilator-associated pneumonia: a randomized study.', *Critical care medicine*, 34(2), pp. 396–402.

Nseir, S., Favory, R., Jozefowicz, E., Decamps, F., Dewavrin, F., Brunin, G., Di Pompeo, C., Mathieu, D. and Durocher, A. (2008) 'Antimicrobial treatment for ventilator-associated tracheobronchitis: a randomized, controlled, multicenter study.', *Critical care (London, England)*, 12(3), p. R62. doi: 10.1186/cc6890.

Nseir, S., Martin-Loeches, I., Makris, D., Jaillette, E., Karvouniaris, M., Valles, J., Zakynthinos, E. and Artigas, A. (2014) 'Impact of appropriate antimicrobial treatment on transition from ventilator-associated tracheobronchitis to ventilator-associated pneumonia.', *Critical care (London, England)*, 18(3), p. R129. doi: 10.1186/cc13940.

O'Grady, N. P., Murray, P. R. and Ames, N. (2012) 'Preventing ventilator-associated pneumonia: does the evidence support the practice?', *JAMA : the journal of the American Medical Association*, 307(23), pp. 2534–9.

O'Neill, J. (2014) 'Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations', *Review on Antimicrobial Resistance*, (December), pp. 1–16.

O'Neill, J. (2016) 'Tackling Drug-Resistant Infections Globally: Final Report and Recommendations the Review on Antimicrobial Resistance', (May).

Ochoa-Ardila, M. E., García-Cañas, A., Gómez-Mediavilla, K., González-Torralba, A., Alía, I., García-Hierro, P., Taylor, N., van Saene, H. K. F. and de la Cal, M. A. (2011) 'Long-term use of selective decontamination of the digestive tract does not increase antibiotic resistance: a 5-year prospective cohort study.', *Intensive care medicine*, 37(9), pp. 1458–65. doi: 10.1007/s00134-011-2307-0.

de Oliveira, S., Reyes-Aldasoro, C. C., Candel, S., Renshaw, S. A., Mulero, V. and Calado, A. (2013) 'Cxcl8 (IL-8) Mediates Neutrophil Recruitment and Behavior in the Zebrafish Inflammatory Response', *The Journal of Immunology*. American Association of Immunologists, 190(8), pp. 4349–4359. doi: 10.4049/jimmunol.1203266.

Opal, S. M., Fisher, C. J., Dhainaut, J. F., Vincent, J. L., Brase, R., Lowry, S. F., Sadoff, J. C., Slotman, G. J., Levy, H., Balk, R. A., Shelly, M. P., Pribble, J. P., LaBrecque, J. F., Lookabaugh, J., Donovan, H., Dubin, H., Baughman, R., Norman, J., DeMaria, E., Matzel, K., Abraham, E. and Seneff, M. (1997) ‘Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group.’, *Critical care medicine*, 25(7), pp. 1115–24.

Ordonez, C. L., Shaughnessy, T. E., Matthay, M. A. and Fahy, J. V (2000) ‘Increased Neutrophil Numbers and IL-8 Levels in Airway Secretions in Acute Severe Asthma’, *American Journal of Respiratory and Critical Care Medicine*. American Thoracic SocietyNew York, NY, 161(4), pp. 1185–1190. doi: 10.1164/ajrccm.161.4.9812061.

Ost, D. E., Poch, D., Fadel, A., Wettimuny, S., Ginocchio, C. and Wang, X.-P. (2010) ‘Mini-bronchoalveolar lavage quantitative polymerase chain reaction for diagnosis of methicillin-resistant Staphylococcus aureus pneumonia.’, *Critical care medicine*, 38(7), pp. 1536–41. doi: 10.1097/CCM.0b013e3181e2ca78.

Oudhuis, G. J., Beuving, J., Bergmans, D., Stobberingh, E. E., ten Velde, G., Linssen, C. F. and Verbon, A. (2009) ‘Soluble Triggering Receptor Expressed on Myeloid cells-1 in bronchoalveolar lavage fluid is not predictive for ventilator-associated pneumonia.’, *Intensive care medicine*, 35(7), pp. 1265–70. doi: 10.1007/s00134-009-1463-y.

Özçaka, Ö., Başoğlu, O. K., Buduneli, N., Taşbakan, M. S., Bacakoğlu, F. and Kinane, D. F. (2012) ‘Chlorhexidine decreases the risk of ventilator-associated pneumonia in intensive care unit patients: a randomized clinical trial.’, *Journal of periodontal research*, 47(5), pp. 584–92. doi: 10.1111/j.1600-0765.2012.01470.x.

Palmer, L. B. (1995) ‘Tracheal Aspirates in Long-term Mechanically Ventilated Patients’, *CHEST Journal*. American College of Chest Physicians, 108(5), p. 1326. doi: 10.1378/chest.108.5.1326.

Palmer, L. B., Smaldone, G. C., Chen, J. J., Baram, D., Duan, T., Monteforte, M., Varela, M., Tempone, A. K., O’Riordan, T., Daroowalla, F. and Richman, P. (2008) ‘Aerosolized antibiotics and ventilator-associated tracheobronchitis in the intensive care unit.’, *Critical care medicine*, 36(7), pp. 2008–13. doi: 10.1097/CCM.0b013e31817c0f9e.

Panchabhai, T. S., Dangayach, N. S., Krishnan, A., Kothari, V. M. and Karnad, D. R. (2009) ‘Oropharyngeal cleansing with 0.2% chlorhexidine for prevention of nosocomial

pneumonia in critically ill patients: an open-label randomized trial with 0.01% potassium permanganate as control.', *Chest*, 135(5), pp. 1150–6. doi: 10.1378/chest.08-1321.

Papazian, L., Bregeon, F., Thirion, X., Gregoire, R., Saux, P., Denis, J. P., Perin, G., Charrel, J., Dumon, J. F., Affray, J. P. and Gouin, F. (1996) 'Effect of ventilator-associated pneumonia on mortality and morbidity.', *American journal of respiratory and critical care medicine*, 154(1), pp. 91–7.

Parker, C. M., Kutsogiannis, J., Muscedere, J., Cook, D., Dodek, P., Day, A. G. and Heyland, D. K. (2008) 'Ventilator-associated pneumonia caused by multidrug-resistant organisms or Pseudomonas aeruginosa: prevalence, incidence, risk factors, and outcomes.', *Journal of critical care*, 23(1), pp. 18–26. doi: 10.1016/j.jcrc.2008.02.001.

Parmentier-Decrucq, E., Nseir, S., Makris, D., Desrousseaux, B., Soudan, B., Favory, R. and Mathieu, D. (2014) 'Accuracy of leptin serum level in diagnosing ventilator-associated pneumonia: a case-control study.', *Minerva anestesiologica*, 80(1), pp. 39–47.

Paul, M., Benuri-Silbiger, I., Soares-Weiser, K. and Leibovici, L. (2004) 'Beta lactam monotherapy versus beta lactam-aminoglycoside combination therapy for sepsis in immunocompetent patients: systematic review and meta-analysis of randomised trials.', *BMJ (Clinical research ed.)*, 328(7441), p. 668. doi: 10.1136/bmj.38028.520995.63.

Perez, K. K., Olsen, R. J., Musick, W. L., Cernoch, P. L., Davis, J. R., Peterson, L. E. and Musser, J. M. (2014) 'Integrating Rapid Diagnostics and Antimicrobial Stewardship Improves Outcomes in Patients with Antibiotic-resistant Gram-negative Bacteremia.', *The Journal of infection*. doi: 10.1016/j.jinf.2014.05.005.

Pileggi, C., Bianco, A., Flotta, D., Nobile, C. G. A. and Pavia, M. (2011) 'Prevention of ventilator-associated pneumonia, mortality and all intensive care unit acquired infections by topically applied antimicrobial or antiseptic agents: a meta-analysis of randomized controlled trials in intensive care units.', *Critical care (London, England)*, 15(3), p. R155. doi: 10.1186/cc10285.

Pittet, D., Hugonnet, S., Harbarth, S., Mourouga, P., Sauvan, V., Touveneau, S. and Perneger, T. V (2000) 'Effectiveness of a hospital-wide programme to improve compliance with hand hygiene. Infection Control Programme.', *Lancet*, 356(9238), pp. 1307–12.

Pittet, L. A., Quinton, L. J., Yamamoto, K., Robson, B. E., Ferrari, J. D., Algül, H.,

Schmid, R. M. and Mizgerd, J. P. (2011) ‘Earliest innate immune responses require macrophage RelA during pneumococcal pneumonia.’, *American journal of respiratory cell and molecular biology*. American Thoracic Society, 45(3), pp. 573–81. doi: 10.1165/rcmb.2010-0210OC.

Price, R., Maclennan, G. and Glen, J. (2014) ‘Selective digestive or oropharyngeal decontamination and topical oropharyngeal chlorhexidine for prevention of death in general intensive care: systematic review and network meta-analysis.’, *BMJ (Clinical research ed.)*, 348(mar31_2), p. g2197. doi: 10.1136/bmj.g2197.

Pronovost, P., Needham, D., Berenholtz, S., Sinopoli, D., Chu, H., Cosgrove, S., Sexton, B., Hyzy, R., Welsh, R., Roth, G., Bander, J., Kepros, J. and Goeschel, C. (2006) ‘An intervention to decrease catheter-related bloodstream infections in the ICU.’, *The New England journal of medicine*, 355(26), pp. 2725–32. doi: 10.1056/NEJMoa061115.

Pugin, J., Auckenthaler, R., Mili, N., Janssens, J.-P., P. Daniel Lew and Suter, P. M. (1991) ‘Diagnosis of Ventilator-associated Pneumonia by Bacteriologic Analysis of Bronchoscopic and Nonbronchoscopic “Blind” Bronchoalveolar Lavage Fluid’, *American Journal of Respiratory and Critical Care Medicine*. American Thoracic Society, 143(5 Pt 1), pp. 1121–1129. doi: 10.1164/ajrccm/143.5_Pt_1.1121.

Puskarich, M. A., Trzeciak, S., Shapiro, N. I., Arnold, R. C., Horton, J. M., Studnek, J. R., Kline, J. A. and Jones, A. E. (2011) ‘Association between timing of antibiotic administration and mortality from septic shock in patients treated with a quantitative resuscitation protocol*’, *Critical Care Medicine*, 39(9), pp. 2066–2071. doi: 10.1097/CCM.0b013e31821e87ab.

Rahal, J. J., Urban, C., Horn, D., Freeman, K., Segal-Maurer, S., Maurer, J., Mariano, N., Marks, S., Burns, J. M., Dominick, D. and Lim, M. (1998) ‘Class restriction of cephalosporin use to control total cephalosporin resistance in nosocomial Klebsiella.’, *JAMA*, 280(14), pp. 1233–7.

Raman, K., Nailor, M. D., Nicolau, D. P., Aslanzadeh, J., Nadeau, M. and Kuti, J. L. (2013) ‘Early antibiotic discontinuation in patients with clinically suspected ventilator-associated pneumonia and negative quantitative bronchoscopy cultures.’, *Critical care medicine*, 41(7), pp. 1656–63. doi: 10.1097/CCM.0b013e318287f713.

Du Rand, I. A., Blaikley, J., Booton, R., Chaudhuri, N., Gupta, V., Khalid, S., Mandal, S., Martin, J., Mills, J., Navani, N., Rahman, N. M., Wrightson, J. M. and Munavvar, M.

(2013) ‘British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults: accredited by NICE.’, *Thorax*, 68 Suppl 1(Suppl_1), pp. i1–i44. doi: 10.1136/thoraxjnl-2013-203618.

Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G. M., Bolsover, S., Gabella, G., Potma, E. O., Warley, A., Roes, J. and Segal, A. W. (2002) ‘Killing activity of neutrophils is mediated through activation of proteases by K+ flux’, *Nature*. Nature Publishing Group, 416(6878), pp. 291–297. doi: 10.1038/416291a.

Rello, J., Afonso, E., Lisboa, T., Ricart, M., Balsera, B., Rovira, A., Valles, J. and Diaz, E. (2013) ‘A care bundle approach for prevention of ventilator-associated pneumonia.’, *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 19(4), pp. 363–9. doi: 10.1111/j.1469-0691.2012.03808.x.

Rello, J., Ollendorf, D. A., Oster, G., Vera-Llonch, M., Bellm, L., Redman, R. and Kollef, M. H. (2002) ‘Epidemiology and outcomes of ventilator-associated pneumonia in a large US database.’, *Chest*, 122(6), pp. 2115–21.

Rello, J., Sa-Borges, M., Correa, H., Leal, S. R. and Baraibar, J. (1999) ‘Variations in etiology of ventilator-associated pneumonia across four treatment sites: implications for antimicrobial prescribing practices.’, *American journal of respiratory and critical care medicine*, 160(2), pp. 608–13. doi: 10.1164/ajrccm.160.2.9812034.

Rello, J., Soñora, R., Jubert, P., Artigas, A., Rué, M. and Vallés, J. (1996) ‘Pneumonia in intubated patients: role of respiratory airway care.’, *American journal of respiratory and critical care medicine*, 154(1), pp. 111–5. doi: 10.1164/ajrccm.154.1.8680665.

Rennard, S. I., Basset, G., Lecossier, D., O’Donnell, K. M., Pinkston, P., Martin, P. G. and Crystal, R. G. (1986) ‘Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution.’, *Journal of applied physiology (Bethesda, Md. : 1985)*, 60(2), pp. 532–8.

Restrepo, M. I., Anzueto, A., Arroliga, A. C., Afessa, B., Atkinson, M. J., Ho, N. J., Schinner, R., Bracken, R. L. and Kollef, M. H. (2010) ‘Economic burden of ventilator-associated pneumonia based on total resource utilization.’, *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 31(5), pp. 509–15. doi: 10.1086/651669.

Rios-Licea, M. M., Bosques, F. J., Arroliga, A. C., Galindo-Galindo, J. O. and Garza-Gonzalez, E. (2010) ‘Quadruplex real-time quantitative PCR assay for the detection of

pathogens related to late-onset ventilator-associated pneumonia: A preliminary report', *Journal of Microbiological Methods*, 81(3), pp. 232–234. doi: 10.1016/j.mimet.2010.03.018.

Rotstein, C., Evans, G., Born, A., Grossman, R., Light, R. B., Magder, S., McTaggart, B., Weiss, K. and Zhanel, G. G. (2008) 'Clinical practice guidelines for hospital-acquired pneumonia and ventilator-associated pneumonia in adults.', *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie médicale / AMMI Canada*, 19(1), pp. 19–53.

Rouby, J. J., Laurent, P., Gosnach, M., Cambau, E., Lamas, G., Zouaoui, A., Leguillou, J. L., Bodin, L., Khac, T. D. and Marsault, C. (1994) 'Risk factors and clinical relevance of nosocomial maxillary sinusitis in the critically ill.', *American journal of respiratory and critical care medicine*, 150(3), pp. 776–83. doi: 10.1164/ajrccm.150.3.8087352.

Rouby, J. J., Martin De Lassale, E., Poete, P., Nicolas, M. H., Bodin, L., Jarlier, V., Le Charpentier, Y., Grosset, J. and Viars, P. (1992) 'Nosocomial bronchopneumonia in the critically ill. Histologic and bacteriologic aspects.', *The American review of respiratory disease*, 146(4), pp. 1059–66.

Roustan, J. P., Kienlen, J., Aubas, P., Aubas, S. and du Cailar, J. (1992) 'Comparison of hydrophobic heat and moisture exchangers with heated humidifier during prolonged mechanical ventilation.', *Intensive care medicine*, 18(2), pp. 97–100.

Ruiz, M., Torres, A., Ewig, S., Marcos, M. A., Alcón, A., Lledó, R., Asenjo, M. A. and Maldonaldo, A. (2000) 'Noninvasive versus invasive microbial investigation in ventilator-associated pneumonia: evaluation of outcome.', *American journal of respiratory and critical care medicine*, 162(1), pp. 119–25.

Safdar, N., Dezfulian, C., Collard, H. R. and Saint, S. (2005) 'Clinical and economic consequences of ventilator-associated pneumonia: a systematic review.', *Critical care medicine*, 33(10), pp. 2184–93.

Safdar, N., Handelsman, J. and Maki, D. G. (2004) 'Does combination antimicrobial therapy reduce mortality in Gram-negative bacteraemia? A meta-analysis.', *The Lancet. Infectious diseases*, 4(8), pp. 519–27. doi: 10.1016/S1473-3099(04)01108-9.

Salord, F., Gaussorgues, P., Marti-Flich, J., Sirodot, M., Allimant, C., Lyonnet, D. and Robert, D. (1990) 'Nosocomial maxillary sinusitis during mechanical ventilation: a prospective comparison of orotracheal versus the nasotracheal route for intubation.',

Intensive care medicine, 16(6), pp. 390–3.

Sanchez-Nieto, J. M., Torres, A., Garcia-Cordoba, F., El-Ebiary, M., Carrillo, A., Ruiz, J., Nuñez, M. L. and Niederman, M. (1998) ‘Impact of invasive and noninvasive quantitative culture sampling on outcome of ventilator-associated pneumonia: a pilot study.’, *American journal of respiratory and critical care medicine*, 157(2), pp. 371–6.

Sánchez García, M., Cambronero Galache, J. A., López Diaz, J., Cerdá Cerdá, E., Rubio Blasco, J., Gómez Aguinaga, M. A., Núnez Reiz, A., Rogero Marín, S., Onoro Canaveral, J. J. and Sacristán del Castillo, J. A. (1998) ‘Effectiveness and cost of selective decontamination of the digestive tract in critically ill intubated patients. A randomized, double-blind, placebo-controlled, multicenter trial.’, *American journal of respiratory and critical care medicine*. American Thoracic SocietyNew York, NY, 158(3), pp. 908–16. doi: 10.1164/ajrccm.158.3.9712079.

Sarkar, M., Hennessy, S. and Yang, Y.-X. (2008) ‘Proton-pump inhibitor use and the risk for community-acquired pneumonia.’, *Annals of internal medicine*, 149(6), pp. 391–8.

Scannapieco, F. A., Yu, J., Raghavendran, K., Vacanti, A., Owens, S. I., Wood, K. and Mylotte, J. M. (2009) ‘A randomized trial of chlorhexidine gluconate on oral bacterial pathogens in mechanically ventilated patients.’, *Critical care (London, England)*, 13(4), p. R117. doi: 10.1186/cc7967.

Schuetz, P., Christ-Crain, M., Thomann, R., Falconnier, C., Wolbers, M., Widmer, I., Neidert, S., Fricker, T., Blum, C., Schild, U., Regez, K., Schoenenberger, R., Henzen, C., Bregenzer, T., Hoess, C., Krause, M., Bucher, H. C., Zimmerli, W., Mueller, B. and ProHOSP Study Group (2009) ‘Effect of procalcitonin-based guidelines vs standard guidelines on antibiotic use in lower respiratory tract infections: the ProHOSP randomized controlled trial.’, *Jama*, 302(10), pp. 1059–66. doi: 10.1001/jama.2009.1297.

Schulz, K. F., Altman, D. G. and Moher, D. (2010) ‘CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials.’, *BMJ (Clinical research ed.)*, 340(mar23_1), p. c332. doi: 10.1136/bmj.c332.

Schurink, C. A. M., Van Nieuwenhoven, C. A., Jacobs, J. A., Rozenberg-Arska, M., Joore, H. C. A., Buskens, E., Hoepelman, A. I. M. and Bonten, M. J. M. (2004) ‘Clinical pulmonary infection score for ventilator-associated pneumonia: accuracy and inter-observer variability.’, *Intensive care medicine*, 30(2), pp. 217–24. doi:

10.1007/s00134-003-2018-2.

Seegobin, R. D. and van Hasselt, G. L. (1986) ‘Aspiration beyond endotracheal cuffs.’, *Canadian Anaesthetists’ Society journal*, 33(3 Pt 1), pp. 273–9.

Segal, A. W. (2005) ‘How neutrophils kill microbes.’, *Annual review of immunology*, 23(3), pp. 197–223. doi: 10.1146/annurev.immunol.23.021704.115653.

Segers, P., Speekenbrink, R. G. H., Ubbink, D. T., van Ogtrop, M. L. and de Mol, B. A. (2006) ‘Prevention of nosocomial infection in cardiac surgery by decontamination of the nasopharynx and oropharynx with chlorhexidine gluconate: a randomized controlled trial.’, *JAMA : the journal of the American Medical Association*. American Medical Association, 296(20), pp. 2460–6. doi: 10.1001/jama.296.20.2460.

Seguin, P., Tanguy, M., Laviolle, B., Tirel, O. and Mallédant, Y. (2006) ‘Effect of oropharyngeal decontamination by povidone-iodine on ventilator-associated pneumonia in patients with head trauma.’, *Critical care medicine*, 34(5), pp. 1514–9. doi: 10.1097/01.CCM.0000214516.73076.82.

Selective Decontamination of the Digestive Tract Trialist’ Collaborative Group (1993) ‘Meta-analysis of randomised controlled trials of selective decontamination of the digestive tract. Selective Decontamination of the Digestive Tract Trialists’ Collaborative Group.’, *BMJ*, 307(6903), pp. 525–32.

Seligman, R., Meisner, M., Lisboa, T. C., Hertz, F. T., Filippin, T. B., Fachel, J. M. G. and Teixeira, P. J. Z. (2006) ‘Decreases in procalcitonin and C-reactive protein are strong predictors of survival in ventilator-associated pneumonia.’, *Critical care (London, England)*, 10(5), p. R125. doi: 10.1186/cc5036.

Seligman, R., Papassotiriou, J., Morgenthaler, N. G., Meisner, M. and Teixeira, P. J. Z. (2008a) ‘Copeptin, a novel prognostic biomarker in ventilator-associated pneumonia.’, *Critical care (London, England)*, 12(1), p. R11. doi: 10.1186/cc6780.

Seligman, R., Papassotiriou, J., Morgenthaler, N. G., Meisner, M. and Teixeira, P. J. Z. (2008b) ‘Prognostic value of midregional pro-atrial natriuretic peptide in ventilator-associated pneumonia.’, *Intensive care medicine*, 34(11), pp. 2084–91. doi: 10.1007/s00134-008-1173-x.

Shakoory, B., Carcillo, J. A., Chatham, W. W., Amdur, R. L., Zhao, H., Dinarello, C. A., Cron, R. Q. and Opal, S. M. (2016) ‘Interleukin-1 Receptor Blockade Is Associated With Reduced Mortality in Sepsis Patients With Features of Macrophage Activation

Syndrome: Reanalysis of a Prior Phase III Trial.', *Critical care medicine*, 44(2), pp. 275–81. doi: 10.1097/CCM.0000000000001402.

Shapiro, D. J., Hicks, L. A., Pavia, A. T. and Hersh, A. L. (2014) 'Antibiotic prescribing for adults in ambulatory care in the USA, 2007-09', *Journal of Antimicrobial Chemotherapy*, 69(1), pp. 234–240. doi: 10.1093/jac/dkt301.

Shehabi, Y., Sterba, M., Garrett, P. M., Rachakonda, K. S., Stephens, D., Harrigan, P., Walker, A., Bailey, M. J., Johnson, B., Millis, D., Ding, G., Peake, S., Wong, H., Thomas, J., Smith, K., Forbes, L., Hardie, M., Micallef, S., Fraser, J. F., Cheng, R., Howe, B., Obrien, R., Janu, M., Ihle, B., Pather, V., Robertson, M., Pollock, E., Ketchley, I., Srinivasan, S., Brailsford, J., Spooner, A., Sharpe, N., Thomas, P., Arora, S., Flynn, G., Tey, W. Y., Williams, P., Kurenda, C. and Geng, W. (2014) 'Procalcitonin algorithm in critically ill adults with undifferentiated infection or suspected sepsis: A randomized controlled trial', *American Journal of Respiratory and Critical Care Medicine*, 190(10), pp. 1102–1110. doi: 10.1164/rccm.201408-1483OC.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B. and Fridkin, S. (2012) 'Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010'. University of Chicago PressChicago, IL.

Silvestri, L., van Saene, H. K. F., Weir, I. and Gullo, A. (2009) 'Survival benefit of the full selective digestive decontamination regimen.', *Journal of critical care*. Elsevier Inc., 24(3), p. 474.e7-14. doi: 10.1016/j.jcrc.2008.11.005.

Sims, J. E. and Smith, D. E. (2010) 'The IL-1 family: regulators of immunity.', *Nature reviews. Immunology*. Nature Publishing Group, 10(2), pp. 89–102. doi: 10.1038/nri2691.

Singal, A. G., Higgins, P. D. R. and Waljee, A. K. (2014) 'A primer on effectiveness and efficacy trials.', *Clinical and translational gastroenterology*. Nature Publishing Group, 5(2), p. e45. doi: 10.1038/ctg.2013.13.

Singh, N., Falestiny, M. N., Rogers, P., Reed, M. J., Pularski, J., Norris, R. and Yu, V. L. (1998) 'Pulmonary infiltrates in the surgical ICU: prospective assessment of predictors of etiology and mortality.', *Chest*, 114(4), pp. 1129–36.

Singh, N., Rogers, P., Atwood, C. W., Wagener, M. M. and Yu, V. L. (2000) 'Short-course empiric antibiotic therapy for patients with pulmonary infiltrates in the intensive

care unit. A proposed solution for indiscriminate antibiotic prescription.', *American journal of respiratory and critical care medicine*. American Thoracic Society, 162(2 Pt 1), pp. 505–11.

Skrupky, L. P., McConnell, K., Dallas, J. and Kollef, M. H. (2012) 'A comparison of ventilator-associated pneumonia rates as identified according to the National Healthcare Safety Network and American College of Chest Physicians criteria.', *Critical care medicine*, 40(1), pp. 281–4. doi: 10.1097/CCM.0b013e31822d7913.

de Smet, A. M. G. A., Kluytmans, J. A. J. W., Blok, H. E. M., Mascini, E. M., Benus, R. F. J., Bernards, A. T., Kuijper, E. J., Leverstein-van Hall, M. A., Jansz, A. R., de Jongh, B. M., van Asselt, G. J., Frenay, I. H. M. E., Thijsen, S. F. T., Conijn, S. N. M., Kaan, J. A., Arends, J. P., Sturm, P. D. J., Bootsma, M. C. J. and Bonten, M. J. M. (2011) 'Selective digestive tract decontamination and selective oropharyngeal decontamination and antibiotic resistance in patients in intensive-care units: an open-label, clustered group-randomised, crossover study.', *The Lancet infectious diseases*, 11(5), pp. 372–80. doi: 10.1016/S1473-3099(11)70035-4.

de Smet, A. M. G. A., Kluytmans, J. A. J. W., Cooper, B. S., Mascini, E. M., Benus, R. F. J., van der Werf, T. S., van der Hoeven, J. G., Pickkers, P., Bogaers-Hofman, D., van der Meer, N. J. M., Bernards, A. T., Kuijper, E. J., Joore, J. C. A., Leverstein-van Hall, M. A., Bindels, A. J. G. H., Jansz, A. R., Wesselink, R. M. J., de Jongh, B. M., Dennesen, P. J. W., van Asselt, G. J., te Velde, L. F., Frenay, I. H. M. E., Kaasjager, K., Bosch, F. H., van Iterson, M., Thijsen, S. F. T., Kluge, G. H., Pauw, W., de Vries, J. W., Kaan, J. A., Arends, J. P., Aarts, L. P. H. J., Sturm, P. D. J., Harinck, H. I. J., Voss, A., Uijtendaal, E. V, Blok, H. E. M., Thieme Groen, E. S., Pouw, M. E., Kalkman, C. J. and Bonten, M. J. M. (2009) 'Decontamination of the digestive tract and oropharynx in ICU patients.', *The New England journal of medicine*, 360(1), pp. 20–31. doi: 10.1056/NEJMoa0800394.

Smith, R. and Coast, J. (2013) 'The true cost of antimicrobial resistance', *BMJ*, 346(March), p. 5. doi: 10.1136/bmj.f1493.

Solé Violán, J., Fernández, J. A., Benítez, A. B., Cardeñosa Cendrero, J. A. and Rodríguez de Castro, F. (2000) 'Impact of quantitative invasive diagnostic techniques in the management and outcome of mechanically ventilated patients with suspected pneumonia.', *Critical care medicine*, 28(8), pp. 2737–41.

Souweine, B., Veber, B., Bedos, J. P., Gachot, B., Dombret, M. C., Regnier, B. and

Wolff, M. (1998) ‘Diagnostic accuracy of protected specimen brush and bronchoalveolar lavage in nosocomial pneumonia: impact of previous antimicrobial treatments.’, *Critical care medicine*, 26(2), pp. 236–44.

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G. and Edwards, J. E. (2004) ‘Trends in antimicrobial drug development: implications for the future.’, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. Oxford University Press, 38(9), pp. 1279–86. doi: 10.1086/420937.

Stillie, R., Farooq, S. M., Gordon, J. R. and Stadnyk, A. W. (2009) ‘The functional significance behind expressing two IL-8 receptor types on PMN.’, *Journal of leukocyte biology*, 86(3), pp. 529–43. doi: 10.1189/jlb.0208125.

Stolz, D., Smyrnios, N., Eggimann, P., Pargger, H., Thakkar, N., Siegemund, M., Marsch, S., Azzola, A., Rakic, J., Mueller, B. and Tamm, M. (2009) ‘Procalcitonin for reduced antibiotic exposure in ventilator-associated pneumonia: a randomised study.’, *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 34(6), pp. 1364–75. doi: 10.1183/09031936.00053209.

Stoutenbeek, C. P., Saene, H. K. F., Miranda, D. R. and Zandstra, D. F. (1984) ‘The effect of selective decontamination of the digestive tract on colonisation and infection rate in multiple trauma patients’, *Intensive Care Medicine*, 10(4), pp. 185–192. doi: 10.1007/BF00259435.

Stoutenbeek, C. P., van Saene, H. K. F., Little, R. A. and Whitehead, A. (2007) ‘The effect of selective decontamination of the digestive tract on mortality in multiple trauma patients: a multicenter randomized controlled trial.’, *Intensive care medicine*, 33(2), pp. 261–70. doi: 10.1007/s00134-006-0455-4.

Subirana, M., Solà, I. and Benito, S. (2007) ‘Closed tracheal suction systems versus open tracheal suction systems for mechanically ventilated adult patients.’, *The Cochrane database of systematic reviews*, (4), p. CD004581. doi: 10.1002/14651858.CD004581.pub2.

Tang, B. M. P., Eslick, G. D., Craig, J. C. and McLean, A. S. (2007) ‘Accuracy of procalcitonin for sepsis diagnosis in critically ill patients: systematic review and meta-analysis.’, *The Lancet infectious diseases*, 7(3), pp. 210–7. doi: 10.1016/S1473-3099(07)70052-X.

Tantipong, H., Morkchareonpong, C., Jaiyindee, S. and Thamlikitkul, V. (2008) ‘Randomized controlled trial and meta-analysis of oral decontamination with 2%

chlorhexidine solution for the prevention of ventilator-associated pneumonia.', *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*. The University of Chicago Press, 29(2), pp. 131–6. doi: 10.1086/526438.

Team, R. C. (2013) *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*.

Tejerina, E., Esteban, A., Fernandez-Segoviano, P., Frutos-Vivar, F., Aramburu, J., Ballesteros, D. and Rodriguez-Barbero, J. M. (2010) 'Accuracy of clinical definitions of ventilator-associated pneumonia: comparison with autopsy findings', *J Crit Care*. 2009/07/14, 25(1), pp. 62–68. doi: 10.1016/j.jcrc.2009.05.008.

Textoris, J., Loriod, B., Benayoun, L., Gourraud, P.-A., Puthier, D., Albanèse, J., Mantz, J., Martin, C., Nguyen, C. and Leone, M. (2011) 'An evaluation of the role of gene expression in the prediction and diagnosis of ventilator-associated pneumonia.', *Anesthesiology*, 115(2), pp. 344–52. doi: 10.1097/ALN.0b013e318225ba26.

Thomachot, L., Leone, M., Razzouk, K., Antonini, F., Vialet, R. and Martin, C. (2002) 'Randomized clinical trial of extended use of a hydrophobic condenser humidifier: 1 vs. 7 days.', *Critical care medicine*, 30(1), pp. 232–7.

Thorley, A. J., Ford, P. A., Giembycz, M. A., Goldstraw, P., Young, A. and Tetley, T. D. (2007) 'Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 178(1), pp. 463–73.

Timsit, J. F., Misset, B., Renaud, B., Goldstein, F. W. and Carlet, J. (1995) 'Effect of previous antimicrobial therapy on the accuracy of the main procedures used to diagnose nosocomial pneumonia in patients who are using ventilation.', *Chest*, 108(4), pp. 1036–40.

Tkalcevic, J., Novelli, M., Phylactides, M., Iredale, J. P., Segal, A. W. and Roes, J. (2000) 'Impaired Immunity and Enhanced Resistance to Endotoxin in the Absence of Neutrophil Elastase and Cathepsin G', *Immunity*, 12(2), pp. 201–210. doi: 10.1016/S1074-7613(00)80173-9.

Torres, A., El-Ebiary, M., Padro, L., Gonzalez, J., de la Bellacasa, J. P., Ramirez, J., Xaubet, A., Ferrer, M. and Rodriguez-Roisin, R. (1994) 'Validation of different techniques for the diagnosis of ventilator-associated pneumonia. Comparison with

immediate postmortem pulmonary biopsy', *Am J Respir Crit Care Med*. 1994/02/01, 149(2 Pt 1), pp. 324–331.

Torres, A., Serra-Batlles, J., Ros, E., Piera, C., Puig de la Bellacasa, J., Cobos, A., Lomeña, F. and Rodríguez-Roisin, R. (1992) 'Pulmonary aspiration of gastric contents in patients receiving mechanical ventilation: the effect of body position.', *Annals of internal medicine*, 116(7), pp. 540–3.

Trouillet, J. L., Chastre, J., Vuagnat, A., Joly-Guillou, M. L., Combaux, D., Dombret, M. C. and Gibert, C. (1998) 'Ventilator-associated pneumonia caused by potentially drug-resistant bacteria.', *American journal of respiratory and critical care medicine*, 157(2), pp. 531–9. doi: 10.1164/ajrccm.157.2.9705064.

Umscheid, C. A., Mitchell, M. D., Doshi, J. A., Agarwal, R., Williams, K. and Brennan, P. J. (2011) 'Estimating the proportion of healthcare-associated infections that are reasonably preventable and the related mortality and costs.', *Infection control and hospital epidemiology*, 32(2), pp. 101–14. doi: 10.1086/657912.

Uzzan, B., Cohen, R., Nicolas, P., Cucherat, M. and Perret, G.-Y. (2006) 'Procalcitonin as a diagnostic test for sepsis in critically ill adults and after surgery or trauma: a systematic review and meta-analysis.', *Critical care medicine*, 34(7), pp. 1996–2003. doi: 10.1097/01.CCM.0000226413.54364.36.

Vandenbroucke-Grauls, C. M. and Vandenbroucke, J. P. (1991) 'Effect of selective decontamination of the digestive tract on respiratory tract infections and mortality in the intensive care unit.', *Lancet*, 338(8771), pp. 859–62.

Vanspauwen, M. J., Linssen, C. F. M., Bruggeman, C. A., Jacobs, J. A., Drent, M., Bergmans, D. C. J. J. and van Mook, W. N. K. A. (2011) 'Clara cell protein in bronchoalveolar lavage fluid: a predictor of ventilator-associated pneumonia?', *Critical care (London, England)*, 15(1), p. R14. doi: 10.1186/cc9418.

Versporten, A., Bolokhovets, G., Ghazaryan, L., Abilova, V., Pyshnik, G., Spasojevic, T., Korinteli, I., Raka, L., Kambaralieva, B., Cizmovic, L., Carp, A., Radonjic, V., Maqsudova, N., Celik, H. D., Payerl-Pal, M., Pedersen, H. B., Sautenkova, N. and Goossens, H. (2014) 'Antibiotic use in eastern Europe: a cross-national database study in coordination with the WHO Regional Office for Europe.', *The Lancet. Infectious diseases*, 14(5), pp. 381–7. doi: 10.1016/S1473-3099(14)70071-4.

Verwaest, C., Verhaegen, J., Ferdinande, P., Schetz, M., Van den Berghe, G., Verbist, L. and Lauwers, P. (1997) 'Randomized, controlled trial of selective digestive

decontamination in 600 mechanically ventilated patients in a multidisciplinary intensive care unit.', *Critical care medicine*, 25(1), pp. 63–71.

Vincent, J.-L., Brealey, D., Libert, N., Abidi, N. E., O'Dwyer, M., Zacharowski, K., Mikaszewska-Sokolewicz, M., Schrenzel, J., Simon, F., Wilks, M., Picard-Maureau, M., Chalfin, D. B., Ecker, D. J., Sampath, R. and Singer, M. (2015) 'Rapid Diagnosis of Infection in the Critically Ill, a Multicenter Study of Molecular Detection in Bloodstream Infections, Pneumonia, and Sterile Site Infections', *Critical Care Medicine*, p. 1. doi: 10.1097/CCM.0000000000001249.

Vincent, J.-L., Moreno, R., Takala, J., Willatts, S., Mendonça, A., Bruining, H., Reinhart, C. K., Suter, P. M. and Thijs, L. G. (1996) 'The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure', *Intensive Care Medicine*, 22(7), pp. 707–710. doi: 10.1007/BF01709751.

Vincent, J.-L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., Moreno, R., Lipman, J., Gomersall, C., Sakr, Y. and Reinhart, K. (2009) 'International study of the prevalence and outcomes of infection in intensive care units.', *JAMA : the journal of the American Medical Association*, 302(21), pp. 2323–9. doi: 10.1001/jama.2009.1754.

Van der Waaij, D., Berghuis-de Vries, J. M. and Lekkerkerk-van der Wees, J. E. C. (1971) 'Colonization resistance of the digestive tract in conventional and antibiotic-treated mice', *Journal of Hygiene*. Cambridge University Press, 69(3), p. 405. doi: 10.1017/S0022172400021653.

Wacker, C., Prkno, A., Brunkhorst, F. M. and Schlattmann, P. (2013) 'Procalcitonin as a diagnostic marker for sepsis: a systematic review and meta-analysis.', *The Lancet. Infectious diseases*, 13(5), pp. 426–35. doi: 10.1016/S1473-3099(12)70323-7.

Walden, A. P., Bonten, M. J. and Wise, M. P. (2012) 'Should selective digestive decontamination be used in critically ill patients?', *BMJ (Clinical research ed.)*, 345(oct09_1), p. e6697. doi: 10.1136/bmj.e6697.

Walsh, T. S., Morris, A. C. and Simpson, A. J. (2013) 'Ventilator associated pneumonia: can we ensure that a quality indicator does not become a game of chance?', *British Journal of Anaesthesia*, 111(3), pp. 333–337. doi: 10.1093/bja/aet131.

Wang, F., Bo, L., Tang, L., Lou, J., Wu, Y., Chen, F., Li, J. and Deng, X. (2012) 'Subglottic secretion drainage for preventing ventilator-associated pneumonia: an updated meta-analysis of randomized controlled trials.', *The journal of trauma and acute care surgery*, 72(5), pp. 1276–85.

Ward, C., Duddridge, M., Fenwick, J., Williams, S., Gardiner, P. V, Hendrick, D. J. and Walters, E. H. (1992) 'The origin of water and urea sampled at bronchoalveolar lavage in asthmatic and control subjects.', *The American review of respiratory disease*, 146(2), pp. 444–7. doi: 10.1164/ajrccm/146.2.444.

Ward, C., Thien, F., Secombe, J., Gollant, S. and Walters, E. H. (2000) 'Bronchoalveolar lavage fluid urea as a measure of pulmonary permeability in healthy smokers.', *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology*, 15(2), pp. 285–90.

Warren, D. K., Shukla, S. J., Olsen, M. A., Kollef, M. H., Hollenbeak, C. S., Cox, M. J., Cohen, M. M. and Fraser, V. J. (2003) 'Outcome and attributable cost of ventilator-associated pneumonia among intensive care unit patients in a suburban medical center.', *Critical care medicine*, 31(5), pp. 1312–7. doi: 10.1097/01.CCM.0000063087.93157.06.

Wiener, J., Itokazu, G., Nathan, C., Kabins, S. A. and Weinstein, R. A. (1995) 'A Randomized, Double-Blind, Placebo-Controlled Trial of Selective Digestive Decontamination in a Medical-Surgical Intensive Care Unit', *Clinical Infectious Diseases*, 20(4), pp. 861–867. doi: 10.1093/clinids/20.4.861.

Wilkinson, T. S., Morris, A. C., Kefala, K., O'Kane, C. M., Moore, N. R., Booth, N. A., McAuley, D. F., Dhaliwal, K., Walsh, T. S., Haslett, C., Sallenave, J.-M. and Simpson, A. J. (2012) 'Ventilator-associated pneumonia is characterized by excessive release of neutrophil proteases in the lung.', *Chest*, 142, pp. 1425–32. doi: 10.1378/chest.11-3273.

Williams, S. E., Brown, T. I., Roghanian, A., Sallenave, J.-M., Pancer, Z., Amemiya, C. T., Ehrhardt, G. R. A., Matzinger, P., Dallegri, F., Ottonello, L., McElvaney, N. G., Crystal, R. G., Sallenave, J. M., Schalkwijk, J., Wiedow, O., Hirose, S., Hochstrasser, K., Albrecht, G. J., Schonberger, O. L., Rasche, B., Lempart, K., Kramps, J. A., Klasen, E. C., Sallenave, J. M., Ryle, A. P., Wiedow, O., Schroder, J. M., Gregory, H., Young, J. A., Christophers, E., Sallenave, J. M., Silva, A., Molhuizen, H. O., Alkemade, H. A., Zeeuwen, P. L., Schalkwijk, J., de Roo, C., de Jongh, G. J., Sallenave, J. M., Shulmann, J., Crossley, J., Jordana, M., Gauldie, J., Brown, T. I., Mistry, R., Collie, D. D., Tate, S., Sallenave, J. M., Alkemade, J. A., Molhuizen, H. O., Ponec, M., Betsuyaku, T., Takeyabu, K., Tanino, M., Nishimura, M., Kuijpers, A. L., Zeeuwen, P. L., de Jongh, G. J., Kuijpers, A. L., Schalkwijk, J., Rulo, H. F., Lin, A. L., Johnson, D. A., Stephan, K. T., Yeh, C. K., Zelvyte, I., Wallmark, A., Piitulainen, E., Westin, U., Janciauskiene, S., Ilzecka, J., Stelmasiak, Z., Hiemestra, P. S., Fernie-King, B. A., McMichael, J.,

Lachmann, P. J., Sallenave, J. M., Zitnik, R. J., Zhang, J., Kashem, M. A., Song, X. Y., Zeng, L., Wenwen, J., Farmer, S. J., Fliss, A. E., Simmen, R. C., Brown, T. I., Mistry, R., Gray, R., Imrie, M., Collie, D. D., Sallenave, J.-M., Grütter, M. G., Fendrich, G., Huber, R., Bode, W., Saitoh, H., Masuda, T., Shimura, S., Fushimi, T., Shirato, K., Si-Tahar, M., Merline, D., Sitaraman, S., Madara, J. L., Zhang, Q., Shimoya, K., Moriyama, A., Sallenave, J. M., Silva, A., Marsden, M. E., Ryle, A. P., Westin, U., Polling, A., Ljungkrantz, I., Ohlsson, K., Sallenave, J. M., Si-Tahar, M., Cox, G., Chignard, M., Gauldie, J., Mihaila, A., Tremblay, G. M., Maruyama, M., Hay, J. G., Yoshimura, K., Chu, C. S., Crystal, R. G., Jaumann, F., Elssner, A., Mazur, G., Dobmann, S., Vogelmeirer, C., Sorensen, O. E., Cowland, J. B., Theilgaard-Monch, K., Liu, L., Ganz, T., Borregaard, N., van Wetering, S., van der Linden, A. C., van Sterkenburg, M. A., Rabe, K. F., Schalkwijk, J., Hiemestra, P. S., van Wetering, S., van der Linden, A. C., van Sterkenburg, M. A., Jin, F., Nathan, C. F., Radzioch, D., Ding, A., Fleming, D. C., King, A. E., Williams, A. R., Critchley, H. O., Kelly, R. W., King, A. E., Morgan, K., Sallenave, J.-M., Kelly, R. W., Boudier, C., Bieth, J. G., Kramps, J. A., Te Boekhorst, A. H., Fransen, A. H., Ginsel, L. A., Dijkman, J. H., Wingens, M., van Bergen, B. H., Hiemstra, P. S., Cavarra, E., Lucattelli, M., Gambelli, F., Ayad, M. S., Knight, K. R., Burdon, J. G., Brenton, S., Taggart, C. C., Lowe, G. J., Greene, C. M., Sponer, M., Nick, H. P., Schnebli, H. P., Saheki, T., Ito, F., Hagiwara, H., Francart, C., Dauchez, M., Alix, A. J., Lippens, G., Nara, K., Ito, S., Ito, T., Tremblay, G. M., Vachon, E., Larouche, C., Bourbonnais, Y., Tremblay, G. M., Wolbink, A. M., Cormier, Y., Hack, C. E., Pfundt, R., van Ruissen, F., van Vlijment-Willems, I. M., Nonomura, K., Yamanishi, K., Yasuno, H., Nara, K., Hirose, S., Sallenave, J. M., Marsden, M. D., Ryle, A. P., Suzuki, Y., Furukawa, M., Abe, J., Kasiwagi, M., Hirose, S., King, A. E., Critchley, H. O., Sallenave, J. M., Kelly, R. W., Tanaka, N., Fujioka, A., Tajima, S., Ishibashi, A., Hirose, S., Pfundt, R., van Vlijmen-Willems, I., Bergers, M., Wingens, M., Cloin, W., Schalkwijk, J., Pfundt, R., Wingens, M., Bergers, M., Zweers, M., Frenken, M., Schalkwijk, J., Bingle, T., Tetley, T. D., Bingle, C. D., van Seuningen, I., Audie, J. P., Gosselin, B., Lafitte, J. J., Davril, M., Reid, P. T., Marsden, M. E., Cunningham, G. A., Haslett, C., Sallenave, J. M., Zeeuwen, P. L., Hendriks, W., de Jong, W. W., Schalkwijk, J., Furutani, Y., Kato, A., Yasue, H., Furukawa, M., Suzuki, Y., Ghoneim, M. A., Tachibana, S., Hirose, S., Tamechika, I., Itakura, M., Saruta, Y., Gipson, T. S., Bless, N. M., Shanley, T. P., Tralau, T., Meyer-Hoffert, U., Schroder, J. M., Wiedow, O., Hiemstra, P. S., Maassen, R. J., Stolk, J., Simpson, A. J., Wallace, W. A., Marsden, M. E., Simpson, A. J., Maxwell, A. I., Govan, J. R., Haslett,

C., Sallenave, J. M., Wiedow, O., Harder, J., Bartels, J., Streit, V., Christophers, E., Fernie-King, B. A., Seilly, D. J., Davies, A., Lachmann, P. J., Tomee, J. F., Hiemstra, P. S., Heinzel-Wieland, R., Kauffman, H. F., Meyer-Hoffert, U., Wichmann, N., Schwichtenberg, L., White, P. C., Wiedow, O., Shugars, D. C., Wahl, S. M., McNeely, T. B., Janoff, E. N., McNeely, T. B., Dealy, M., Dripps, D. J., McNeely, T. B., Shugars, D. C., Rosendahl, M., Ma, G., Greenwell-Wild, T., Lei, K., King, A. E., Fleming, D. C., Critchley, H. O., Kelly, R. W., Levy, O., Chattopadhyay, A., Gray, L. R., Patton, L. L., Farquhar, C., VanCott, T. C., Mbori-Ngacha, D. A., Pillay, K., Coutsoudis, A., Agadzi-Naqvi, A. K., Gompertz, S., Bayley, D. L., Hill, S. L., Stockley, R. A., Draper, D. L., Landers, D. V., Krohn, M. A., Duffy, C. L., Phillips, S. L., Klingelhutz, A. J., Ohta, K., Nakajima, T., Cheah, A. Y., O'Blenes, S. B., Zaidi, S. H., Cheah, A. Y., McIntyre, B., Kaneda, Y., Rabinovitch, M., Zaidi, S. H., Hui, C., Cheah, A. Y., Mulligan, M. S., Desrochers, P. E., Chinnaiyan, A. M., Rudolphus, A., Stolk, J., Dijkman, J. H., Kramps, J. A., Mitsuhashi, H., Asano, S., Nonaka, T., Hamamura, I., Masuda, K. I., Kiyoki, M., Wex, T., Treiber, G., Nilius, M., Vieth, M., Roessner, A., Malfertheiner, P., Grobmyer, S. R., Barie, P. S., Nathan, C. F., Wang, X., Li, X., Xu, L., Vachon, E., Bourbonnais, Y., Bingle, C. D., Rowe, S. J., Janelle, M. F., Tremblay, G. M., Zaidi, S. H., You, X. M., Ciura, S., O'Blenes, S., Husain, M., Rabinovitch, M., Cowan, B., Baron, O., Crack, J., Coulber, C., Wilson, G. J., Rabinovitch, M., Murata, E., Sharmin, S., Shiota, H., Shiota, M., Yano, M., Kido, H., Sehnert, B., Cavcic, A., Bohm, B., He, S. H., Xie, H., Zhang, X. J., Wang, X. J., Haslett, C., Odaka, C., Mizuochi, T., Yang, J., Ding, A., Henriksen, P. A., Devitt, A., Kotelevtsev, Y., Sallenave, J. M., Sano, C., Shimizu, T., Sato, K., Kawauchi, H., Tomioka, H., Sano, C., Shimizu, T., Tomioka, H., Ding, A., Thieblemont, N., Zhu, J., Jin, F., Zhang, J., Wright, S., Zhu, J., Nathan, C., Ding, A., Lentsch, A. B., Ward, P. A., Jin, F. Y., Nathan, C., Radzioch, D., Ding, A., Henriksen, P. A., Hitt, M., Xing, Z., Lentsch, A. B., Jordan, J. A., Czermak, B. J., Taggart, C. C., Greene, C. M., McElvaney, N. G., O'Neill, S., Mulligan, M. S., Lentsch, A. B., Huber-Lang, M., Simpson, A. J., Cunningham, G. A., Porteous, D. J., Haslett, C., Sallenave, J. M., Sallenave, J. M., Cunningham, G. A., James, R. M., McLachlan, G., Haslett, C., McMichael, J. W., Roghanian, A., Jiang, L., Rammage, R., Sallenave, J. M., Lee, S. K., Lee, S. S., Hirose, S., Yoshida, N., Egami, H., Yamashita, J., Westin, U., Nystrom, M., Ljungcrantz, I., Eriksson, B., Ohlsson, K., van Bergen, B. H., Andriessen, M. P., Spruijt, K. I., van de Kerkhof, P. C., Schalkwijk, J., Ashcroft, G. S., Lei, K., Jin, W., Angelov, N., Moutsopoulos, N., Jeong, M. J., Nares, S., Ashcroft, G., Wahl, S. M., Zhu, J., Nathan, C., Jin, W., Sumi, Y., Muramatsu, H., Hata, K., Ueda, M., Muramatsu, T.,

Zhang, D., Simmen, R. C., Michel, F. J., Zhao, G., Vale-Cruz, D., Simmen, F. A., Kikuchi, T., Abe, T., Yaekashiwa, M., Vassiliou, E., Sharma, V., Jing, H., Sheibanie, F., Ganea, D., Katamura, K., Shintaku, N., Yamauchi, Y., Jing, H., Yen, J. H., Ganea, D., Zhang, Y., DeWitt, D. L., McNeely, T. B., Wahl, S. M., Wahl, L. M., Tremblay, G. M., Sallenave, J. M., Israel-Assayag, E., Cormier, Y., Gauldie, J., Schalkwijk, J., van Vligmen, I. M., Alkemade, J. A., de Jongh, G. J., Schutte, B. C., McCray, P. B., Ganz, T., Aarbiou, J., Rabe, K. F., Hiemstra, P. S., Wilson, C. L., Ouellette, A. J., Satchell, D. P., Duits, L. A., Ravensbergen, B., Rademaker, M., Hiemstra, P. S., Nibbering, P. H., Zanetti, M., Gennaro, R., Romeo, D., Sorensen, O., Arnljots, K., Cowland, J. B., Bainton, D. F., Borregaard, N., Bals, R., Wang, X., Wu, Z., Frohm, M., Agerberth, B., Ahangari, G., Agerberth, B., Charo, J., Werr, J., Di Nardo, A., Vitiello, A., Gallo, R. L., Sorensen, O. E., Follin, P., Johnsen, A. H., Yang, D., Chertov, O., Bykovskaia, S. N., Biragyn, A., Ruffini, P. A., Leifer, C. A., Yang, D., Chen, Q., Schmidt, A. P., Davidson, D. J., Currie, A. J., Reid, G. S. D., Colaco, C. A., Jego, G., Knight, S. C., Burke, F., Bedford, P. A., Cespedes, I. S., Toka, F. N., Schollenberger, A., Gierynska, M., Niemialtowski, M., Matzinger, P., Stumbles, P. A., Thomas, J. A., Pimm, C. L., Dodge, I. L., Carr, M. W., Cernadas, M., Brenner, M. B., de Jong, E. C., Vieira, P. L., Kalinski, P., Pulendram, B., Gan, Y., Shi, Y. E., Bu, L. Y., Hogg, K. G., Kumkate, S., Mountford, A. P., Zhu, Y., Da'dara, A., Harn, D., Bennouna, S., Bliss, S. K., Curiel, T. J., Denkers, E. Y., Lu, H., Xing, Z., Brunham, R. C., Biragyn, A., Surenhu, M., Yang, D., Liu, D. W., Chang, J. L., Tsao, Y. P., Wang, J., Zganiacz, A., Xing, Z., Xing, Z., Tremblay, G. M., Sime, P. J., Gauldie, J., Koczulla, A. R., Bals, R., McMichael, J. W., Maxwell, A. I. and Hayashi, K. (2006) 'SLPI and elafin: one glove, many fingers.', *Clinical science*. Portland Press Limited, 110(1), pp. 21–35. doi: 10.1042/CS20050115.

Williamson, D. R., Albert, M., Perreault, M. M., Delisle, M.-S., Muscedere, J., Rotstein, C., Jiang, X. and Heyland, D. K. (2011) 'The relationship between Candida species cultured from the respiratory tract and systemic inflammation in critically ill patients with ventilator-associated pneumonia', *Canadian Journal of Anesthesia/Journal canadien d'anesthésie*. Springer-Verlag, 58(3), pp. 275–284. doi: 10.1007/s12630-010-9439-5.

World Health Organisation (2014) *Antimicrobial resistance: Global report on surveillance*.

Wu, C.-L., Lu, Y.-T., Kung, Y.-C., Lee, C.-H. and Peng, M.-J. (2011) 'Prognostic value of dynamic soluble triggering receptor expressed on myeloid cells in bronchoalveolar

lavage fluid of patients with ventilator-associated pneumonia.', *Respirology (Carlton, Vic.)*, 16(3), pp. 487–94. doi: 10.1111/j.1440-1843.2011.01945.x.

Wunderink, R. G., Woldenberg, L. S., Zeiss, J., Day, C. M., Ciemins, J. and Lacher, D. A. (1992) 'The radiologic diagnosis of autopsy-proven ventilator-associated pneumonia', *Chest*. 1992/02/01, 101(2), pp. 458–463.

Youden, W. J. (1950) 'Index for rating diagnostic tests.', Cancer, 3(1), pp. 32–5.

Young, D., Harrison, D. A., Cuthbertson, B. H. and Rowan, K. (2013) 'Effect of early vs late tracheostomy placement on survival in patients receiving mechanical ventilation: the TracMan randomized trial.', *JAMA : the journal of the American Medical Association*, 309(20), pp. 2121–9. doi: 10.1001/jama.2013.5154.

Zhou, X., Fragala, M. S., McElhaney, J. E. and Kuchel, G. A. (2010) 'Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research.', *Current opinion in clinical nutrition and metabolic care*, 13(5), pp. 541–7. doi: 10.1097/MCO.0b013e32833cf3bc.

Zielińska-Borkowska, U., Skirecki, T., Złotorowicz, M. and Czarnocka, B. (2012) 'Procalcitonin in early onset ventilator-associated pneumonia.', *The Journal of hospital infection*, 81(2), pp. 92–7. doi: 10.1016/j.jhin.2012.02.011.