

**CHARACTERISATION OF INNATE IMMUNITY IN
PATIENTS WITH
VENTILATOR-ASSOCIATED PNEUMONIA**

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DECLARATION

The work described in this thesis was my own unless otherwise acknowledged. No part of this work has been submitted in candidature for another degree.

Kallirroi Kefala
Edinburgh, December 2008

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ABSTRACT

Ventilator-associated pneumonia (VAP) is the most common ICU-acquired infection in patients receiving mechanical ventilation, associated with excess mortality, morbidity and cost. The innate immune system is considered to be responsible for the rapid initial response in eradicating invading micro-organisms. Neutrophils are the professional phagocytes of innate immunity and are mobilised rapidly towards the site of inflammation where they will eliminate pathogens through phagocytosis and killing. Unregulated responses however can lead to neutrophil-mediated tissue injury. Secretory leukocyte protease inhibitor (SLPI) and elafin are considered to play a pivotal role in modulating innate responses whilst providing an antiprotease shield towards the influx of neutrophil-dependent proteases.

The hypothesis of this work was that an exaggerated pro-inflammatory response is present in patients with VAP while the local antiprotease/antimicrobial expression is impaired, potentially facilitating neutrophil-mediated tissue injury. In the same context, neutrophils in patients with VAP may have impaired capacity to eliminate pathogens whilst having the potential to cause tissue damage.

Healthy volunteers from primary care and patients from a general ICU with clinically suspected VAP were recruited in the study. Bronchoscopy and bronchoalveolar lavage (BAL) was performed and in the patients the presence or absence of pathogens at a concentration $\geq 10^4$ cfu/ml of BAL fluid (BALF) defined the VAP group and the NON-VAP group respectively. Blood was also sampled and freshly prepared circulating neutrophils were used in *in vitro* assays. Serum and supernatants from BALF were used for quantification of cytokines and antiproteases.

BALF from patients with VAP displayed a trend towards a high number of neutrophils although the difference was not statistically significant. VAP was associated with a florid pro-inflammatory response as demonstrated by significantly elevated CXCL8 and interleukin-1 β (IL-1 β) levels.

Circulating neutrophils from both groups of critically ill patients exhibited significantly impaired phagocytic capacity and appeared to have the potential, upon contact with bacterial products, to cause damage to the membrane of epithelial cells.

BALF SLPI levels were similar in both groups of critically ill patients while elafin levels were significantly elevated in the BALF of VAP patients compared with healthy volunteers. However the excess of HNE does support an inherent imbalance in protease-antiprotease in the alveolar space in VAP.

The above findings provide more information in our understanding of the biology of VAP. A florid inflammatory response is present along with an impaired phagocytic capacity of neutrophils that simultaneously exhibit the potential to cause tissue damage. The antiprotease shield may also be impaired facilitating further host tissue injury.

Further research is needed to elucidate pathways that may govern the observed deficiencies and/or dysregulations and may provide the basis for novel therapeutic strategies targeting neutrophil-mediated lung injury.

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ABBREVIATIONS

<i>A. baumannii</i> :	<i>Acinetobacter baumannii</i>
ALI:	Acute Lung Injury
APACHE:	Acute Physiology and Chronic Health Evaluation
AP-1:	Activator Protein 1
ARDS:	Adult Respiratory Distress Syndrome
<i>Asp. fumigatus</i> :	<i>Aspergillus fumigatus</i>
BAL:	Bronchoalveolar Lavage
BALF:	Bronchoalveolar Lavage Fluid
bp:	Base pairs
BPI:	Bactericidal/Permeability-Increasing protein
BSA:	Bovine Serum Albumin
CAAA:	Combined Admissions Assessment Area
<i>C. albicans</i> :	<i>Candida albicans</i>
CAP:	Community Acquired Pneumonia
CF:	Cystic Fibrosis
cfu:	Colony forming units
CGD:	Chronic Granulomatous Disease
<i>C. freundii</i> :	<i>Citrobacter freundii</i>
<i>C. koseri</i> :	<i>Citrobacter koseri</i>
CLD:	Chronic Liver Disease
<i>Cl. difficile</i> :	<i>Clostridium difficile</i>
CLP:	Caecal Ligation and Puncture
COPD:	Chronic Obstructive Pulmonary Disease
CPIS:	Clinical Pulmonary Infection Score
CSU:	Catheter Specimen Urine
CXR:	Chest Radiograph
C5a:	activated complement factor 5
DM:	Diabetes Mellitus
DMEM:	Dulbecco`s Modified Eagles Medium
ECG:	Electrocardiogram
ED:	Emergency Department
EDTA:	Ethylene Diamine Tetraacetic Acid
EGF:	Epidermal Growth Factor
EIA:	Elastase Inhibitory Activity
ELF:	Epithelial Lining Fluid
ELISA:	Enzyme-linked Immunosorbent Assay
ENA-78:	Epithelial Cell Neutrophil Activator
<i>E. aerogenes</i> :	<i>Enterobacter aerogenes</i>
<i>E. cloacae</i> :	<i>Enterobacter cloacae</i>
ERK:	Extracellular signal-Regulating Kinase
<i>E coli</i>	<i>Escherichia coli</i>
ETA:	Endotracheal Aspirate
ETT:	Endotracheal Tube

FCS:	Foetal Calf Serum
fMLP:	Formyl Methionyl Leucyl Phenylalanine
FOB:	Fibre-optic Bronchoscopy
GM-CSF:	Granulocyte-macrophage Colony Stimulating Factor
GPCR:	G-protein Coupled Receptors
HAP:	Hospital-acquired Pneumonia
HDU:	High Dependency Unit
HELICS:	Hospitals in Europe Link for Infection Control through Surveillance
HGF:	Hepatocyte Growth Factor
<i>H. influenzae:</i>	<i>Haemophilus influenzae</i>
HIV:	Human Immunodeficiency Virus
HMG-1:	High-mobility Group protein
HNE:	Human Neutrophil Elastase
HV:	Healthy Volunteers
ICAM-1:	Intercellular Adhesion Molecule 1
ICU:	Intensive Care Unit
Ig:	Immunoglobulin
IFN- γ :	Interferon-gamma
IGFBP-3:	Insulin-like Growth Factor-binding Protein-3.
IHD:	Ischaemic Heart Disease
I κ B:	inhibitor κ B
IL:	Interleukin
IL-1Ra:	IL-1 Receptor antagonist
IMDM:	Iscove's Dulbecco Modified Medium
IQR:	Interquartile Range
IRAK:	Interleukin-1 Receptor Associated Kinase
i.v.:	intravenous
Kb:	kilobase
KC:	Keratinocyte-derived Chemokine
kDa:	Kilodaltons
<i>Kl. Pneumoniae:</i>	<i>Klebsiella pneumoniae</i>
kPa:	kilopascal
LDH:	Lactate Dehydrogenase
LDL:	Low Density Lipoprotein
LPS:	Lipopolysaccharide
LREC:	Local Research Ethics Committee
LTA:	Lipoteichoic Acid
LTB4:	Leukotriene B4
MAPK:	Mitogen Activated Protein Kinase
MBL:	Mannose Binding Lectin
MCP-1:	Monocyte Chemoattractant Protein-1
MIDAS:	Metal Ion-dependent Adhesion Site
MIP-2:	Metalloproteases
<i>M. catarrhalis:</i>	<i>Moraxella catarrhalis</i>
MPO:	Myeloperoxidase

mRNA:	messenger ribonucleic acid
MRSA:	Methicillin resistant <i>Staphylococcus Aureus</i>
MSSA:	Methicillin Sensitive <i>Staphylococcus Aureus</i>
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NE:	Neutrophil Elastase
NF-κB:	Nuclear Factor-kappaB
NK cells:	Natural killer cells
NPV:	Negative Predictive Value
N/S:	Normal (isotonic) Saline
OD:	Optical Density
OLB:	Open Lung Biopsy
PAF:	Platelet-activating Factor
PAOP:	Pulmonary Artery Occlusion Pressure
PAR-1:	Proteinase-activated Receptor -1
PIA:	Pseudomonas Isolation Agar
PBS:	Phosphate Buffered Saline
PECAM-1:	Platelet Endothelial Cell Adhesion Molecule-1
PEEP:	Positive End-expiratory Pressure
PGE2:	Prostaglandin E2
PGLYRP:	Peptidoglycan Recognition Protein
PMNs:	Polymorphonuclear cells
PPV:	Positive Predictive Value
<i>Pr. mirabilis</i> :	<i>Proteus mirabilis</i>
PRP:	Platelet Rich Plasma
<i>Ps. aeruginosa</i> :	<i>Pseudomonas aeruginosa</i>
PSB:	Protected Specimen Brush
PSGL:	P-selectin Glycoprotein Ligand
REST:	Rapidly Evolving Seminal vesicle-transcribed
ROS:	Reactive Oxygen Species
RNS:	Reactive Nitrogen Species
rSLPI:	recombinant SLPI
SaO ₂ :	Saturation of Oxygen
SBP:	Systolic Blood Pressure
<i>S. marcescens</i> :	<i>Serratia marcescens</i>
sIL-1RII:	soluble IL-1 Receptor II
SKALP:	Skin-derived Anti-leucoproteinase
SLPI:	Secretory Leukocyte Protease Inhibitor
SMR:	Standardized Mortality Rates
SP-D:	Surfactant Protein D
<i>St. aureus</i> :	<i>Staphylococcus aureus</i>
<i>St. epidermidis</i> :	<i>Staphylococcus epidermidis</i>
<i>St. maltophilia</i> :	<i>Stenotrophomonas maltophilia</i>
<i>Str. morbilloforum</i> :	<i>Streptococcus morbilloforum</i>
<i>Str. pneumoniae</i> :	<i>Streptococcus pneumoniae</i>
<i>Str. pyogenes</i> :	<i>Streptococcus pyogenes</i>
TBB:	Transbronchial Biopsy

TGF- β :	Transforming Growth Factor-beta
TLR:	Toll-like Receptors
TM:	Thrombomodulin
TNF- α :	Tumour Necrosis Factor-alpha
VAP:	Ventilator-associated Pneumonia
VLA:	Very Late Antigen
WAP:	Whey Acidic Protein

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GENERAL INTRODUCTION

The thesis aims to characterize the lung's reactivity of patients with moderate-to-severe asthma (VAE) with focus on respiratory function and airway reactivity. The overall hypothesis is that VAE is associated with impaired physiological lung function, structural airway inflammation and defective airway homeostasis. It is hypothesized that in a subgroup of these individuals, VAE will be associated with airway hyper-responsiveness to bronchodilator challenge. It is further hypothesized that VAE

is the result of an imbalance between airway hyper-responsiveness and airway homeostasis. The overall hypothesis is that VAE is associated with airway hyper-responsiveness to bronchodilator challenge. It is further hypothesized that VAE is the result of an imbalance between airway hyper-responsiveness and airway homeostasis. It is further hypothesized that VAE is the result of an imbalance between airway hyper-responsiveness and airway homeostasis.

The thesis is divided into four parts. The first part is a review of the literature on asthma and airway hyper-responsiveness. The second part is a study on airway hyper-responsiveness in patients with moderate-to-severe asthma. The third part is a study on airway hyper-responsiveness in patients with moderate-to-severe asthma. The fourth part is a study on airway hyper-responsiveness in patients with moderate-to-severe asthma.

CHAPTER I: INTRODUCTION

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GENERAL INTRODUCTION

This thesis aims to characterise the innate immunity of patients with ventilator-associated pneumonia (VAP), with focus on neutrophil function and protease inhibition. The central hypothesis is that VAP is associated with impaired neutrophil phagocytosis, enhanced neutrophil activation and deficient antiprotease production. It is postulated that as a consequence of these abnormalities, VAP will be associated with enhanced lung inflammation as compared with critically ill patients who do not have evidence for VAP.

VAP is the most common ICU-acquired infection among patients who are mechanically ventilated and its impact in morbidity and cost is widely recognised. Ongoing controversy surrounds the best diagnostic methodology for VAP. The lack of agreement and the resulting variety in diagnostic tools impacts on variations in incidence and perhaps outcome described in various studies. These problems will be discussed in more detail in section 1 of the introduction along with an overview of the incidence, mortality and morbidity, risk factors, pathogenesis and microbiology of VAP.

VAP refers to a hospital-acquired pulmonary infection in a subgroup of patients that are critically ill and in need of at least a single organ support, namely ventilatory support. The role of neutrophils as the first responders in the invasion of pathogens remains of crucial importance. Neutrophils are mobilised very quickly towards the site of inflammation and attempt to eliminate pathogens through phagocytosis and microbial killing. The mechanisms that govern the steps from recruitment to bacterial killing will be discussed in section 2 of the introduction. Emphasis will also be given to the potential of these mechanisms to contribute to host tissue damage with reference to a clinical example high relevant to critically ill patients: the acute respiratory distress syndrome (ARDS) and its less severe form acute lung injury (ALI).

Following the concept of neutrophil dysregulation and neutrophil-mediated tissue damage, the role of compensatory mechanisms and the expression of protease inhibitors (namely alpha1-antitrypsin, elafin and SLPI) will be discussed in the final part (section 3) of the introduction. Elafin and SLPI not only provide an antiprotease shield but are capable of exhibiting antimicrobial and potentially anti-inflammatory functions. They therefore have the intriguing potential to enhance bacterial clearance and/or attenuate neutrophil-mediated tissue damage.

1. VENTILATOR-ASSOCIATED PNEUMONIA

1.1 Definition

Hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) are major causes of morbidity and mortality despite advances in supportive care and antimicrobial therapy.

HAP is a pulmonary infection that occurs more than 48 hours after hospital admission (American Thoracic Society 2005). HAP is the second most common hospital-acquired infection in the United States but remains the leading cause of death from nosocomial infections (Emori et al. 1991; Rello et al. 2001).

VAP is a subset of HAP and refers to pneumonia that occurs more than 48 hours after hospital admission, endotracheal intubation and initiation of mechanical ventilation (American Thoracic Society 2005). Among intensive care unit (ICU) patients nearly 90% of episodes of HAP occur during mechanical ventilation.

1.2 Incidence

VAP is the most common ICU-acquired infection among patients receiving mechanical ventilation, with an incidence reported to range from 8-28% at a variety of institutions (Rello et al. 2001; Chastre and Fagon 2002). The variation in incidence can be explained by the variation in diagnostic methods employed for the case definition of pneumonia and also by the differences in population being evaluated.

Despite variation among institutes it has been recognised that the incidence of VAP in mechanically ventilated patients increases with time (Cook et al. 1998). However as most cases of mechanical ventilation are of short duration, approximately half of all episodes of VAP occur within 4 days of mechanical ventilation.

The time of onset also has important epidemiological and therapeutic implications as early-onset VAP (within the first 4 days) carries a better prognosis as it is generally attributed to antibiotic-sensitive bacteria. In contrast late-onset VAP (arising after 5 days or more) is associated with increased morbidity and mortality as multi-resistant pathogens are more commonly involved.

Reports also suggest that VAP can commonly complicate the course of adult respiratory distress syndrome (ARDS), affecting between 34%-70% of patients at some stage. However the diagnosis of pulmonary infection in patients with ARDS remains difficult without the use of bronchoalveolar lavage (Andrews et al. 1981; Bell et al. 1983; Chastre et al. 1998; Meduri et al. 1998; Markowicz et al. 2000).

1.3 Mortality, morbidity and cost

In contrast to infections of other frequently involved organs (e.g. urinary tract and skin) acquired in ICU, which carry a low mortality ranging from 1-4%, the crude mortality rate for VAP varies from 24-50% and can rise up to 76% in specific population groups and when the pulmonary infection is attributed to high-risk pathogens (Fagon et al. 1989; Fagon et al. 1996; Rello et al. 1997; Chastre and Fagon 2002; Kollef et al. 2005).

The variation shown above partially reflects differences between institutions and study populations. Increased mortality rates have been associated with infection due to *Pseudomonas aeruginosa*, *Acinetobacter* species and *Stenotrophomonas maltophilia*, inappropriate antibiotic therapy and medical rather than surgical illness (Heyland et al. 1999a; Chastre and Fagon 2002). Cardiac surgery patients (Kollef et al. 1995; Leal-Noval et al. 2000), immunocompromised patients with acute leukaemia (Randle, Jr. et al. 1996), lung transplantation (Egan et al. 1995) and bone marrow transplantation (Lossos et al. 1995) are other subgroups associated with a higher mortality.

Case control studies have shown that VAP is associated with a significant attributable mortality although multivariate analysis has not consistently identified VAP as an independent risk factor for death (Chastre and Fagon 2002). Stepwise logistic regression to control for effects of confounding variables has shown VAP to significantly increase the risk of death in the ICU (Vincent et al. 1995). Case control studies using a rigorous method of matching patients with pneumonia to patients without pneumonia concluded conflicting results showing that VAP probably does increase the risk of death (Fagon et al. 1993b), whereas in another study the opposite was found (Papazian et al. 1996).

The interpretation of mortality studies in VAP remains challenging as multiple variables can influence the extent to which VAP influences mortality. Variations in patients' population and causative organism have already been mentioned. The timing of onset of pneumonia (early versus late, with multi-resistant pathogens involved more commonly in late than early VAP), the diagnostic tools employed in establishing a diagnosis ('clinical' empirical approach versus 'bacteriological' approach), the sensitivity and specificity of the each diagnostic strategy, timing of antibiotics, and appropriateness of antibiotics vary hugely among studies. Taking into account all the existing difficulties and limitations, VAP seems to independently increase the risk of death up to 20-30% compared to that due to the underlying illness alone, at least in subgroups of patients.

Whilst the debate on attributable mortality continues, most investigators agree that VAP is associated with longer duration of mechanical ventilation, ICU stay and hospital stay. Patients with VAP stayed in the ICU 4.3 days longer than control subjects (Cook et al. 1998; Heyland et al. 1999a; Chastre and Fagon 2002; Rello et al. 2002).

The financial burden of the prolonged hospitalisation cannot be underestimated. Extra hospital charges attributed to nosocomial pneumonia in a large dataset of US ICU trauma patients were evaluated to be more than \$40,000 per patient (Baker et al. 1996).

Recent studies have suggested that the attributable costs range from \$20,000-\$40,000 per case of VAP in the ICU (Rello et al. 2002; Warren et al. 2003). The cost of antibiotic treatment contributes to the above as it has been reported that approximately 50% of all antibiotics prescribed in ICU are administered for the treatment of lower respiratory tract infection (Warren et al. 2003).

1.4 Risk factors

Various risk factors have been identified for VAP. Among them intubation and mechanical ventilation is an independent risk factor that increases the risk of developing nosocomial pneumonia 6- to 21-fold (Weinstein 1991; Torres et al. 1995; Craven and Steger 1996; Tablan et al. 2004). This is supported by increasing evidence including randomised trials showing that avoidance of intubation and mechanical ventilation is associated with much lower rates of nosocomial pneumonia and reduction in mortality (Bott et al. 1993; Brochard et al. 1995; Antonelli et al. 1998; Lightowler et al. 2003; Hess 2005).

Other risk factors emphasise airway/ventilator circuit manipulation (i.e. reintubation, tracheotomy, low intracuff pressure and leakage, type of endotracheal tube, frequent ventilator circuit change, failed subglottic drainage), or the role of the gastrointestinal system (i.e. witnessed aspiration, stress-ulcer prophylaxis with gastric pH-altering agents and enteral feeding) (reviewed by Chastre and Fagon 2002).

Host factors such as age, male gender, chronic obstructive pulmonary disease (COPD) or other pulmonary disease, ARDS, trauma, coma or impaired consciousness, burns, organ failure, severity of illness and cardiorespiratory arrest have also been identified as independent factors by multivariate analysis in selected studies (reviewed by Chastre and Fagon 2002).

A variety of interventions have also been associated with an increased risk of VAP including paralytic agents, transport out of ICU, nasotracheal tubes, nasogastric tubes, sinusitis, supine head position, and prior use or no use of antibiotics (Chastre and Fagon 2002). Although prior use of antibiotics remains rather controversial as an independent risk factor for development of VAP (Kollef 1993; Cook et al. 1998), it is more evident that prior use of antibiotics increases the risk of superinfection with multiresistant pathogens e.g. *Pseudomonas* or *Acinetobacter* spp (Fagon et al. 1989; Rello et al. 1993). It has also been shown that patients with late onset VAP due to high-risk pathogens have an increased hospital mortality rate when compared with patients with VAP due to non high-risk pathogens (Kollef et al. 1995). Risk factors for VAP are summarised in Table 1.

	Airways/ventilator manipulation	Gastro-intestinal System	Host factors	Interventions
Risk factors	Intubation and re-intubation,	Witnessed aspiration	Age, male gender	Transfer out of ICU
	Low intracuff pressure and ETT leakage	pH-altering agents	Underlying lung disease, ARDS	Neuromuscular relaxants
	Ventilator circuit change	Enteral feeding	Trauma, burns	Nasotracheal tubes
			Organ failure and severity of illness	Sinusitis
				Supine head position
			Prior use of antibiotics	

Table 1: Risk factors for VAP.

1.5 Pathogenesis

Multiple host defense mechanisms are capable of eliminating microbes and maintaining a sterile lower respiratory tract. These include anatomic and mechanical barriers (ciliated epithelium and mucus, mucociliary clearance, cough reflexes). Below the terminal bronchioles humoral (complement and antibodies) and cellular components of the innate immune system (polymorphonuclear leukocytes, macrophages and their soluble mediators), play a fundamental role in host defenses.

Pneumonia develops from invasion of the lower respiratory tract and lung parenchyma by pathogens. For pneumonia to occur the balance between host defenses and microbial propensity for colonisation and invasion must shift in favour of pathogens. In order for the bacterial burden to overcome host defense an overwhelming inoculum or a challenge by an extremely virulent pathogen or a defect in host defense or a combination of the above has to arise.

In the mechanically ventilated patient, a combination of factors compromises host defenses e.g. co-morbidity and malnutrition (Niederman et al. 1989). Endotracheal intubation itself bypasses the anatomical barriers, eliminates cough reflexes, compromises mucociliary clearance, injures the tracheal epithelium and provides pathogens with direct access into the lower respiratory system.

It is also known that critical illness itself results not only in increased bacterial adherence (Niederman 1989) but also in colonisation of the oral cavity with a predominance of aerobic Gram-negative bacilli and *Staphylococcus aureus* (Niederman et al. 1989; Scannapieco et al. 1992; Cardenosa Cendrero et al. 1999). Oropharyngeal colonisation has been reported to be an independent predictor of subsequent tracheobronchial colonisation and a risk factor for VAP (George et al. 1998).

Therefore whilst oropharyngeal colonisation with predominantly Gram-negative bacilli may begin as early as 12 hours, being most abundant at 96 hours of ICU stay (Feldman et al. 1999), at the same time an endotracheal tube (ETT) in place serves as a pool of contaminated secretions, facilitating direct access of pathogens into the lower airways. The ETT also serves as a reservoir of pathogens which adhere to the inner surface of the tube producing a biofilm. Biofilms are relatively resistant to eradication and may represent another site of persistent colonisation with high risk nosocomial microorganisms (Adair et al. 1999; Feldman et al. 1999).

Microaspiration of contaminated oropharyngeal secretions, leakage around the tube cuff or embolisation of pathogens from the contaminated biofilm have been implicated as key factors in the development of VAP.

The rate of aspiration in critically ill patients is much higher given the impaired level of consciousness, an abolished gag reflex, delayed gastric emptying and/or decreased gastrointestinal motility. Gastro-oesophageal reflux and aspiration of gastric contents is another possible mechanism of entry of bacteria into the lower airways although this appears to be less important (Bonten et al. 1994).

Finally other much less common routes of lung infection in mechanically ventilated patients include haematogenous spread of pathogens to the lung from distal sites of infection, inhalation of contaminated medical aerosols or air, and direct extension of infection from a pleural infection.

1.6 Microbiology

The aetiology of VAP can differ depending on the methods and criteria used for the diagnosis, the characteristics of the patient population studied, the duration of hospitalisation and mechanical ventilation prior to the onset of the pneumonia, and prior use of antibiotics.

Studies have reported that aerobic Gram-negative bacteria are responsible for VAP in up to 58 % of cases and Gram-positive cocci up to 35 % of the cases (reviewed by Chastre and Fagon 2002). Pooled data from studies across the world confirm that among the Gram-negative bacteria, *P. aeruginosa* accounts for half of them with an incidence approaching 24 % of all isolates, whilst Gram-positive bacteria and in particular *S. aureus* are involved in 20 % of the cases.

Of all *S. aureus* isolates, 56 % of them were methicillin resistant strains (MRSA). *Enterobacteriaceae* (*Escherichia coli*, *Proteus* spp., *Enterobacter* spp., *Serratia* spp., *Klebsiella* spp in roughly equal numbers and *Citrobacter* spp. and *Hafnia* in small numbers) make up the third most common group of pathogens accounting for 14 % of isolates. *Haemophilus* spp account for the fourth most common group with an incidence up to 9.8%, followed by non pneumococcal Streptococci (8.0 %), *Acinetobacter* spp (7.9 %), *Streptococcus pneumoniae* (4 %), *Neisseria* spp (2.6 %), *Stenotrophomonas maltophilia* (1.7 %), coagulase-negative Staphylococci (1.4 %) and various other organisms (<1%) including anaerobic bacteria, fungi, *Corynebacterium* spp, *Moraxella* spp and enterococci.

More recent studies have reported that Gram-positive bacteria have become increasingly common with *S. aureus* accounting for up to 42 % of cases. Again, among Gram-negative bacteria *P. aeruginosa* is the most common isolate accounting for 21 % of all cases (Kollef et al. 2005).

It has been recognised that underlying diseases may predispose patients to infections with specific pathogens. For example patients with COPD are more susceptible to infections with *Moraxella catarrhalis*, *Haemophilus influenzae* or *S. pneumoniae* whilst patients with trauma or neurological patients are at higher risk of infections with *S. aureus*. Patients with head trauma and neurosurgical patients or patients with large volume aspiration also appear to be at higher risk of VAP due to *Acinetobacter baumannii* (Chastre and Fagon 2002).

However the most important factor in determining the aetiology of VAP and the risk of multiple-drug-resistant pathogens is the duration of hospitalisation and mechanical ventilation prior to the onset of pneumonia.

It has been recognised that the microbiology of early-onset VAP is different from that of late-onset VAP. High rates of *H. influenzae*, *S. pneumoniae* and MSSA, are more common in early-onset VAP whereas *P. aeruginosa*, *Acinetobacter* spp., MRSA and multiresistant Gram-negative bacteria are significantly more frequent in late-onset VAP. Other risk factors for multiresistant VAP pathogens include recent antimicrobial treatment (in the preceding 90 days), recent hospitalisation (for 2 days or more in the preceding 90 days), high frequency of antibiotic resistance in the community or the specific hospital unit, residence in a nursing home or extended-care facility, chronic dialysis (within 30 days), home wound care, family member with a multiresistant pathogen and immunosuppressive disease or therapy (Park 2005).

1.7 Diagnosis

It is important at this stage to recognise that the aims of any diagnostic tool are to identify patients with true pulmonary infection early, collect appropriate samples for culture and initiate early, adequate and appropriate antibiotic therapy.

It has been shown that initial inappropriate therapy with antibiotics is associated with poor prognosis and increased hospital mortality (Alvarez-Lerma 1996; Kollef et al. 1999; Iregui et al. 2002; American Thoracic Society 2005). Furthermore prior use of inadequate antibiotics is a risk factor for development of antibiotic-resistant pathogens (Fagon et al. 1989; Kollef 1993; Rello et al. 1993; Goldmann et al. 1996; Trouillet et al. 1998; Kollef and Fraser 2001).

It is equally important, before diagnostic techniques are discussed, to keep in mind that many studies have compared different techniques with the accepted “gold standard” for the diagnosis of nosocomial pneumonia, which for many years has been the histological diagnosis of pneumonia, often in post mortem tissue. Even this ‘gold standard’ comparator poses difficulties when it comes to interpreting results. For example one may be asked to assume that the patient did not develop pneumonia before the episode studied, that no new antibiotics were introduced between microbiological sampling and the autopsy, and that the involved area of the lung has been biopsied (some studies contradict the dogma that pneumonia spreads into every pulmonary lobe) (Rouby et al. 1992; Fabregas et al. 1996; Marquette et al. 1996).

VAP is suspected when a patient who has been receiving mechanical ventilation for more than 48 hours develops new or progressive pulmonary infiltrates along with clinical signs of infection (fever, leukocytosis and purulent tracheal secretions). A high index of suspicion should also be raised in patients who have unexplained haemodynamic instability or deterioration of oxygenation during mechanical ventilation (American Thoracic Society 2005) .

The diagnosis is usually based on the above clinical criteria and on bacteriological evidence of pulmonary infection based on microscopic evaluation and culture of predominantly lower respiratory tract samples, and much less commonly on blood or pleural fluid cultures.

Two main diagnostic strategies for the diagnosis of VAP have been suggested and debated among experts over many years: the “clinical” (or empirical) approach and the “bacteriological” approach.

The clinical approach employs clinical criteria and non-invasive qualitative or semi-quantitative cultures of endotracheal aspirates (ETA) in order to define the aetiological cause of pneumonia.

The bacteriological approach uses quantitative cultures of samples from the distal airways acquired by:

a) non-invasive techniques leading to quantitative cultures of ETA and non-bronchoscopic sampling of the distal airways with a variety of techniques such as mini bronchoalveolar lavage (mini BAL) and blinded sampling with protected specimen brush (PSB) and

b) invasive techniques referring to bronchoscopic sampling of the distal airways using either a double-lumen catheter with a protected specimen brush (PSB) or bronchoalveolar lavage (BAL) and less commonly to techniques such as transbronchial biopsy (TBB) and open lung biopsy (OLB). From all the aforementioned techniques, the diagnostic value of quantitative cultures of ETA and BAL will be discussed in more detail below.

1.7.1 Clinical approach

The diagnosis of pneumonia in the context of the clinical approach is defined as the presence of new or worsening radiographic pulmonary infiltrates plus at least two of three clinical features: body temperature greater than 38°C; leukocytosis or leukopenia; and purulent secretions. However clinical criteria alone, while sensitive, have a very low specificity for diagnosis of pulmonary infection in the ICU setting, as both infective and non-infective systemic inflammatory responses share similar clinical findings, all representing the downstream result of cytokine release. In fact it has reported that among all patients with “suspected VAP” based on clinical criteria, only a third will meet microbiological criteria for infection (as defined by PSB cultures $\geq 10^2$ cfu/ml), resulting in two thirds of these patients being treated with antibiotics unnecessarily (Fagon et al. 1988; Meduri et al. 1994).

In a prospective study evaluating the accuracy of the clinical criteria comparing predicted versus PSB-documented VAP, clinical diagnosis was accurate in 60% of cases and the proposed therapeutic plan was adequate in only 33% of cases. As such more than a third of patients were treated inappropriately with antibiotics in the absence of pneumonia and two thirds of those with bacteriologically confirmed pneumonia were treated with inappropriate therapy (Fagon et al. 1993a).

Similar limitations apply to radiographic evidence of pulmonary infiltrates as infiltrates are evident not only in infection but also in atelectasis, alveolar haemorrhage, cardiogenic and non cardiogenic pulmonary oedema, pulmonary infarction or contusion (Meduri et al. 1994). In one post mortem study the last radiograph prior to autopsy was interpreted and signs (air bronchograms, alveolar infiltrates, atelectasis, cavities, silhouette signs and asymmetric infiltrates superimposed on diffuse bilateral infiltrates) were correlated with autopsy evidence of pneumonia. All signs had a diagnostic accuracy less than 68%, with the presence of air bronchograms being the only sign that correlated well with VAP, correctly predicting 64% of pneumonias in the entire group (Wunderink et al. 1992).

In order to identify a causative pathogen for pneumonia the clinical approach uses qualitative or semi-quantitative cultures of ETA. This is a widely used and easily applicable technique that does not require expertise or expensive equipment but is severely limited by the very high number of false positive results. It has been mentioned earlier that tracheal colonisation occurs within 12 hours of admission and intubation in ICU (Feldman et al. 1999), and qualitative cultures of ETA cannot discriminate between colonisation of the upper airways and invasion of the pulmonary parenchyma.

A postmortem study evaluating clinical parameters and several invasive techniques in the diagnosis of pneumonia confirmed specificity of 55% for the presence of fever, 33% for presence of purulent secretions and 42% for radiological changes. The combination of pulmonary infiltrates, leukocytosis and the presence of purulent secretion produced a 70% sensitivity and 45% specificity (Torres et al. 1994). In another postmortem study the combination of radiological changes and two of the clinical criteria was associated with 30 % false-negative and 25% false-positive results.

If all three criteria were used in combination with radiological changes specificity increased up to 92% at the expense of sensitivity which dropped to 23%, potentially resulting in 70% of patients with pneumonia not being diagnosed and treated (Fabregas et al. 1999).

More detailed and quantitative clinical scores have been employed such as the clinical pulmonary infection score (CPIS) which is based on 6 variables (temperature, blood leukocyte count, volume and purulence of tracheal secretions, oxygenation, pulmonary radiography and semi-quantitative culture of the tracheal aspirate). A score more than 6 correlated well with quantitative bronchoalveolar lavage fluid (BALF) culture in one study (Pugin et al. 1991). However as the initial score proved difficult to use in clinical practice, it has been modified and used in several studies (all somewhat different from each other) with a wide range of sensitivity and specificity (Niederman 2005).

A recent study of 200 patients compared CPIS collected retrospectively and bronchoscopically obtained quantitative cultures. Results showed that while the sensitivity of a CPIS >6 on day 3 for identifying VAP was 89%, its specificity was only 47%, leading to potentially unnecessary treatment of 60 (53%) of the 113 patients without VAP as diagnosed by bronchoscopy (Luyt et al. 2004).

The above studies suggest that the clinical approach itself carries a variable but low sensitivity and/or specificity. The main risk should this diagnostic tool be employed, is of potentially unnecessary treatment of patients without VAP and/or inadequate treatment of patients with VAP. These are the pitfalls that studies of a bacteriological approach, discussed next, have addressed.

1.7.2 Bacteriological approach

When the bacteriological approach is used, different ways of sampling the distal airways have been proposed as mentioned earlier and have fuelled another debate among experts between the non-invasive (quantitative cultures of ETA, non-bronchoscopic sampling of distal airways) and the invasive (bronchoscopic) approach (quantitative cultures of BAL or PSB). The basic principle behind all the different techniques is appropriate sampling of lower airways and accurate separation between colonisation and infection in order to avoid overtreatment with antibiotics. Studies have suggested that when this approach is engaged fewer patients have been treated with antibiotics (Heyland et al. 1999b).

1.7.2.1 Non-invasive techniques

As it became clear that qualitative cultures of ETA exhibit a high number of false-positive results in the diagnosis of pneumonia due to bacterial colonisation, many researchers looked at quantitative cultures of endotracheal aspirates and compared them with either bronchoscopic techniques or pathological post mortem diagnosis or both.

Studies have evaluated a range of cutoff values for VAP (from 10^3 to 10^7 colony forming units (cfu)/ml) for ETA and there are suggestions in some studies, that when the threshold of 10^6 is used, acceptable sensitivity and specificity can be achieved (el-Ebiary et al 1993; Marquette et al. 1993; Torres et al. 1993; Maquette et al. 1995;). Sensitivity varied from 38 to 82% (mean of 76 %) and specificity ranged from 72 to 85% (mean of 75 %) (Cook and Mandell 2000). However in one of the studies only 40% of micro-organisms cultured in the ETA correlated with those from a PSB (Jourdain et al. 1995) and in one post mortem study only 53% of the micro-organisms isolated from ETA were also found in quantitative lung tissue cultures (Borderon et al. 1981). This implies that when decisions to treat are based on ETA cultures then the risk of either treating unnecessarily with antibiotics and/or using much broader spectrum of antibiotics emerges.

In clinical practice non-invasive techniques are immediately available, do not require expensive equipment (endobronchial catheters as opposed to fibre-optic bronchoscope), and they can be performed by trained nursing staff, physiotherapists or physicians not qualified to perform a bronchoscopy. They are less invasive, are associated with less compromise of gas exchange during the sampling, can potentially avoid contamination by the bronchoscopic channel and can be performed in patients intubated with small endotracheal tubes. However they are blind procedures without airway visualisation and despite the 80% concordance between bronchoscopic and non-bronchoscopic technique described by some investigators, the diagnosis can potentially be missed by a blind technique.

1.7.2.2 Invasive /Bronchoscopic techniques

Fibre-optic bronchoscopy (FOB) and BAL have been used widely in the diagnosis of lung diseases and are among the most valuable research tools for studying inflammatory diseases.

a) Diagnostic value of BAL in VAP

In the context of VAP, many groups have evaluated the diagnostic accuracy of quantitative BAL culture (Chastre et al. 1988; Torres et al. 1989; Meduri et al. 1991; Pugin et al. 1991; el-Ebiary et al. 1993; Torres et al. 1994; Chastre et al. 1995; Marquette et al. 1995; Papazian et al. 1995).

Most of these studies have used a diagnostic threshold of 10^4 cfu/ml, which seemed to correlate best with histological evidence of pneumonia and lung tissue cultures (Chastre et al. 1995). When results of 23 studies were pooled together the calculated mean sensitivity was $73 \pm 18 \%$ and the calculated mean specificity was $82 \pm 19 \%$ (Torres and el-Ebiary 2000).

One of the factors that accounts for the variation noted in many studies is the recent use of antibiotics. The accuracy of BAL culture is questionable for patients who have received new antibiotics after the onset of signs and before the collection of samples, when a high rate of false-negative results has been reported (Montravers et al. 1993a; Souweine et al. 1998). In two different studies, when antibiotics had been introduced in the 72 hours prior to sampling, the sensitivities of BAL cultures dropped to only 38 % (Montravers et al. 1993a; Souweine et al. 1998).

Also, diagnostic accuracy depends on proper selection of the sampling area. Usually this is selected upon visualisation of a segment with purulent secretion during FOB or on the basis of the location of the infiltrate on the chest radiograph.

In the case of diffuse infiltrates and when no segment with the maximum endobronchial abnormality (in this case purulent secretions) can be identified, the posterior segment of the right lower lobe is usually sampled (Chastre and Fagon 2002). Posterior segments of the lower lobes have been proposed as the segments of choice in the case of diffuse infiltrates as post mortem studies have shown that histological lesions of pneumonia were preferentially found in dependent segments (Rouby et al. 1992).

In addition to the diagnostic accuracy, studies have looked at the impact of the diagnostic strategy on antibiotic use and outcome. In one of the first studies to address this question, mortality among patients who underwent bronchoscopy was significantly lower than control patients (19% versus 35% respectively). In the same study, patients who underwent bronchoscopy received fewer antibiotics and more patients had their antibiotics discontinued as clinicians' confidence was strengthened (Heyland et al. 1999b). Similar results were reported in a large randomised trial of 413 patients. Again, apart from a significant decrease in mortality at 28 days, unnecessary use of antibiotics was also minimised and also more non-pulmonary infections were diagnosed (Fagon et al. 2000).

The same effects on mortality have not been reproduced in other studies with smaller number of patients (Sanchez-Nieto et al. 1998; Ruiz et al. 2000). A recent study also failed to show an impact on clinical outcome between quantitative BAL samples and nonquantitative ETA sampling (The Canadian Critical Care Trials Gp 2006). It is worth bearing in mind that in this study a large number of patients with previous *MRSA* or *Pseudomonas aeruginosa* colonisation and/or infection had been excluded and de-escalation of treatment may have not taken place in all cases.

In conclusion, the argument regarding the best diagnostic strategy should perhaps move away from focusing purely on the impact on mortality given the difficulties that interpretation of these studies pose at present. Clinical strategies suffer from high sensitivity and low specificity while arguably improved but less repeatable results have been shown with the non invasive bacteriological approach. A highly specific approach such as the bronchoscopic approach is supported by evidence that shows increased confidence and comfort level of health care workers in managing patients with suspected nosocomial pneumonia when this strategy was employed (Heyland et al. 1999b). There is evidence to suggest that the bronchoscopic approach results in more targeted use of antibiotics in critically ill patients that could reduce overall costs, despite the expense of FOB and quantitative cultures, and minimise antibiotic-related toxicity. More importantly, the targeted use of antibiotics could minimise the incidence of multiresistant pathogens (Fagon et al. 1988), a task that is of high priority in ICUs and therefore shifts strongly the argument towards the use of an invasive bronchoscopic approach whenever possible.

b) BAL as a research tool: technical considerations

BAL has been used for many years as a widely acceptable and valuable technique in studying lung inflammation. In this context it should be remembered that BAL is designed to investigate diseases that predominantly affect the alveolar structures and therefore aims to sample material from the alveoli and reduce contamination from the lower airways.

The first problem that arises is avoidance of contamination as the working channel passes through the oropharyngeal or nasopharyngeal cavity, the trachea and the segmental bronchi before it is carefully wedged in the lumen of a subsegmental bronchus. It has been recommended that avoidance of suctioning before retrieval of specimens and aggressive suctioning before the procedure with a separate suction catheter can minimise contamination (Baselski et al. 1992). It is widely accepted that the return from the first 20 mls has a higher concentration of epithelial cells and should be discarded as "bronchial" rather than bronchoalveolar sample, especially in studying infective processes such as VAP (Rennard et al. 1990; Rennard et al. 1998). Another index of contamination that has been introduced is the percentage of bronchial and squamous epithelial cells present in BAL, accepting that if this does not exceed 5% of total cells in BAL, then BAL is considered satisfactory (Haslam and Baughman 1999).

The second problem is that of adequate sampling of the alveoli. In order to minimise variability a standard introduction volume of lavage fluid (normally sterile isotonic saline) of at least 100 mls and a standard number of aliquots (usually 4 aliquots of 40-50 mls each) should be used (Baselski et al. 1992; Meduri and Chastre 1992; Haslam and Baughman 1999).

It is estimated that the alveolar surface area distal to the wedged bronchoscope is 100 times greater than that of the peripheral airway and that approximately 1×10^6 alveoli (approximately 1% of the lung surface area) are sampled with 1 ml of actual lung secretions (or epithelial lining fluid (ELF)) retrieved (Chastre and Fagon 2002).

This raises 2 further considerations. The first is related to the amount of fluid retrieved. The returned volume can vary considerably depending on the site (returns from middle lobe/lingula can be greater than returns from lower lobe/posterior samples as a result of gravity), dwell time, permeability, and underlying lung disease such as emphysema. A very small return may contain only diluted material from the working channel and the bronchi resulting in false negative results (Meduri and Chastre 1992; Rennard et al. 1998).

The second consideration directly related to the amount of fluid retrieved and the adequate sampling of the alveoli, is the dilution factor of the ELF. This is of particular importance when measurements of the acellular components are performed as different proportions of cells are unaffected by variable BAL dilutions.

The question of the most appropriate marker of dilution to correct for the recovery of the ELF remains unresolved. Many markers have been proposed and studied. External markers are limited by concerns about safety, resistance to biodegradation, homogeneous distribution, potential for transepithelial migration and feasibility.

The proposed internal markers have to be present at constant levels in body fluids so that comparison of lavage with blood levels can give an accurate measure of dilution. Protein and albumin have both been studied, however their usefulness as dilution markers is questionable in BAL from lungs with increased permeability. Urea continues to be used widely with the limitation that because it diffuses very quickly from the blood to the lung, the need for a minimum dwell time is mandatory (Haslam and Baughman 1999).

Finally when considering FOB and BAL as a diagnostic and/or research tool it is worth mentioning that the risk of FOB in critically ill patients requiring mechanical ventilation remains low (Trouillet et al. 1990; Montravers et al. 1993b).

The same applies in patients with ARDS, where in one study a 4.5% transient desaturation to <90% during bronchoscopy was reported without prolonged episodes of severe hypoxaemia (Steinberg et al. 1993). The above findings support the safety of FOB in mechanically ventilated patients taking into consideration individuals' clinical state and level of support.

In summary this section reviewed the current concept of VAP discussing in detail the problems and the controversies that surround the subject. Emphasis was given to the ongoing discussion among investigators regarding the existing variety of diagnostic strategies which has posed difficulties in interpreting results of studies. Regardless of the above, VAP remains the most common hospital-acquired infection among ventilated patients with significant impact on morbidity and cost.

The work presented in Chapter III, section 1, will characterise the population of VAP defined in our ICU, keeping in mind the controversies described above.

VAP is a pulmonary infection and in the context of invasion of pathogens, recruitment of neutrophils is crucial. The various stages from neutrophil recruitment to bacterial elimination will be discussed in the next section, with emphasis on the potential dysregulation of the above mechanisms that could contribute to host tissue damage. This is of particular relevance to the hypothesis of this thesis which has impaired neutrophil function and enhanced neutrophil activation at its core (Figure 1).

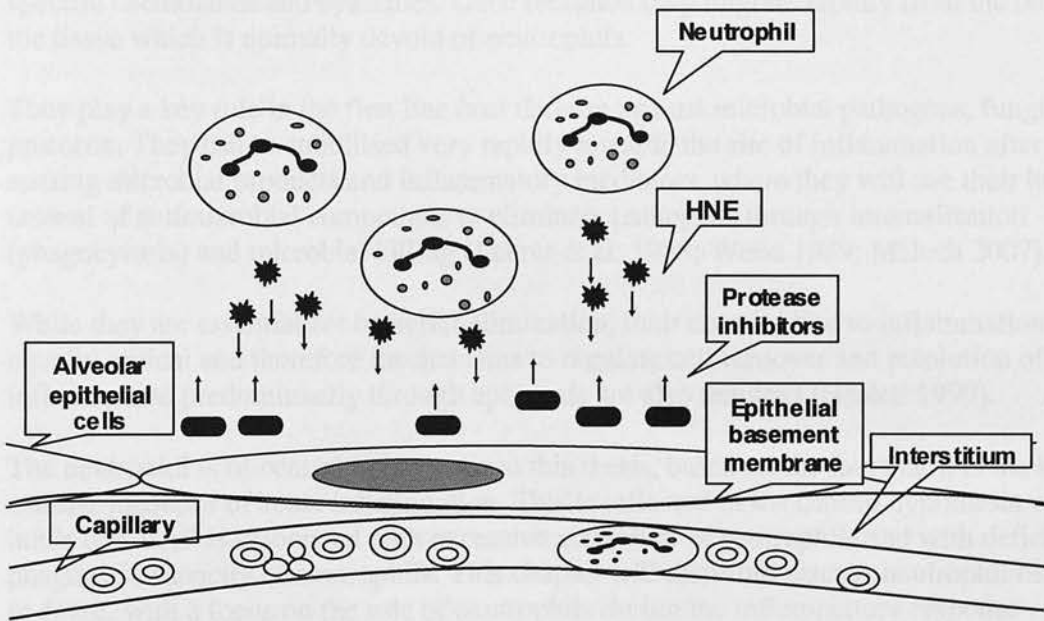


Figure 1: Neutrophil interaction with epithelial cells.

2. NEUTROPHILS AND NEUTROPHIL BIOLOGY

2.1 Introduction

Neutrophils are specialised white blood cells, comprising over 95% of the circulating granulocytes. They are produced in the bone marrow and are of myeloid origin. They are characterised by a multilobed nucleus and a granular cytoplasm, and are 10-20 µm in diameter.

They are normally abundant in the bloodstream in a quiescent state, where they will circulate for an average of 6.5 hours if not recruited to a specific site of inflammation by specific chemokines and cytokines. Once recruited they migrate rapidly from the blood to the tissue which is normally devoid of neutrophils.

They play a key role in the first line host defense against microbial pathogens, fungi and protozoa. They can be mobilised very rapidly towards the site of inflammation after sensing microbial products and inflammatory mediators, where they will use their huge arsenal of antimicrobial compounds to eliminate pathogens through internalisation (phagocytosis) and microbial killing (Lehrer et al. 1988; Weiss 1989; Malech 2007).

While they are essential for bacterial elimination, their contribution to inflammation is equally critical and therefore mechanisms to regulate cell turnover and resolution of the inflammation predominantly through apoptosis are also required (Haslett 1999).

The neutrophil is of central importance to this thesis, based on the fact that it is the key cellular mediator of acute inflammation. This is reflected in the central hypothesis which holds that VAP is associated with excessive activation of neutrophils and with deficient phagocytic capacity of neutrophils. This chapter will therefore discuss neutrophil biology in detail, with a focus on the role of neutrophils during the inflammatory response starting from the very early stages of neutrophil recruitment and leading to neutrophil degranulation and phagocytosis/killing of pathogens. Emphasis will also be given to the unregulated response that can lead to neutrophil-mediated tissue damage with reference to acute lung injury (ALI).

2.2 Neutrophil recruitment

Neutrophil recruitment involves a multistep adhesion cascade between leukocyte and endothelial cell adhesion molecules that mediate leukocyte attachment and rolling, subsequent leukocyte stable adhesion and, ultimately, transmigration across the endothelium. Subsequent retention in the inflamed tissue is critical in addition to transepithelial cell migration that will allow circulating neutrophils to access the inflammatory focus and elicit a response (Springer 1994; Liu et al. 2004).

In order for neutrophil recruitment to be initiated signals that induce the release of chemoattractants and the up-regulation of adhesion molecules are required. These signals will guide the migration of neutrophils that express receptors for these molecules (Carlos and Harlan 1994; Luster 1998).

2.3 Rolling and adhesion

Rolling is the first step in neutrophil recruitment to tissues and is mediated by selectins and selectin-ligand interactions under conditions of flow.

Selectins (L-, P-, and E- for leukocyte, platelet and endothelial respectively) are type 1 membrane glycoproteins that bind to cell-surface ligands rapidly but briefly (McEver et al. 1995). L-selectin is localised on the surface of the neutrophil microvilli and is constitutively expressed. L-selectin-dependent leukocyte rolling and attachment relies on the activation of endothelial cells by proinflammatory mediators. This induces expression of ligands on vascular endothelium that show increased adhesiveness during inflammation (Jutila et al. 1989; Smith et al. 1991a; Spertini et al. 1991).

Studies indicate that E- and P-selectin also support leukocyte rolling under conditions of flow playing a complementary role in regulating leukocyte-endothelial interactions (Lawrence and Springer 1991; Abbassi et al. 1993). It is now recognised that selectins allow interaction, not only with endothelial cells, but also with other neutrophils via the P-selectin glycoprotein ligand (PSGL-1) (Diacovo et al. 1996; McEver and Cummings 1997). In addition to facilitating rolling, selectins may also contribute to signaling regulating adhesion-dependent leukocyte functions, β 2-integrin activation, and O_2^- production (Crockett-Torabi 1998).

As circulating neutrophils are exposed to chemoattractant gradients produced locally in the site of inflammation, they move from a rolling state to a state of tight adhesion. Primary adhesion or tethering mediated by selectins allows the neutrophil to enter a transient contact with the endothelial wall and "search" for factors that will activate the secondary phase of tight and strong adhesion.

The molecules primarily responsible for adhesion to the endothelium and extracellular matrix are members of the β 2 integrin family. Integrins are heterodimeric adhesion molecules composed of transmembrane α and β chains. The β 2 chain (CD18) is expressed exclusively by leukocytes and pairs with 1 of the chains of the CD11 family (CD11a, -b, -c).

The two most important β 2-integrins on the neutrophil are CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1, CR3). They both mediate firm adhesion to endothelial cells by binding diverse ligands such as intracellular adhesion molecule 1 (ICAM 1) and 2 for CD11a/CD18 and CD11b/CD18 respectively, and also fibrinogen, heparin and Factor X for CD11b/CD18 (Luscinskas et al. 1991).

Both adhesion molecules display a domain with a metal ion-dependent adhesion site (MIDAS) that contributes not only to binding but also to a conformational change of the receptor to a high affinity state (Diamond and Springer 1994; McDowall et al. 1998). Stimulation of neutrophils with inflammatory mediators such as tumour necrosis factor- α (TNF- α), activated complement factor 5 (C5a), platelet-activating factor (PAF), or formyl methionyl leucyl phenylalanine (fMLP) can induce upregulation of the surface membrane content of CD11b/CD18 (Hughes et al. 1992).

In vivo studies have shown that both integrins and their counterligands are upregulated by inflammatory stimuli. ICAM-1, normally expressed at low levels on pulmonary capillary endothelial cells, is upregulated by TNF- α and interleukin-1(IL-1) and also in response to lipopolysaccharide (LPS) instillation in the lungs facilitating neutrophil migration to inflammatory stimuli (Dustin et al. 1986; Springer 1990; Burns et al. 1994; Beck-Schimmer et al. 1997). Interaction between CD11/CD18 adhesion molecules and their ligands contributes to neutrophil migration to rodent lungs after instillation of *E. coli*, LPS or *P. aeruginosa* as evidenced by various studies using blocking antibodies against CD11/CD18 (Doerschuk et al. 1990; Qin et al. 1996; Ramamoorthy et al. 1997). However it is worth keeping in mind that the same effect was not observed for *S. pneumoniae*, Group G Streptococci or *S. aureus* (Doerschuk et al. 1990; Sherman et al. 1992; Ramamoorthy et al. 1997). This is suggestive of the presence of at least two different migration mechanisms: CD18-dependent and CD18-independent. Human *in vitro* studies have confirmed similar findings (Issekutz et al. 1995).

Additional adhesion molecules also contribute to neutrophil migration elicited by bacterial stimuli in the lungs. *In vivo*, neutrophil recruitment in rodent lungs in response to LPS is diminished by blocking antibodies against CD29, CD49e, or CD49f, suggesting that the β 1 integrins very late antigen (VLA)-5 (CD49e/CD29) and VLA-6 (CD49f/CD29) contribute to this process, by binding fibronectin and laminin and facilitating transit through the interstitium (Ridger et al. 2001).

2.4 The role of chemokines

Migration can be modulated by the secretion of chemokines that play a critical role in the onset of cellular inflammation. They are structurally related cytokines that stimulate chemotaxis or directed migration of cells expressing specific cell surface receptors (Luster 1998).

They are expressed in a wide variety of cell types and tissues, can be secreted by most cell types and are classified according to their structure. Peripheral neutrophils express receptors for the alpha (CXC) family that contains the glutamic acid-leucine-arginine (ELR) motif, with CXCL8 (previously named interleukin-8, IL-8) being the prototype (Simpson et al. 2003).

CXC chemokines are not normally expressed in the quiescent state but can be rapidly up-regulated on activation, where the chemokine messenger ribonucleic acid (mRNA) may comprise up to 1% of total mRNA (Adams and Loyd 1997). IL-1, interleukin-2 (IL-2), TNF- α , γ -interferon (IFN- γ), C5a, leukotriene B4 (LTB4) and bacterial products such as LPS can very promptly up-regulate chemokine secretion (Baggiolini et al. 1994) whilst transforming growth factor beta (TGF- β), interleukin-4 (IL-4) and interleukin-10 (IL-10) inhibit production (Adams and Lloyd 1997) .

In vivo experiments have shown that neutrophil migration post intrapulmonary LPS instillation in rats can be inhibited by blocking either rat keratinocyte-derived chemokine (KC) or macrophage inflammatory protein-2 (MIP-2) (Frevort et al. 1995; Schmal et al. 1996). Similar results were observed in a mouse model of *K. pneumoniae* (Greenberger et al. 1996b) suggesting that multiple ELR+CXC chemokines may play an independent role in neutrophil migration induced by bacterial stimuli.

Different microenvironments can perhaps be created with differential chemokine expression, facilitating the neutrophil's journey from the intravascular space to the inflamed tissue (Middleton et al. 1997; Kuschert et al. 1999). On the same note, recent work suggests that whilst bound chemokines are considered responsible for promoting adhesion and the presence of a chemoattractant gradient (Rot 1993; Weber et al. 1999), surface chemokines can promote migration in the absence of a gradient suggesting a role for additional factors in this process (Cinamon et al. 2001).

It has been recognised that chemokines not only drive neutrophil recruitment but they are also capable of inducing transient increases in leukocyte adhesion to integrin ligands (Weber et al. 1996; Weber et al. 1999; Constantin et al. 2000; Chan et al. 2001) by upregulating affinity through conformational changes (Weber et al. 1996; Bazzoni and Hemler 1998; Weber et al. 1999; Constantin et al. 2000; Chan et al. 2001). Other studies have shown that the increased affinity state can also be achieved through changes in integrin avidity which can occur through integrin clustering (Stewart et al. 1996; Weber et al. 1996; Yauch et al. 1997; Stewart et al. 1998; Constantin et al 2000).

In addition to the above, chemokines may also play an important role in regulating neutrophil activation. For example CXCL8 has been shown to increase neutrophils' ability to kill bacteria through enhancement of phagocytosis, superoxide generation and granule release (Baggiolini et al. 1989a; Baggiolini et al. 1989b).

Other classes of chemokines such as the beta family (CC) may play a complementary role in neutrophil recruitment, elicited by bacterial products in the lungs (Shanley et al. 1995). Receptors for CC chemokines can increase in neutrophils after treatment with IFN- γ , TNF- α , or granulocyte-macrophage colony stimulating factor (GM-CSF) (Bonicchi et al. 1999; Yamashiro et al. 2000; Cheng et al. 2001). It is also worth noting that other cytokines can regulate neutrophil recruitment such as interleukin-17 (IL-17) (facilitating) (Ye et al. 2001), or interleukin-6 (IL-6) (limiting) (Ulich et al. 1991).

Finally in the context of infection, whilst chemokines will drive neutrophils in the general vicinity of infection, once there the neutrophils will encounter a series of chemoattractants, including bacterial products and complement fragments that will lead to the final site of infection. Indeed these signals (which may include fMLP, C5a, LTB₄, and CXCL8) may be present simultaneously and the hypothesis of an existing hierarchy of chemokines has been proposed. Numerous investigators have demonstrated preferential migration towards end-target chemoattractants such as fMLP and C5a despite the presence of high concentrations of “intermediary” or host-derived chemoattractants such as CXCL8 and LTB₄ (Campbell et al. 1997; Foxman et al. 1997; Foxman et al. 1999; Shen et al. 2000). More recently it has been proposed that this phenomenon is effected through selective activation of specific signal transduction (Heit et al. 2002).

It has therefore become clear that chemokines, induced early by LPS or early proinflammatory cytokines e.g. TNF- α and IL-1, are orchestrating a coordinated response that includes driving neutrophils rapidly to the site of inflammation or injury, facilitating adhesion and transendothelial migration and also promoting neutrophil activation which is crucial in the battle against invading pathogens but potentially detrimental in acute lung injury (Downey and Granton 1997).

With a segregation of 50 human chemokines that has emerged since their discovery (Charo and Ransohoff 2006) it is apparent that one has to be cautious when interpreting current evidence remembering that a diversity in the molecular pathways involved in initiating an early or a late response to a wide variety of stimuli does exist. That diversity depends on the cell type, tissue, stimulus and host specificity and many mechanisms are not fully elucidated (Mizgerd 2002).

An example of the variations between different tissues can be found in the lung itself where important observations relevant to lung pathology are worth mentioning. Even in a normal lung, the concentration of neutrophils within the pulmonary capillary blood is 35-100 times greater than in the large vessels of the systemic circulation (Doerschuk et al. 1987; Doerschuk et al. 1993; Gee and Albertine 1993).

In the systemic circulation, neutrophils are concentrated in the postcapillary venules and sequestration is L- and P-selectin mediated (Butcher 1991; Lawrence and Springer 1991; Spertini et al. 1991). However in the pulmonary circulation the site of sequestration is usually the capillary network where 97% of neutrophils are found (Doerschuk et al. 1999).

Neutrophils will normally roll along in the endovascular space, however in the lung, rolling does not occur within the capillary bed (Gebb et al. 1995) where vessels are too small to allow rolling (average 5-6 μm , and as small as 2 μm) of the spherical neutrophils (Doerschuk et al. 1993; Wiggs et al. 1994). Studies have shown that because 40-60% of the pulmonary capillary segments are smaller than neutrophils, most neutrophils will encounter a capillary segment narrower than the spherical diameter and that they need to deform in order to get through (Wiggs et al. 1994; Gebb et al. 1995; Hogg and Doerschuk 1995).

The changes in neutrophil shape take much longer than those in erythrocytes and this is reflected in their longer pulmonary transit time compared with erythrocytes (2.7 versus 1.3s) (Hogg et al. 1988). This suggests that neutrophil transit through the pulmonary capillaries is dependent on cellular deformation. Inflammatory stimuli will increase neutrophil retention by decreasing neutrophil deformability, resulting in stiffening (Downey and Worthen 1988; Worthen et al. 1989; Drost and MacNee 2002). This process, in addition to upregulated adhesion, will result in sequestration of the neutrophils to the pulmonary circulation.

2.5 Neutrophil emigration and phagosome formation

Following adhesion to the endothelial surface neutrophils will transmigrate to extravascular sites of inflammation/infection either between (Del et al. 1996) or directly through endothelial cells (Feng et al. 1998). This can be blocked *in vitro* by blocking platelet endothelial cell adhesion molecule-1 (PECAM-1), a member of the immunoglobulin (Ig) superfamily, which is expressed on both endothelial cells and neutrophils (Muller et al. 1993). Integrin-associated protein (CD 47) also supports transmigration independent of chemotactic gradient (Cooper et al. 1995).

Recent studies have provided evidence for a novel form of communication between neutrophils and endothelial cells through the formation of gap junctions, with the interesting observation that when the formation of the gap junctions was inhibited transmigration was enhanced (Zahler et al. 2003).

Continuing the journey onwards, neutrophils will migrate from the interstitium and will emerge into the alveolar air spaces between the type II pneumocytes and their neighbouring type I cells (Damiano et al. 1980).

This step of transepithelial migration depends upon epithelial characteristics, with alveolar epithelium appearing more permissive than bronchial epithelium (Carolan and Casale 1996), and seems to be largely but not entirely CD11b/CD18 dependent (Parkos et al. 1991). Transmigration also occurs most efficiently in the basolateral to apical direction (Liu et al. 1996) and is dependent upon the nature and the concentration of chemoattractant molecules (Casale et al. 1992; Smart and Casale 1993).

Once neutrophils reach the site of infection, they can directly recognise invading microbes through pattern recognition molecules that are present on the surface of neutrophils. For example, peptidoglycan recognition protein (PGLYRP) (Worthen et al. 1992; Liu et al. 2000), interacts with peptidoglycan produced by Gram-positive bacteria, including *Streptococcus pyogenes* and *S. aureus*. CD14 and Toll-like receptors (Kurt-Jones et al. 2002) interact with LPS generated by Gram-negative microorganisms, such as *Salmonella typhimurium* and *E.coli*.

Neutrophils will bind and engulf pathogens into a vacuole called the phagosome.

Binding and ingestion is greatly enhanced if bacteria are opsonised with antibodies or complement fragments such as C3bi which is recognised by the activated β 2-integrin CD11b/CD18 (Cunnion et al. 2003). Once formed the phagosome will undergo a rapid series of remodeling events termed phagosome maturation which will enable the phagosome to kill pathogens and dispose of the debris. The phagosome will acquire its antimicrobial activity through a series of fusion events with secretory vesicles and granules, forming the phagolysosome.

2.6 Neutrophil granules and contents

Neutrophil granules are formed sequentially during myeloid cell differentiation and contain a multitude of moieties that include antimicrobial peptides, proteases, and components of the respiratory burst oxidase system, and also a wide range of membrane bound receptors for endothelial adhesion molecules, extracellular matrix proteins, bacterial products and soluble mediators of inflammation. This machinery will be delivered to the phagosome during maturation or to the exterior of the cell following degranulation. Its controlled mobilisation will transform the neutrophil to a potent effector of innate immunity (Burg and Pillinger 2001).

Four categories of granules have been identified and classified as azurophilic granules (primary), specific (secondary), gelatinase (tertiary) and the secretory granules (Moraes et al. 2006).

Azurophilic granules are defined by the presence of myeloperoxidase (MPO) (Bainton and Farquhar 1966; Bainton et al. 1971) which is a microbicidal protein released into the phagosome upon activation. MPO reacts with H_2O_2 formed by the respiratory burst and the H_2O_2 -MPO complex can oxidise a variety of substances including hypochlorous acid, other chlorination products, tyrosine radicals and reactive nitrogen intermediates. These are all powerful oxidants that can attack the membrane of microorganisms leading to a rapid microbicidal effect (Weiss 1989; Klebanoff 1999).

Other major constituents of azurophilic granules are alpha-defensins. These are small cationic antimicrobial peptides (Ganz et al. 1985) that display antimicrobial activity against a broad range of bacteria, fungi, viruses and protozoa (Ganz et al. 1985; Lehrer et al. 1985; Daher et al. 1986) through transmembrane pore formation (Wimley et al. 1994). Bactericidal/permeability-increasing protein (BPI) is another potent antimicrobial peptide of the azurophilic granules that kills Gram-negative bacteria at nanomolar concentration (Elsbach 1998).

Other important constituents of azurophilic granules are three structurally related serprocidins (serine proteases with microbicidal activity): proteinase-3, cathepsin G and human neutrophil elastase (HNE). The serprocidins are cationic polypeptides which display potent proteolytic activity against a diversity of extracellular matrix components such as elastin, fibronectin, laminin and type IV collagen.

Serine proteases are also capable of activating endothelial and epithelial cells, macrophages, lymphocytes and platelets (Owen and Campbell 1999). The most important serine protease, HNE will be discussed in more detail later.

Peroxidase negative granules contain several potent antimicrobial peptides such as lactoferrin, lysozyme and hCAP-18 (the larger specific granules being richer in antimicrobial peptides than the smaller gelatinase granules) and three matrix metalloproteases (MMPs): neutrophil collagenase (MMP-8), gelatinase (MMP-9) and leukolysin (MT6-MMP/MMP-25). MMPs are stored in both subsets of granules in an inactive form. They will undergo proteolytic activation following exocytosis, thereby displaying a powerful proteolytic activity that can degrade major structural components of the extracellular matrix including collagens, fibronectin, proteoglycans, laminin and gelatin. Thus MMPs are now recognised as key mediators of degradation of the vascular basement membrane and interstitial structures that will facilitate neutrophil migration to inflammatory foci (Delclaux et al. 1996; Borregaard and Cowland 1997; Owen and Campbell 1999).

Finally secretory vesicles contain a variety of membrane-associated receptors that are mobilised at the early stages of the inflammatory response following stimulation by various inflammatory mediators, such as CD11b/CD18 (Sengelov et al. 1993), complement receptor 1 (CR1), (Sengelov et al. 1994b), receptors for fMLP (Sengelov et al. 1994a) and CD14 (Detmers et al. 1995).

2.7 Phagocytosis and killing

As mentioned earlier, following the engulfment of pathogens and the formation of the phagosome, granule secretion and fusion of the phagosome with the granules will result in phagolysosome formation.

This process appears to be dependent on the changes in the level of free cytosolic calcium (Lew et al. 1986; Jaconi et al. 1990), whilst other investigators have proposed a role for protein kinases (Mohn et al. 1995), although their targets and possible signaling pathways remain to be explored.

In this way neutrophils will activate the oxygen-dependent nicotinamide adenine dinucleotide phosphate oxidase (NADPH) pathway and oxygen-independent antimicrobial systems by releasing granule proteins into the phagosome or to the exterior of the cell. The oxidative mechanism of killing pathogens is mediated by the production of reactive oxygen species (ROS) generated by activated neutrophils in a process called the respiratory burst. ROS are produced almost exclusively but perhaps not entirely (Blouin et al. 1999) by the NADPH oxidase complex that assembles at the phagosomal membrane upon activation. Its importance has been highlighted by the susceptibility to infections of individuals with chronic granulomatous disease (CGD), a genetic disease in which NADPH is inactive (Smith and Curnutte 1991).

The phagocyte NADPH oxidase is a multicomponent enzyme that transfers electrons from cytoplasmic NADPH onto extracellular or intraphagosomal molecular oxygen therefore generating superoxide (Sbarra and Karnovsky 1959; Babior et al. 1973; Segal and Abo 1993). Superoxide will be rapidly converted to other highly toxic ROS such as hydrogen peroxide and hypochlorous acid that efficiently kill bacteria with the most important and efficient mechanism mediated by myeloperoxidase as has already been mentioned (Klebanoff 1999). As neutrophils can generate an enormous amount of superoxide two important principles arise. The first is that superoxide generation is critical in the defense against pathogens and the second is that this mechanism has to be tightly regulated in order to effect innate immunity but prevent “collateral damage” to the surrounding tissues.

The NADPH oxidase enzyme is composed of different subunits that are located in different parts of the resting neutrophil and upon activation these subunits are brought together and assembled into the active form. This process starts with phosphorylation of cytosolic oxidase components, which introduces conformational changes. These changes allow protein–protein interactions between the cytosolic and the membrane-bound oxidase components (charge-mediated and SH₃-proline-mediated), protein–lipid interactions (through PX domains binding to phosphoinositides) and interactions with small GTPases. Together these changes result in assembly of an active NADPH oxidase in the membrane of the cell. Additional interactions with cytoskeletal proteins lead to proper positioning of the enzyme in the membrane of the phagosome that contains the ingested microbe where the products of this enzyme cooperate with proteases to kill the pathogen (Roos et al. 2003).

Finally, in addition to the important bactericidal mechanism described above, there is recent evidence to suggest that the bactericidal activity of the neutrophils relies heavily on the activity of granule proteases, in a pathway that requires potassium influx and neutral pH (Reeves et al. 2002).

Neutrophil granules also contain antimicrobial peptides which may be concentrated in the phagolysosome. A more detailed discussion of antimicrobial peptides will be reviewed in the next chapter, with particular reference to secretory leukocyte protease inhibitor (SLPI) and elafin, which have dual antimicrobial and protease inhibitory functions. Both are described in neutrophils (Sallenave et al. 1997).

In summary, neutrophils are the professional phagocytes of the innate immune system equipped to sense and destroy invading pathogens. They express membrane receptors that will interact with specific bacterial ligands or inflammatory cytokines and they will trigger a series of coordinated responses including chemotaxis, phagocytosis and release of cytotoxic products. The same cellular and biochemical events, although tightly regulated, can contribute to host tissue damage (Weiss 1989) and a good example of dysregulation of the inflammatory response damage is ARDS and its less severe form, ALI .

2.8 ALI/ARDS and neutrophils

ALI/ARDS is a syndrome characterised by increased alveolar-capillary permeability and hypoxaemia, triggered by an extensive list of pulmonary and extrapulmonary insults. Although mortality has decreased over recent years, this remains high (30-40%) along with significant morbidity in survivors, and continues to affect the lives of approximately 200,000 patients per year in the United States alone (Ware and Matthay 2000; Matthay and Zimmerman 2005).

Since ARDS was first described (Ashbaugh et al. 1967), important discoveries have been made in order to understand the pathophysiological events underlying ARDS/ALI. It became apparent early on that ARDS/ALI are characteristically associated with histological features including accumulation of neutrophils in the pulmonary microvasculature and neutrophil sequestration in the alveoli and pulmonary interstitium that precede the development of lung injury (Bachofen and Weibel 1982; Tate and Repine 1983; Idell and Cohen 1985; Fowler et al. 1987).

These findings were supported by studies of subjects with ARDS/ALI that showed the presence of neutrophils in bronchoalveolar lavage fluid. A strong correlation between neutrophil numbers and severity of ARDS has been described (Lee et al. 1981; Clark et al. 1994; Steinberg et al. 1994; Pittet et al. 1997).

In vivo evidence that neutrophil oxidants and proteases can injure cells of the alveolar-capillary membrane further support the role of neutrophil inflammation in ALI/ARDS (Brigham 1982; Cochrane et al. 1983). The above led to the concept that inflammation is a key feature and this was acknowledged in the 1994 European American Consensus Conference statement on ALI/ARDS (Bernard et al. 1994).

The role of the neutrophil in lung injury has since been investigated extensively and although one has to remember that lung injury can occur in neutropenic patients (Laufe et al. 1986; Ognibene et al. 1986), there is substantial evidence to suggest that neutrophils and their contents do contribute to the epithelial and endothelial injury seen in ARDS/ALI.

2.9 Neutrophil elastase and matrix metalloproteases

HNE has an important place in this thesis on account of the important role of extracellular HNE in mediating tissue injury. It is generally believed that excessive activation of neutrophils leads to dysregulated degranulation and subsequent inappropriate release of HNE into the surrounding milieu. The relevance of this process to conditions like severe pneumonia is such that HNE is discussed in detail (while bearing in mind that other neutrophil granule mediators may play contributory roles and among those the role of matrix metalloproteases will be briefly mentioned). Chapter III, section 4 describes levels of HNE in the alveolar space of patients with VAP.

The role of HNE in the pathogenesis of disease was first highlighted by the recognition of premature emphysema in patients deficient for α 1-antitrypsin, the most abundant circulating elastase inhibitor (Laurell and Eriksson 1964; Eriksson 1964). This was supported by studies that demonstrated free active HNE in airway secretions from patients with a variety of respiratory diseases such as emphysema and chronic bronchitis (Fujita et al. 1990), asthma (Vignola et al. 1998), cystic fibrosis (CF) (Goldstein and Doring 1986; Nunley et al. 1999), non-cystic fibrosis bronchiectasis (Stockley et al. 1984) and pneumonia (Boutten et al. 1996). Free HNE was also found in BAL fluid (Lee et al. 1981) and in the serum of patients with ARDS (Donnelly et al. 1995).

Neutrophil elastase is a glycoprotein consisting of 218 amino acid residues and contains two asparagine-linked carbohydrate side chains. It is synthesised as a 267 amino acid protein that undergoes cleavage by cathepsin C and transforms to the proteolytically active protein which will be glycosylated and stored in its active form in azurophilic granules (Sinha et al. 1987; McGuire et al. 1993). NE is highly cationic with a strongly basic isoelectric point (pH 10-11) and cleaves bonds that are carboxy-terminal to small hydrophobic residues (particularly those formed by valine or alanine residues) (Bode et al. 1989; Owen and Campbell 1999).

It can degrade a multitude of matrix macromolecules including elastin, fibronectin, laminin, collagen (types III, IV and VI), proteoglycans and also plasma proteins including immunoglobulins, clotting factors and complement components (Owen and Campbell 1999). It is also worth remembering that in marked contrast to "free" NE released from neutrophils, membrane bound HNE is remarkably resistant to inhibition (Owen et al. 1995) and potentially capable of retaining proteolytic activity.

Neutrophil elastase also exhibits antimicrobial properties through direct or indirect mechanisms (Shi and Ganz 1998; Belaouaj et al. 2000) and can cleave mediators such as pro-interleukin 1 β , TNF- α and TNF- β (Porteu et al. 1991; Scuderi et al. 1991). Furthermore HNE can induce secretion of cytokines and chemoattractants from endothelial cells, epithelial cells, and mononuclear phagocytes (Hubbard et al. 1991; Bedard et al. 1993).

More recent studies have demonstrated that neutrophil serine proteases can degrade surfactant proteins D (SP-D) and A (SP-A) (Hirche et al. 2004; Rubio et al. 2004), potentially leading to enhanced susceptibility to infection, increased lung inflammation in response to various stimuli and delayed resolution of inflammation.

Neutrophil elastase can also act in a non degrading way and can activate signal transduction pathways that can alter cell function, with most studies suggesting a harmful role. Among these are studies demonstrating that HNE can promote goblet cell hyperplasia (Voynow et al. 2004) and enhance MUC1 (a member of the mucin gene family) (Kuwahara et al. 2005).

Recently it has been shown that HNE can also stimulate the lung epithelium to release growth factors and proinflammatory cytokines through activation of the mitogen activated protein kinase (MAPK) p38 and extracellular signal-regulating kinase (ERK), kinases necessary for release and gene expression of CXCL8 and other cytokines (Mukaida et al. 1994; Carter et al. 1999). This upregulation can also be effected through activation of nuclear factor-kappaB (NF- κ B) and activator protein 1 (AP-1) (Buczek-Thomas et al. 2004; Chen et al. 2004; Witherden et al. 2004), therefore perpetuating the inflammatory process.

Furthermore it has been shown that HNE released by neutrophils transmigrating across the epithelial layer can cleave e-cadherin, a transmembrane protein important in calcium-dependent epithelial cell-to-cell interaction, resulting in the disruption of the integrity of the epithelial monolayer (Ginzberg et al. 2001).

A similar role has been proposed for the interaction of HNE and endothelial cells. Recent findings suggest that the disruption of cadherins (endothelial junction proteins) is in part responsible for the deterioration of lung microvasculature and that this disruption is due to NE-mediated proteolysis. Fragments of soluble cadherins were also detected in the serum of patients with ARDS (Carden et al. 1998). An increased level of the endothelial cell surface anticoagulant protein thrombomodulin (TM) has also been found in plasma from patients with ARDS and again proteolytic release of TM mediated by HNE and cathepsin G has been suggested as a potential mechanism (MacGregor et al. 1997).

Finally NE has also been linked with the induction of apoptosis of lung epithelial cells *in vitro* and *in vivo*. The *in vivo* effect is mediated by proteinase-activated receptor-1 (PAR-1) (Suzuki et al. 2005). This may lead to disrupted epithelial barrier function and enhanced alveolar oedema, which remains a hallmark of ALI/ARDS.

Interestingly results of a placebo-controlled trial that included 492 patients with ALI found no benefit for the administration of an HNE inhibitor, sivelestat (Zeiber et al. 2004), suggesting that proteinase inhibition may be more complex in practice and emphasising once more a multifactorial pathogenesis of ALI that remains to be fully understood.

In addition other proteases have been implicated in tissue damage and in ALI/ARDS, particularly metalloproteases. Elevated concentrations of gelatinases A (MMP-2) and B (MMP-9) have been found in the epithelial lining fluid of patients with ARDS (Delclaux et al. 1997a) indicating a possible contribution to ALI/ARDS. This was supported by *in vivo* studies inhibiting MMP-2 and MMP-9, which were associated with improvement in measures of ALI (Plitas et al. 2003; Steinberg et al. 2003).

In conclusion, the role of neutrophils has been recognised as central to the pathogenesis of lung injury for many years now. However the list of proposed mediators and mechanisms continues to expand and includes: the role of platelets and the links between the thrombotic and the inflammatory systems; the role of active sodium and chloride transport channels and cAMP across the pulmonary epithelium; the role of circulating monocyte subpopulations and resident alveolar macrophages; the mechanisms that govern apoptosis and necrosis of endothelial and epithelial cells in key phases of lung injury; and the role of mechanical injury. A comprehensive review of all the above is beyond the purpose of this thesis but can be found in (Matthay and Zimmerman 2005).

ALI/ARDS represents a response to a variety of heterogeneous pulmonary and extrapulmonary stimuli, where the initial inflammatory response may well be activated to combat infection. In an uncomplicated pneumonia the pulmonary inflammatory response is limited by counter-regulatory processes that prevent damage.

However, poorly regulated or excessive responses imply an imbalance, be it between pro- and anti-inflammatory cytokines, or oxidants and antioxidants, or procoagulants and anticoagulants, or neutrophil recruitment/activation and mechanisms of neutrophil clearance, or proteases and protease inhibitors, leading to detrimental tissue damage.

To synthesise all of these abnormalities in a unifying model that will allow targeting of a single pathway remains too difficult and perhaps may not be pragmatic. Further research and evidence is needed to refine our knowledge of the role of neutrophils and their contents that will allow us to enhance the beneficial effects of the neutrophil while minimising its detrimental consequences.

The following and final section of the introduction will follow the concept of the neutrophil-mediated tissue damage and focus on the imbalance between proteases and protease inhibitors. The role of the major endogenous HNE inhibitors in the lung (elafin, SLPI and alpha1-antitrypsin) will be discussed in detail, given their direct relevance to the central hypothesis of this thesis, which contends that impairment of 'lung protective' antiproteases is associated with VAP.

3. INHIBITORS OF HUMAN NEUTROPHIL ELASTASE

3.1 Alpha1-antitrypsin

It is more than 40 years since Laurell and Eriksson made the remarkable observation that a band for α -globulin was missing in the protein electrophoresis gel of several individuals who had emphysema at a very young age (Eriksson S. 1964; Laurell C. and Eriksson S. 1964). This was the landmark recognition of the association between α 1-antitrypsin deficiency and emphysema and since then a lot has been learned about α 1-antitrypsin including the full structure of the protein, the mechanism of binding to its main substrate HNE, the clinical manifestations and the natural history of its deficiency, and the genetics of the deficiency.

The α 1-antitrypsin gene is located on the long arm of chromosome 14 and is a 12.2 kilobase (kb) gene composed of 7 exons and 6 introns (Kueppers and Christopherson 1978; Rabin et al. 1986). It encodes a 418 amino-acid protein that includes a signal peptide which is glycosylated and modified in the endoplasmic reticulum before being packaged and released as the final product. This will form a 52-kilodalton (kDa) glycoprotein that belongs to the family of serpins which also include α 1-antichymotrypsin, α 2-antiplasmin, plasminogen activator inhibitor type I, thyroxine binding globulin, cortisol binding globulin, angiotensinogen, kallistatin and leukocyte inhibitor protein (Carrell et al. 1982; Potempa et al. 1994).

The gene is expressed in many cells but the highest expression is described in hepatocytes from where serum α 1-antitrypsin is almost totally derived (Rogers et al. 1983). Serum levels can increase 3- to 4- fold during the host response to inflammation identifying α 1-antitrypsin as a positive "hepatic acute phase reactant" (Wewers et al. 1987). The protein is also expressed and secreted by neutrophils, mononuclear phagocytes and intestinal epithelium (Carlson et al. 1988; Molmenti et al. 1993).

Alpha1-antitrypsin reaches the lungs mainly by diffusion from the systemic and pulmonary circulation and much less by local production from macrophages and bronchial epithelial cells (Lomas and Mahadeva 2002).

Although the protein was named after its ability to inhibit trypsin, α 1-antitrypsin can inactivate virtually all mammalian serine proteinases including pancreatic and neutrophil elastase, proteinase-3, pancreatic trypsin and chymotrypsin, neutrophil cathepsin G, thrombin, plasmin, tissue kallikrein, Factor Xa and XIa, skin and synovial collagenases, urokinase and microbial serine proteinases (Travis and Salvesen 1983). However its most potent and irreversible inhibition is of neutrophil elastase in the lower respiratory tract where it provides more than 90% of the anti-NE protective shield (Travis and Salvesen 1983).

Alpha1-antitrypsin binds HNE through a methionine aminoacid residue (Met³⁵⁸) which is located at the reactive centre of the serpin (Met³⁵⁸-Ser³⁵⁹) and this tight bond will remain stable with a very low dissociation rate constant (Loebermann et al. 1984). Neutrophil elastase will cleave the reactive centre of the molecule releasing it from its metastable high energy state and allows the cleaved reactive loop of α 1-antitrypsin to snap back with the protease in tow into a more stable conformation. This process will distort and inactivate the HNE molecule by squeezing it on the other pole of the α 1-antitrypsin molecule and will inevitably lead to destruction of both molecules (Huntington et al. 2000).

Deficiency of α 1-antitrypsin arises from mutations that have been classified in 4 groups as base substitutions, in-frame deletions, frameshift mutations and exon deletions.

Phenotypes are categorised by a coding system in which the inherited alleles are represented by letters that denote migration of the molecule in an isoelectric pH gradient from A (for anodal gradients) to Z (for slower migrating variants). The MM phenotype indicates the homozygous state of normal individuals with the normal M allele and the ZZ phenotype characterises homozygous Z allele mutation individuals who suffer from the most severe deficiency of α 1-antitrypsin (DeMeo and Silverman 2004).

Alpha1-antitrypsin deficiency is associated with a substantially increased risk for the development of pulmonary emphysema by the 3rd or 4th decades of life but can also predispose to liver disease (chronic hepatitis, cirrhosis and hepatoma), skin disease (panniculitis) and vasculitis. Glomerulonephritis, coeliac disease, cancers (lung, colorectal and bowel), intracranial and intraabdominal aneurysms, and pancreatitis comprise a list of other diseases that have been suggested to be associated with α 1-antitrypsin deficiency although these are not yet well established (Stoller 2004).

The ZZ phenotype accounts for more than 95% of clinically recognised cases of α 1-antitrypsin deficiency and demonstrates low serum levels of α 1-antitrypsin (less than 20 μ mol/L while normal individuals typically have serum values of 20-53 μ mol/L) and also decreased functional activity (Owen et al. 1983). The molecular basis seems to be a substitution of lysine for glutamic acid at position 342 resulting in irreversible structural alterations and polymerisation of the molecule (Yoshida et al. 1976; Dafforn et al. 1999; Devlin et al. 2002; Sivasothy et al. 2000). Polymerisation prevents its secretion by hepatocytes resulting in only 15% of Z-mutated α 1-antitrypsin reaching the circulation.

In the pathogenesis of lung disease, the balance between protease-antiprotease has received a lot of early attention and led to the concept of neutrophil-mediated lung diseases.

Alpha1-antitrypsin deficiency has been historically linked with excess HNE burden leading to uninhibited proteolysis and it is the ongoing elastolytic damage that is believed to result in the destruction of alveolar walls and the development of emphysema (Janoff 1985).



Individuals with the ZZ phenotype not only have much lower circulating levels of α 1-antitrypsin but the molecule itself is five times less effective in HNE inhibition (Ogushi et al. 1987). More recent evidence suggests that, in addition to the quantitative deficiency, Z-type polymers can promote inflammation per se exerting chemoattractant properties for neutrophils (Elliott et al. 1998; Parmar et al. 2002; Mulgrew et al. 2004).

It is also worth remembering that clinically significant disease occurs predominantly in smokers and this phenomenon can be in part explained by the inactivation of the Met³⁵⁸ active site residue by cigarette smoke-derived oxidants. This oxidation results in a 1000-fold decrease in antiprotease activity (Brantly et al. 1988). Whilst NE can still cleave oxidised α 1-antitrypsin at the active site, it is unable to maintain its connection and the dissociation rate is much increased (Johnson and Travis 1979).

A relative imbalance of α 1-antitrypsin and proteases in favour of proteases has been described in other neutrophil-mediated lung diseases such as cystic fibrosis (Birrer et al. 1994) and emphysema not related to α 1-antitrypsin deficiency (Fujita et al. 1990; Yoshioka et al. 1995), implying a role for α 1-antitrypsin in modulating the effects of neutrophils and their products in inflammatory lung diseases.

The observation that α 1-antitrypsin can be secreted by inflammatory cells is suggestive of an important role in the global protection of the lung parenchyma against proteolytic damage in the context of an acute inflammatory milieu, despite the fact that the overall contribution of inflammatory cells is substantially lower in comparison to that of hepatocytes (Knoell et al. 1998).

The same observation on secretion and up-regulation of α 1-antitrypsin from front line alveolar macrophages and peripheral blood monocytes served as the basis for an emerging concept of α 1-antitrypsin being capable of exerting important anti-inflammatory properties in addition to protection against HNE-mediated degradation and injury.

Investigators have shown *in vitro* that α 1-antitrypsin can inhibit neutrophil superoxide production (Bucurenci et al. 1992), induce macrophage-derived interleukin-1 receptor antagonist release (Tilg et al. 1993) and that it can interact with the proteolytic cascade involved in apoptosis (Ikari et al. 2001).

Alpha1-antitrypsin has also been shown to inhibit HNE-mediated production of neutrophil chemoattractants such as LTB₄ and CXCL8 by alveolar macrophages (Spencer et al. 2004), to inhibit LPS-induced synthesis and release of TNF- α , IL-1 β , monocyte chemoattractant protein-1 (MCP-1/CCL2), CXCL8 and IL-6 by human monocytes and enhance the release of the anti-inflammatory cytokine IL-10.

In human studies of α 1-antitrypsin augmentation therapy, not only were α 1-antitrypsin concentrations restored to normal but a reduction in LTB₄ (a known mediator of neutrophil recruitment and activation) release in the larger airways was also noted (Stockley et al. 2002).

The role of α 1-antitrypsin in the context of acute inflammation is therefore evolving as more evidence is gathering on an anti-inflammatory effect that is perhaps independent of the protection against neutrophil-mediated proteolysis.

The same concept will govern ongoing research on the two other main HNE inhibitors, namely secretory leukocyte protease inhibitor (SLPI) and elafin, and will be discussed in the following sections.

3.2 SLPI

In contrast to alpha1-antitrypsin, SLPI and elafin share the characteristics of being cationic low molecular weight inhibitors of HNE which have 'defensin-like' antimicrobial properties and are secreted within the lung in response to inflammatory stimuli. These combined characteristics of SLPI and elafin make them candidate 'lung-protective' molecules in the setting of infective inflammation. For these reasons SLPI and elafin play an important part in this thesis and are reviewed in depth. Indeed, the starting hypothesis of the study postulates that VAP is associated with down-regulation of SLPI and elafin, resulting in relative protease/antiprotease imbalance in favour of biologically active extracellular HNE.

Human SLPI is an 11.7 kDa cationic non-glycosylated protein which contains 107 aminoacid residues. It is a highly basic (pH > 9.5), acid-stable but alkaline-labile protein.

SLPI is the third most abundant innate immunity protein of respiratory secretions after lysozyme and lactoferrin (Rogan et al. 2006). It is estimated that SLPI is present at concentrations of 0.1 to 2 μ g/ml in airway lavage fluid (Kouchi et al. 1993), and 2.5 μ g/ml in nasal secretions (Vogelmeier et al. 1991).

The gene encoding the protein is approximately 2.65 kb long (Seemuller et al. 1986; Thompson and Ohlsson 1986). A promoter region has been identified in a 131 base pair fragment (Maruyama et al. 1994) and within this region, a proximal 41 base pair (bp) region that confers specificity for lung expression has been described (Kikuchi et al. 1997).

The protein consists of two domains each containing four-disulphide cores (or 16 cysteine residues that form eight disulphide bridges). The resulting compact structure of the four-disulphide core is also called a WAP motif which derives from the well-characterised member of the four-disulphide core whey acidic protein family, which is the most abundant protein in rodent milk (Grutter et al. 1988). The NH₂-terminal domain contains heparin-binding sites (Ying et al. 1994; Mellet et al. 1995) and the COOH-terminal domain contains the active site (peptide bond Leu 72-Met73) (Grutter et al. 1988).

SLPI is constitutively expressed at many mucosal surfaces (leading to its alternative name of mucus proteinase inhibitor) and is produced by respiratory (tracheal and bronchial epithelium, bronchiolar Clara cells, type II alveolar cells), intestinal and amniotic epithelium (Sallenave et al. 1993; Si-Tahar et al. 2000; Saitoh et al. 2001; Zhang et al. 2001). Production of SLPI has also been demonstrated in mast cells, neutrophils and alveolar macrophages (Sallenave et al. 1997; Westin et al. 1999; Mihaila and Tremblay 2001).

SLPI has been shown to inhibit HNE, cathepsin G, trypsin, chymotrypsin and mast cell chymase, but not proteinase 3. Its most important action however is considered to be the inhibition of HNE to which it binds tightly in a 1:1 stoichiometric complex (Boudier and Bieth 1992; Sallenave 2000).

It has been suggested from the size and crystal structure of SLPI that it can offer protection within the gap between neutrophils and substrate (Rice and Weiss 1990). This is in direct contrast to the bulkier alpha1-antitrypsin molecule which is considered incapable of accessing this space. In the same context, SLPI has been demonstrated to associate with elastin fibres in the lung (Willems et al. 1986; Kramps et al. 1989) and skin (Wingens et al. 1998) suggesting that SLPI can be concentrated at the site of damaged/denuded epithelium and can play a role in protection of matrix against proteolysis.

SLPI production from alveolar epithelial cells, bronchial epithelial cells and airway submucosal glands is significantly upregulated by alarm signals such as LPS and early proinflammatory cytokines (namely IL-1 and TNF- α) (Sallenave et al. 1993; Maruyama et al. 1994; Sallenave et al. 1997). Similar findings have been observed in SLPI production from amniotic epithelium (Zhang et al. 2001). Epidermal growth factor (EGF) and α -defensins have also been shown to augment SLPI production from epithelial cells (van Wetering S. et al. 2000a; Sorensen et al. 2003).

Interestingly HNE appears to upregulate SLPI mRNA expression from bronchial submucosal glands and epithelial cells but this is not associated with increased SLPI secretion. In fact the opposite has been observed and SLPI secretion is reduced post stimulation with HNE (Abbinante-Nissen et al. 1993; Sallenave et al. 1994; van Wetering S. et al. 2000b; Saitoh et al. 2001).

Expression of SLPI by myeloid cells appears to be subject to additional regulation, providing evidence that SLPI may play an anti-inflammatory role.

SLPI has been demonstrated to inhibit the production of prostaglandin E2 (PGE2) by LPS-stimulated human monocytes, resulting in the suppression of interstitial collagenase (MMP-1) and gelatinase B (MMP-9). The inhibitory effect of SLPI was largely independent of its antiprotease activity as SLPI muteins, with significantly lower antiprotease activity, also suppressed the induction of PGE2 and MMPs (Zhang et al. 1997).

Expression of SLPI by murine macrophages has been shown to be upregulated by stimulation with LPS. However LPS-induced macrophage secretion of IL-1 and TNF- α did not seem to have an effect on SLPI production while SLPI production was induced by IL-6 and IL-10 in a slow and prolonged way suggestive of an autocrine role dampening down macrophages' inflammatory response (Jin et al. 1998).

In other experiments SLPI has been shown to attenuate LPS-induced macrophage responses. Transfection of normal macrophages with SLPI suppressed LPS-induced activation of NF- κ B and production of nitric oxide and TNF- α , rendering them hyporesponsive (a phenomenon that could be reversed by IFN- γ which restored LPS responsiveness and suppressed SLPI production) (Jin et al. 1997). This effect could be in part explained by suggestions that SLPI blocks LPS transfer to soluble CD14 and interferes with the uptake of LPS from LPS-CD14 complexes by macrophages (Ding et al. 1999). Thus, SLPI is an LPS-induced phagocyte product that can be suppressed by IFN- γ and plays an important role in the inhibition of LPS responses at the interface between innate and adaptive immunity.

More evidence on the potential anti-inflammatory role of SLPI has been provided by a number of *in vivo* studies and human observational studies that will be summarised in brief below. With specific regard to lung inflammation:

a) in a model of immune complex/neutrophil-mediated acute alveolitis, recombinant SLPI reduced *in vivo* lung damage as assessed by increased permeability or haemorrhage (Mulligan et al. 1993).

b) intratracheal and intravenous administration of truncated recombinant SLPI significantly attenuated lung injury in a sequential LPS-fMLP model of neutrophil-dependent acute lung injury in hamsters (Mitsuhashi et al. 1997).

c) intraperitoneal recombinant SLPI was also shown to ameliorate injury and lung fibrosis in bleomycin-induced pulmonary fibrosis in hamsters (Mitsuhashi et al. 1996).

d) pre-treatment with SLPI greatly reduced inflammation in both liver and lungs and diminished neutrophil accumulation in a mouse model of hepatic ischaemia/reperfusion injury (Lentsch et al. 1999b)

e) intratracheal recombinant SLPI (rSLPI) significantly diminished the development of LPS-mediated pulmonary emphysema in hamsters (Rudolph et al. 1993).

f) antibodies against endogenous rat SLPI resulted in significant augmentation of lung injury (as revealed by extravascular leak of albumin), substantially increased neutrophil accumulation, and correlated with increased levels of C5a-related chemotactic activity in BAL fluid (Gipson et al. 1999).

g) in an elastase-induced model of hyperresponsiveness, recombinant SLPI significantly inhibited airway constriction, airway hyper-responsiveness and the increase of cells in BALF (Suzuki et al. 1996).

h) in murine and sheep models of atopic asthma, SLPI inhibited eosinophil, lymphocyte and neutrophil influx, the subsequent late-phase bronchoconstriction, and development of hyper-responsiveness (Wright et al. 1999).

The protective effect of SLPI has also been studied in other models outside the lung. SLPI has been shown to have protective effects against HNE-mediated endothelial cell damage and proinflammatory signaling in both endothelial cells and macrophages. Adenovirus-mediated overexpression of SLPI suppressed oxidised low density lipoprotein (LDL)- and LPS-induced activation of NF- κ B (Henriksen et al. 2004b).

SLPI has been shown to reduce eosinophil and neutrophil recruitment in models of eye and joint inflammation (Murata et al. 2003; He et al. 2004a; Sehnert et al. 2004), and to inhibit IgE-dependent histamine release from lung, tonsil, and skin mast cells (He et al. 2004b).

A protective role of SLPI in brain injury has also been demonstrated in a rat model of ischaemic stroke. Administration of a recombinant adenovirus overexpressing SLPI into the cortical tissue resulted in a significant reduction in the ischaemic lesion over controls at the site of SLPI expression and significantly improved functional outcome (Wang et al. 2003).

Finally in observational studies, *Helicobacter pylori*-infected subjects exhibited a strong decline in antral SLPI levels compared to *H. pylori*-negative subjects and subjects from whom *H. pylori* had been eradicated. This reduction was specific for the antrum where SLPI levels were inversely correlated with inflammatory scores of antrum-predominant gastritis (Wex et al. 2004). In patients with ischaemic stroke, SLPI serum levels were increased compared with controls with a significant correlation between SLPI levels and brain tissue damage (Ilzecka and Stelmasiak 2002). Similarly in patients with sepsis circulating SLPI levels were elevated and maximal concentrations of serum SLPI correlated significantly with maximal multiple organ dysfunction scores (Grobmyer et al. 2000).

The above suggest that SLPI expression is up-regulated in the context of an acute inflammatory response. It is tempting to speculate that this represents the host's attempt to attenuate tissue damage. The *in vivo* evidence that accompanies the observational studies support this explanation as opposed to the concept that upregulation of SLPI is of detrimental effect and associated with worse outcome.

The proposed anti-inflammatory role of SLPI is intriguing and much work has been done in order to shed more light into possible mechanisms.

It has already been mentioned that SLPI impairs lipoteichoic acid (LTA)- and LPS-induced pro-inflammatory gene expression in monocytes and macrophages *in vitro* (Jin et al. 1998) through blockade of LPS transfer to soluble CD14 and interference of SLPI with the uptake of LPS from LPS-CD14 complexes by macrophages (Ding et al. 1999).

In more recent years investigators have focused on the interaction between SLPI and NF- κ B, in order to elucidate potential signal transduction pathways. NF- κ B has been recognised as a central mediator that orchestrates the human immune response.

This heterodimeric complex is retained in the cytoplasm bound to inhibitory proteins of the inhibitor κ B ($I\kappa$ B) family. Following cell activation, a number of signal transduction pathways lead to degradation of $I\kappa$ B proteins. This will result in nuclear translocation of NF- κ B and the ensuing transcriptional activation of proinflammatory genes (Lentsch and Ward 2000). NF- κ B is induced by over 150 different stimuli and the active form participates in the control of transcription of over 150 target genes including genes that regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules (Pahl 1999).

SLPI knockout mice (SLPI^{-/-}) showed a higher mortality from endotoxic shock than did wild type mice. This was followed by the observation that SLPI^{-/-} macrophages showed higher IL-6 and high-mobility group (HMG)-1 production, and enhanced NF- κ B activity after LPS treatment, as compared with SLPI^{+/+} macrophages (Nakamura et al. 2003).

Other investigators have shown that adenovirus-mediated overexpression of SLPI (and elafin) suppresses the oxidised LDL- and LPS-induced activation of NF- κ B in macrophages and endothelial cells (Henriksen et al. 2004b). It has been proposed that SLPI can exert this effect by protecting the NF- κ B inhibitor, $I\kappa$ B β from degradation, as shown in an immune complex model of lung inflammation and lung injury (Lentsch et al. 1999a). Similarly SLPI could inhibit LPS-induced NF- κ B activation, by inhibiting degradation of interleukin-1 receptor associated kinase (IRAK), $I\kappa$ B α and $I\kappa$ B β (Lentsch et al. 1999a; Taggart et al. 2002).

More recently it has been demonstrated that SLPI can enter cells, and is rapidly localised to the cytoplasm and nucleus where it affects NF- κ B activation by binding directly to NF- κ B binding sites in a site-specific manner (Taggart et al. 2005).

However once SLPI is oxidised, the anti-inflammatory and anti-elastase effects are ameliorated. In community-acquired pneumonia (CAP) it was demonstrated that cleavage of SLPI resulted in impaired anti-elastase activity (Greene et al. 2003). The anti-elastase effect of SLPI can be compromised by cleavage or oxidation due to smoking (Cavarra et al. 2001). Cathepsins B, L, and S have also been shown to cleave and inactivate SLPI (Taggart et al. 2001). Similar observations have been made in asthma where it has been demonstrated that serine and cysteine proteases produced by the house dust mite can cleave SLPI (Brown et al. 2003).

Along with the potential anti-inflammatory role of SLPI, other properties have been proposed and will be mentioned briefly below:

a) resolution of inflammation.

Murine macrophages synthesise and secrete SLPI during clearance of apoptotic cells leading to suppression of TNF- α production. This may suggest that SLPI, produced by existing macrophages after contact with apoptotic cells, serves to prevent overproduction of TNF- α by incoming macrophages (Odaka et al. 2003).

b) repair processes and wound healing

SLPI can up-regulate LPS-stimulated production of anti-inflammatory/repair type cytokines TGF- β and IL-10 by macrophages (Sano et al. 2000). At the same time TGF- β has been shown to downregulate SLPI suggesting perhaps a feedback loop (Jaumann et al. 2000).

SLPI has been shown to play an important role in cutaneous and oral mucosal wound healing (Ashcroft et al. 2000; Angelov et al. 2004). In an *in vitro* model of wound healing and scar formation, SLPI inhibited fibroblast-mediated collagen gel contraction (Sumi et al. 2000). However it is worth mentioning that studies have also suggested a pro-proliferative role. SLPI selectively up-regulated proliferative signals (cyclin D1 gene) in endometrial epithelial cell lines and negatively influenced anti-proliferative and pro-apoptotic protein expression (insulin-like growth factor-binding protein-3 (IGFBP-3)). Lysyl oxidase, a phenotypic inhibitor of the ras oncogenic pathway and a tumour suppressor whose expression is up-regulated by TGF- β , was also suppressed by SLPI (Zhang et al. 2002) and SLPI positively regulated hepatocyte growth factor (HGF) production in human lung fibroblasts (Kikuchi et al. 2000).

c) protection against a variety of pathogens

SLPI has been shown to display antimicrobial properties that have been attributed at least in part to the cationic properties of the molecule. Current theories suggest that cationic 'defensin-like' molecules can punch holes in the cell wall of bacteria, critically altering the flux of ions and solutes exchanged with the surrounding environment (Hancock and Diamond 2000; Hale and Hancock 2007) Studies have demonstrated inhibition of the antimicrobial activities of SLPI by higher salt concentrations (Hiemstra et al. 1996). This is in keeping with reports showing inhibition of antimicrobial activity at higher ionic strength of a variety of other antimicrobial proteins (Lehrer and Ganz 1990; Martin et al. 1995).

SLPI displays antibacterial activity (located in the NH₂ domain), at concentrations found in various secretions, against Gram-negative (*E.coli*) and Gram-positive (*S. aureus*) pathogens (Hiemstra et al. 1996). The group of pathogens that SLPI exerts its antibacterial activity on has expanded to include *P. aeruginosa*, *S. epidermidis* (Wiedow et al. 1998a), group A Streptococci, and fungi in particular *Aspergillus fumigatus* and *Candida albicans* (Tomee et al. 1997; Wiedow et al. 1998; Fernie-King et al. 2002).

Finally SLPI has been shown to protect adherent monocytes and activated peripheral blood mononuclear cells against human immunodeficiency virus-1 (HIV-1) infection (McNeely et al. 1995; Wahl et al. 1997; Shugars 1999), and to interrupt the interaction of HIV with receptors on the host cell (McNeely et al. 1997). This protective effect is supported by human observational studies which demonstrated that increased infant levels of SLPI in salivary fluid are associated with reduced maternal-infant HIV transmission (Farquhar et al. 2002). Similarly increased vaginal fluid levels are associated with reduced HIV transmission at childbirth (Pillay et al. 2001).

d) Regulation of adaptive immune response

Dendritic cells, upon maturation, will act as antigen presenting cells and will initiate an adaptive immune response (Banchereau et al. 2003).

It has been proposed that SLPI regulates cellular activation of dendritic cells in response to LPS orchestrating tolerance towards protective LPS signals and setting off an inflammatory immune response in the presence of pathogenic levels of LPS (Samsom et al. 2007).

3.3 Elafin

Elafin was first identified as a “non-SLPI low molecular weight anti-elastase” (Hochstrasser et al. 1981; Kramps and Klasen 1985). It was isolated and purified a few years afterwards in sputum from patients with chronic bronchitis (Sallenave and Ryle 1991), and also from psoriatic skin and human keratinocytes (Schalkwijk et al. 1990; Wiedow et al. 1990; Schalkwijk et al. 1991), and named elafin and skin-derived anti-leucoproteinase (SKALP) respectively.

The gene is approximately 2.3 kb long (Sallenave and Silva 1993), is located on chromosome 20 (Molhuizen et al. 1994), and contains 3 exons and 2 introns. Elafin and SLPI are co-localised (within 75 kb) on chromosome 20q12-13.12, where they reside in close proximity to two rapidly evolving seminal vesicle-transcribed (REST) proteins, semenogelin I and II, that encode transglutaminase substrates (Schalkwijk et al. 1999). The elafin gene encodes a 9.9 kDa protein consisting of 117 amino acids, including a hydrophobic signal peptide of 22 amino acids (Sallenave and Silva 1993) and shares approximately 40% sequence homology with SLPI (Wiedow et al. 1990; Sallenave and Silva 1993).

Elafin is a cationic molecule that can be divided into two distinct domains. The NH₂-terminal domain, also referred to as “cementoin”, provides a substrate for the enzyme transglutaminase and contains characteristic VKGQ sequences that allow elafin to form polymers through transglutamination (Nara et al. 1994) therefore enabling the molecule to anchor to extracellular matrix. The COOH domain, which contains the active antiproteinase site, contains 8 cysteine residues that form 4 disulphide bonds (one WAP domain). The COOH domain contains the Ala-Met bond which represents the active inhibitory site (Tsunemi et al. 1996; Francart et al. 1997).

The presence of these 2 structural domains (WAP domain and a transglutaminase substrate domain) characterises not only elafin but a family of proteins called the trappin family (trappin being an acronym for TRansglutaminase substrate and wAP domain containing ProteIN, referring to its property of getting ‘trapped’ in a tissue and thereby functioning as an anchored protein) (Schalkwijk et al. 1999). Members of this family are characterised by containing two functional domains: a transglutaminase substrate domain composed of multiple hexapeptide repeats with the consensus sequence GQDPVK, and a C-terminal inhibitor domain that folds into the four-disulphide core structure.

WAP-containing trappins have been described in various species (Schalkwijk et al. 1999) and five human trappins have now been identified (including elafin that has also been named trappin-2), but little is known about the rest of them (Furukawa et al. 1996; Zeeuwen et al. 1997). In the current nomenclature a lot of confusion exists as more recently the term trappin-2 has been reserved for the whole molecule along with the term pre-elafin whilst the term elafin is mainly used to refer to the COOH terminal. For the purpose of this thesis the term elafin will be used to represent the whole molecule.

Elafin is an inhibitor of neutrophil serine proteases such as HNE and proteinase 3 but not cathepsin G. Elafin contains motifs (substrates for transglutaminases), that may enable the molecule to be cross-linked to extracellular matrix proteins, thus anchoring the inhibitor at its site of action (Guyot et al. 2005). As described for SLPI previously, this theoretically allows elafin to be concentrated in matrix in the event of epithelial damage/denudation.

Elafin inhibits HNE forming a reversible 1:1 stoichiometric complex, although at a slow rate of dissociation (Ying and Simon 1993; Tsunemi et al. 1996). It also binds and inhibits proteinase-3 in a fully reversible but rapidly dissociated 1:1 complex (Ying and Simon 1993; Ying and Simon 2001) and porcine pancreatic elastase. Therefore elafin appears to have a more restricted spectrum of inhibition than SLPI and neither of the two antiproteases have an activity against granzyme (Tremblay et al. 2000).

Once oxidised however elafin, like SLPI, exerts diminished elastolytic activity against HNE and almost completely loses its capacity to inhibit proteinase-3 (Nobar et al. 2005).

Elafin was first isolated in bronchial secretions (Sallenave and Ryle 1991; Sallenave and Silva 1993) and has been present in tracheal biopsies and bronchoalveolar lavage both from patients and healthy individuals (Sallenave et al. 1999). Its production has been attributed to bronchial epithelial cells (Sallenave et al. 1994), alveolar type II epithelial cells (Sallenave et al. 1993), and also more recently to alveolar macrophages (Mihaila and Tremblay 2001). Elafin is also present in skin where its expression is constitutive in the squamous epithelium (Wiedow et al. 1990; Pfundt et al. 1996), and is upregulated in inflammatory conditions such as psoriasis (Alkemade et al. 1994; Nonomura et al. 1994). Elafin is also produced by epithelium in the large intestine (Suzuki et al. 2000) and in endometrial epithelial cells (King et al. 2003).

Elafin expression and secretion from pulmonary epithelial cells is upregulated by IL-1 and TNF- α . Recent studies suggest that this effect is regulated by a functional NF- κ B site located within the first 100 base pairs of the elafin gene promoter (Sallenave et al. 1994; Tanaka et al. 2000a; Bingle et al. 2001). IL-1 and TNF- α have similar effects on the production of elafin from keratinocytes where the p38MAPK pathway has been implicated (Pfundt et al. 2000; Tanaka et al. 2000b). This can be downregulated by the application of topical corticosteroids (Kuijpers et al. 1997). In mammary epithelial cells the transcription factor activating protein-1 (AP-1) mediates activation (Zhang et al. 1995) suggesting that transcriptional activation of elafin is cell specific.

Neutrophil elastase, in a similar fashion to that discussed in the section for SLPI, seems to upregulate elafin mRNA expression in A549 cells but elafin secretion is down-regulated (Reid et al. 1999). It is of interest that the cytokine-mediated upregulation of elafin production is greater than for SLPI production (Sallenave et al. 1994). Therefore whilst SLPI may offer a baseline antiprotease shield, being present in bronchoalveolar lavage of healthy individuals, elafin could play an important role in airway protection at the very early phases of the inflammatory response.

The observation that elafin, like SLPI, is highly cationic and shows selective expression at mucosal surfaces suggested that it may possess antimicrobial properties.

Indeed elafin has been shown to have antimicrobial activity against *S. aureus* and *P. aeruginosa*. For optimum activity to be exerted, the full length of the molecule is required as antimicrobial activity was not localised specifically to either domain (Simpson et al. 1999; Meyer-Hoffert et al. 2003; McMichael et al. 2005). More recently it has been shown that elafin appears to opsonise *P. aeruginosa* for more efficient, CD14-dependent clearance by macrophages (Wilkinson et al. 2009).

Finally genetic augmentation of elafin not only enhanced bacterial clearance but effected protection against acute inflammatory lung injury in a model of *P. aeruginosa* pneumonia (Simpson et al. 2001). This effect shifted attention to the potential anti-inflammatory properties of elafin and there is emerging evidence from various models of inflammation to support this role:

a) In a model of atherosclerosis, genetic augmentation of elafin (like SLPI) has been shown to have protective effects against HNE-mediated endothelial cell damage and proinflammatory signaling in both endothelial cells and macrophages (Henriksen et al. 2004b;).

b) Similar results were reported in other *in vivo* cardiovascular studies. Recombinant elafin conferred protection in post-cardiac transplant coronary arteriopathy and myocardial necrosis induced by rejection in a rabbit model of cardiac transplantation (Cowan et al. 1996). In elafin-expressing transgenic mice, elafin appears to suppress inflammation, cardiac dilatation and dysfunction after myocardial infarct (Ohta et al. 2004). Elafin also reduces mortality in association with better cardiac function and less myocardial inflammatory damage following viral myocarditis (Zaidi et al. 1999). In another model of arterial injury, elafin-transgenic mice showed reduced inflammatory cell infiltration in the injured arteries and preserved positive remodeling after injury (Zaidi et al. 2000).

c) *In vivo* studies of lung injury showed that recombinant elafin can reduce neutrophil influx in the alveolar spaces in an LPS-induced model, and can downregulate mRNA levels of early proinflammatory cytokines such as IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), and TNF- α . In the same experiments elafin inhibited LPS-induced production of gelatinase (MMP-9) and MIP-2/CCL1, suggesting an immunomodulatory role that can protect against NE-mediated lung injury (Vachon et al. 2002).

In similar studies in hamsters, recombinant human elafin has been shown to exert a significant protective effect against HNE-induced acute lung injury as demonstrated by a dose-dependent decrease in the BAL fluid haemoglobin content of treated animals (Tremblay et al. 2002).

In addition, *in vitro*, elafin inhibits LPS-induced production of MCP-1/CCL2 from a monocytic cell line by preventing the LPS-induced activation of AP-1 and NF- κ B. This appears to be dependent upon an effect of elafin on the ubiquitin-proteasome pathway (Butler et al. 2006).

Other effects proposed for the protective role of elafin are related to

a) interaction with neutrophil chemotaxis.

Both elafin and SLPI were able to reverse the complete inhibition of C5a –dependent neutrophil migration noted upon incubation of neutrophils with neutrophil lysates or neutrophil degranulation supernatants (Tralau et al. 2004).

b) resolution of inflammation

Elafin has been shown to inhibit cleavage of macrophage CD14 by HNE and therefore facilitate clearance of apoptotic neutrophils by phagocytosis (Henriksen et al. 2004a).

Finally attention has been shifted recently to the regulation of dendritic cells by elafin. Elafin has been recently shown to increase the number, and enhance activation, of the dendritic cells *in vivo* promoting a Th-1 type immune response (Roghianian et al. 2006), thus providing a potential role for elafin as a bridge between innate and adaptive immunity.

In summary, the innate immune response is considered to be responsible for the rapid initial eradication of invading microorganisms. However it is also recognised that the same system with its impressive collection of weapons, exhibits destructive potential for the integrity of the host tissue. Excessive release of HNE and other proteolytic enzymes as a result of neutrophil degranulation, has been implicated in the extensive tissue damage seen in a variety of inflammatory conditions.

Both SLPI and elafin, while historically identified and characterised as antiproteases, are now regarded as proteins in possession of antimicrobial activities against a variety of bacteria, fungi and potentially viruses. They are expressed by a number of cells including alveolar epithelial cells, neutrophils, monocytes and alveolar macrophages and are up-regulated by early inflammatory cytokines such as IL-1 and TNF- α . These characteristics not only provide an antiprotease shield against neutrophil proteases but also anti-inflammatory properties which are regulated by mechanisms that have not been fully elucidated.

Several hypotheses related to the anti-inflammatory role of SLPI and elafin have emerged as described earlier, involving inhibition of neutrophil influx, inhibition of inflammatory cells, resolution of inflammation via macrophage-mediated phagocytosis of apoptotic neutrophils, and upregulation of anti-inflammatory responses by augmenting production of anti-inflammatory repair type cytokines IL-10 and TGF- β .

Furthermore SLPI and elafin can regulate extracellular matrix production, cell growth and proliferation playing a role not only in resolution of inflammation but also in subsequent repair processes. Emerging evidence suggests that they may be functional in adaptive immunity facilitating Th1-type immune responses (Schalkwijk et al. 1990; Tremblay et al. 1996; Zhang et al. 1997).

They are now recognised as key mediators in innate host defense and perhaps act as a crosslink between innate and adaptive immunity. The above characteristics are attractive for use *in vivo* to modulate neutrophil-mediated lung injury.

3.4 Summary of introduction

This introduction has attempted to outline the current controversies faced in the study of VAP, with particular emphasis on the heterogenous populations in which VAP has been studied, and the relative merits of diagnostic techniques available. I have tried to stress the importance of vigorous characterisation of VAP in the patient population the population studied, a principle that dominates Chapter III, section 1. The introduction has also outlined the importance of the neutrophil in acute inflammation and has considered some of the key cytokines involved in the recruitment of neutrophils during pneumonia, a subject of importance to Chapter III, section 2 and 3. Furthermore, the biology of neutrophil activation and neutrophil phagocytosis have been reviewed, based on the fact that the central hypothesis of this work contends that both processes may be aberrant in association with VAP, subjects dealt with in Chapter III, section 5 and 6. Among the many potentially harmful mediators released by neutrophils, HNE has a key role and was discussed in depth because of its relevance to Chapter III, section 4. Finally, the introduction considered the lung's main endogenous inhibitors of HNE which are revisited in Chapter III, section 4, specifically seeking whether production of SLPI and elafin are deficient in VAP.

4. CENTRAL HYPOTHESES

The central hypothesis of this thesis is that VAP is associated with impaired neutrophil phagocytosis, enhanced neutrophil activation and deficient antiprotease production resulting in an exaggerated inflammatory response (Figure 2).

The testing of this hypothesis is critically dependent on rigorous characterisation of patients with 'clinically suspected VAP' such that this population (common in the intensive care unit) could be confidently separated into VAP and NON VAP groups, within the considerations surrounding diagnostic methodologies.

Despite the wide acknowledgement that VAP remains the most common ICU-acquired infection in ventilated patients, associated with excess mortality, morbidity and cost, very little data are available regarding possible unrecognised deficiencies of the innate immunity specific to VAP. Studying these was a novel concept aiming to identify defects and perhaps suggest new therapeutic targets not only in the context of infection but also in other neutrophil-mediated diseases. In order to achieve the aims of the study patients with clinically suspected VAP were recruited from a general ICU. Strict, pre-defined criteria were used to separate this population into VAP and 'NON VAP' groups, the latter acting as a control group. A group of healthy volunteers served as a reference group.

In any study of VAP it is crucial to define the demography and microbiology of the population studied. Analysis of the demographic and microbiological data are therefore presented in detail in Chapter III, section 1. The main purpose of this analysis is to identify how well matched the VAP and NON VAP groups were and if any differences in age, sex, comorbidities, severity of illness etc could bias the interpretation of the results. Presentation of the microbiological data allows a detailed analysis of the local epidemiology of VAP with emphasis on the diagnostic methodology used in the study. The diagnostic utility of BAL and ETA is briefly considered given the ongoing controversy in the literature.

In order to assess the presence of increased inflammation in the alveolar space of these patients BAL was performed. The cellular composition of BAL in patients and healthy volunteers and the degree of neutrophilia noted is discussed in Chapter III, section 2. A detailed quantification of inflammatory markers namely CXCL8, IL-1 β , TNF- α , IL-6, IL-10 and IL-12p70, in the lung compartment and in the systemic circulation was also performed using cytokine bead array kits and the results are presented in Chapter III, section 3. Along with neutrophil chemoattractant mediators and cytokines, MCP-1/CCL2 (as a monocyte chemoattractant that is implicated not only in bacterial clearance but also in monocyte activation and potentially in exaggerating host tissue damage) was measured in BALF and serum. Data are discussed in the same section.

Along with the presence of a pro-inflammatory profile in patients with VAP, an impaired local antiprotease/antimicrobial expression was anticipated allowing or facilitating neutrophil-mediated tissue injury.

This was tested with quantification of the endogenous HNE inhibitors in the lung: elafin and SLPI (two low molecular weight cationic inhibitors of HNE which are produced locally in the lung in response to inflammatory stimuli) and alpha1-antitrypsin (a larger molecule produced by the liver and which is thought to diffuse into the lung passively via the circulation). In addition the lung's 'global' capacity to neutralise HNE was quantified by testing the capacity of the BALF to inhibit HNE using an EIA assay. Finally, the level of HNE antigen in BALF was measured. Data are presented in Chapter III, section 4.

Along with the hypothesis of excessive inflammatory response and the impaired antiprotease expression, neutrophil dysfunction and enhanced neutrophil activation was anticipated in VAP. It was hypothesised that neutrophils, abundant in the lungs of patients with VAP, will display an impaired capacity to eliminate pathogens. The phagocytic capacity of peripheral blood neutrophils and alveolar phagocytes was tested in a phagocytosis assay. These data are described in Chapter III, section 5. Neutrophil activation and the capacity of peripheral neutrophils to cause alveolar epithelial damage was tested in *in vitro* experiments with A549 cells. The model of assessing epithelial damage using A549 cells derives from the morphological features that A549 cells share with alveolar type II epithelial cells and the difficulties that human alveolar cell culture models have encountered. Results from these experiments are described in Chapter III, section 6.

CHAPTER II: MATERIALS AND METHODS

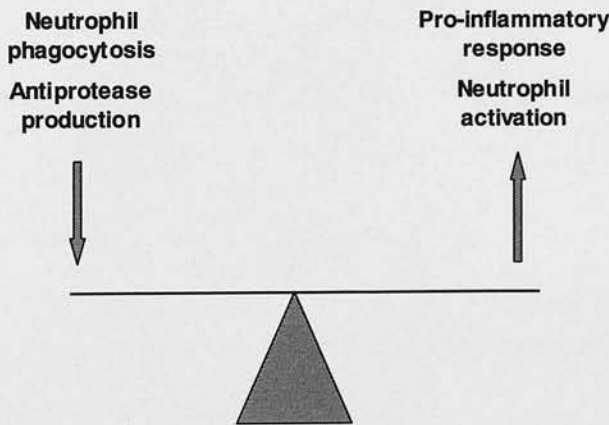


Figure 2: Central hypothesis

II. MATERIALS

II.1 Chemicals and reagents

All chemicals were from Sigma-Aldrich Company Ltd, Irvine, Ayrshire, UK (including Zymogen A and Zymogen B), and LPS from *E. coli* strain O127:H6, and BCA assay for protein quantification, except:

1. Agar (Columbia agar with blood) from Oxoid Ltd, Basingstoke, UK
2. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
3. Antibodies for ELISA from BD Biosciences, Franklin Lakes, NJ, USA
4. Anti-human α_1 -antitrypsin (AAT) from Calbiochem, La Jolla, CA, USA
5. AAT from human plasma from Plasmatron, St. Mary's Priory, Poole, Dorset, UK
6. Cytokine bead array (CBA) system from BD Biosciences, CA, USA
7. Dextran 70 from Pharmacia, Biotech, Sweden
8. E-Cell Quick Kill from Celltech, Technical Services, Slough, UK
9. Fetal calf serum (FCS) from Gibco, Grand Island, NY, USA
10. Human IgG from Gibco, Grand Island, NY, USA
11. Human IgM from Gibco, Grand Island, NY, USA
12. Human IgG1 from Gibco, Grand Island, NY, USA
13. Human IgG2 from Gibco, Grand Island, NY, USA
14. Human IgG3 from Gibco, Grand Island, NY, USA
15. Human IgA from Gibco, Grand Island, NY, USA

CHAPTER II: MATERIALS AND METHODS

1. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
2. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
3. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
4. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
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13. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
14. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA
15. Biotinylated anti-CD14 from BD Biosciences, Franklin Lakes, NJ, USA

II.2 Methods

Recombinant human α_1 -antitrypsin (AAT) was from BD Biosciences, NJ, USA. All other reagents were from Gibco, Grand Island, NY, USA.

1. MATERIALS

1.1 Chemicals and reagents

All chemicals were from Sigma Aldrich Company Ltd, Irvine, Ayrshire, UK (including Zymosan A from *Saccharomyces cerevisiae*, and LPS E Coli: Serotype 0127:B8, and BCA assay for protein estimation), except:

1. Agars (Columbia agar with Horse blood, Columbia Chocolate Blood agar with Bacitracin, McConkey agar without Salt, MALT agar) from Oxoid Ltd, Basingstoke, Hampshire, UK.
2. Antibodies for ELISAs from R&D Systems, Abingdon, UK.
3. Anti-human α 1-antitrypsin (peroxidase conjugate) from The Binding Site Limited, Birmingham, UK.
4. API identification system from Biomérieux sa, Marcy l'Etoile, France.
5. Cytokine Bead Assay (CBA) system from BD Bioscience, CA, USA.
6. Dextran/Percoll from Pharmacia, Uppsala, Sweden.
7. Diff-Quick Kit from Gamidor, Technical Services Ltd, Abingdon, UK.
8. Foetal Calf Serum (Heat Inactivated) from Biosera, Ringmer, East Sussex, UK.
9. Human Elafin and HNE ELISA kit from High Cult, Bioscience, Cambridge, UK.
10. Human SLPI ELISA kit from R&D Systems, Abingdon, UK.
11. Human Sputum Leukocyte Elastase from Elastin Product Company, Owensville, Missouri, USA.
12. IMDM (Iscove's Dulbecco Modified Eagle's medium) from GIBCO, Invitrogen, Paisley, Scotland, UK.
13. LDH assay from Biovision Research Products, CA, USA
14. Trypsin-Ethylene Diamine Tetraacetic Acid (EDTA) from GIBCO Invitrogen, Paisley, Scotland, UK.
15. Urea Assay from Bioassay Systems, Hayward, CA, USA.

1.2 Plastic-ware

Tissue culture plates and other plastic ware WERE from BD Falcon, NJ, USA. ELISA plates were from Costar, Corning Incorporated, NY, USA.

2. METHODS

2.1 Recruitment of patients/controls

2.1.1 Recruitment of patients: inclusion-exclusion criteria

Patients were recruited from a general ICU with a mixture of general surgical and medical admissions while cardiothoracic surgical patients and neurosurgical patients based at separate specialists ICUs were not included. This large mixed general and surgical ICU admits normally > 1000 patients annually of whom 50% stay more than 48 hours. Ventilation technology and strategy remained unchanged during the period of the study.

The attending clinicians on the general ICU were asked to contact the study team if he/she felt a patient had a clinical suspicion of VAP. Posters were displayed in the ICU to maintain awareness of the study. After such identification the study team applied pre-defined criteria for clinically suspected VAP to establish whether the patient was eligible, i.e. patients were recruited if they satisfied the following, in accordance with existing literature (Chastre and Fagon 2002).

Intubation and mechanical ventilation for at least 48 hours and:

-*New and persistent infiltrates on chest radiograph (CXR)* (these were defined as infiltrates that were not present at intubation or if present had worsened in appearance since intubation. The term persistent infiltrates was implemented to exclude the likelihood of left ventricular failure where radiological appearances resolve rapidly with treatment), and at least one of the following:

-*Temperature > 38°C,*

-*Purulent tracheal secretions,*

-*White cell count > 11x10⁹ /L*

In the above system I assessed all criteria personally, but as an additional quality control, new changes on CXR had to be independently verified by a consultant respiratory physician (Dr J. Simpson) for eligibility to be confirmed.

Patients were excluded if:

A) any of the following risk factors for adverse effects and complications at bronchoscopy of ventilated patients were present (Meduri and Chastre 1992):

-*PaO₂ < 8kPa on FiO₂ > 0.7,*

-*PEEP > 15 cmH₂O,*

-*Active bronchospasm (in keeping with findings of wheeze on auscultation of the chest),*

-*Recent (within 3 months) acute myocardial infarction,*

-*Unstable tachy- or bradyarrhythmia with cardiovascular compromise,*

- Mean arterial pressure <65mmHg on vasopressor therapy and,
- Bleeding diathesis including platelet count <20 x 10⁹ /L.

B) initiation or modification of antibiotics for new clinical features had taken place in the preceding 72 hours.

These patients were excluded as recent institution of antibiotics prior to bronchoscopy can potentially lead to false-negative culture (Montravers et al. 1993a; Baselski and Wunderink 1994; Torres and el-Ebiary 1998; Prats et al. 2002).

Patients with known ARDS/ALI were also included when they developed the entry criteria for clinically suspected VAP mentioned above.

The diagnosis of ARDS/ALI was made by the attending intensivist according to the American-European Consensus Conference (AECC) definitions of ARDS/ALI (Bernard et al. 1994a) that include the following:

- Acute onset,
- Bilateral infiltrates on CXR,
- Pulmonary artery occlusion pressure (PAOP) ≤18 mmHg or absence of clinical signs of left atrial hypertension,
- Hypoxaemia (defined for ARDS at PaO₂/ FiO₂ <200 mmHg or < 26 kPa, defined for ALI at PaO₂/ FiO₂ <300 mmHg or < 40 kPa).

An estimate of patients screened is not available for the exact period of recruitment as infection surveillance was introduced at least 6 months after the introduction of the study. Data from the Hospitals in Europe Link for Infection Control through Surveillance (HELICS) system are available for the period of January '05 until July '08 (when study ended). During this period, there were 309 suspected episodes of VAP, of whom 35 had contraindications to bronchoscopy, 25 had had antibiotics started/changed within 72 hours, 175 occurred at a time when the investigating team were unavailable, 1 relative declined consent - leaving 72 patients enrolled in the study (data kindly provided by Dr Andy Conway Morris).

Informed written or verbal consent was obtained by the next of kin and written and verbal information was given in agreement with and approval by the Local Research Ethics Committee (LREC). Verbal information was given through a phone call conversation and verbal consent was witnessed by a member of the nursing staff of the ICU available at the time.

Demographic and clinical data were collected from the patients' medical notes, laboratory results and the daily monitoring charts in the ICU. The Acute Physiology and Chronic Health Evaluation II (APACHE II) score was used in order to compare severity of illness between the two groups of critically ill patients. Data were collected from the ICU database.

The APACHE is a widely accepted and applicable scoring system that focuses on using physiological data to assess the severity of illness and to stratify patients prognostically on the basis of the risk of death.

The APACHE II score was derived from the relatively complex APACHE score that used 34 physiological parameters and is a simplified version widely used in ICUs. It is based on 12 physiological parameters measured on admission or during the first 24 hours, added on to a score based on age and a chronic health evaluation score derived from the state of health prior to admission (Knaus et al. 1985).

Severity scores such as APACHE II have been widely used not only to predict outcome and compare standardised mortality rates (SMR) of units but also to describe patient populations in clinical trials and studies.

2.1.2 Recruitment of healthy volunteers

Healthy volunteers (n=15) were recruited through advertisement mainly in primary care Penicuik Health Centre, Penicuik and also within the University of Edinburgh. The subjects from primary care were recruited with the aim of obtaining an age- and sex matched population.

Exclusion criteria included: hypoxia (saturation of oxygen (SaO₂) <92% on air), bleeding diathesis, anticoagulant therapy, insulin-dependent diabetes mellitus, arrhythmia, bronchospasm not responding to nebulised beta-2 agonist, or clinical evidence of respiratory tract infection

Informed written consent was obtained by the volunteer and verbal and written information was given in agreement with and approval by the LREC.

2.2 Preparation of the study patients/volunteers

2.2.1 Patients

Patients were prepared and premedicated by the attending anaesthetist prior to bronchoscopy with the following as directed:

-FiO₂: 1.0

-Short acting sedative eg. midazolam or propofol

-Synthetic narcotics for analgesia e.g.: alfentanil and/or muscle relaxant e.g. atracurium, if appropriate.

Topical lignocaine was not used in ventilated patients.

In ventilated patients, ETA was also collected by aspiration prior to bronchoscopy where possible.

The sample was used for microbiological analysis as part of studying the sensitivity and specificity of the ETA in comparison with BAL. This also allowed better visualisation of the bronchial tree upon insertion of the bronchoscope.

The internal diameter of the endotracheal tube was taken into account ensuring that the external diameter of the bronchoscope was at least 1.5mm smaller than the internal diameter of the endotracheal tube in order to maintain adequate ventilation throughout the procedure.

The bronchoscope was inserted via the endotracheal tube or tracheostomy tube through a special swivel connector. Physiological monitoring of electrocardiogram (ECG), arterial blood pressure and SaO₂ were recorded continuously before, during and after the procedure.

2.2.2 Healthy Volunteers

Intravenous access was established before bronchoscopy. Intravenous sedative (short acting benzodiazepines e.g. midazolam) was administered in incremental doses to achieve adequate sedation. Nasal anaesthesia was achieved with lignocaine gel (2%) and pharyngeal anaesthesia with lignocaine spray (2%) at a dose not exceeding 8.2 mg/kg in adults according to bronchoscopy guidelines (British Thoracic Society guidelines 2001b). Nasal or oral intubation was performed, the decision based on nasal anatomy and individual preferences. Lignocaine was not routinely instilled in the bronchial tree.

Oxygen supplementation via a nasal cannula was provided before, during and up to 2 hours after the procedure at a rate of 2-4 l/min. Physiological monitoring of ECG, and SaO₂ were recorded continuously before, during and after the procedure.

2.3 Flexible bronchoscopy and BAL

Flexible bronchoscopy was performed in accordance with the existing British Thoracic Society guidelines (2001).

Sampling and processing of the samples were performed according to the existing literature (Rennard et al. 1990; Baselski et al. 1992; Baselski and Wunderink 1994; Meduri and Chastre 1992; Torres and el-Ebiary 1998).

In brief BAL was performed as described below:

In patients with suspected VAP, CXR was reviewed and the decision about the segment to be lavaged was based on the following:

- segment involved on CXR or,
- segment seen to have purulent secretions during bronchoscopy or,

-posterior segment of the right lower lobe if none of the above were available or there were diffuse and bilateral progressive infiltrates on CXR.

In all healthy volunteers the posterior segment of the right lower lobe was lavaged.

Sterile normal (isotonic) saline (N/S) was used for lavage and was prewarmed to 37 °C prior to instillation.

The tip of the bronchoscope was carefully wedged into the lumen of the segment involved. 20 mls of sterile N/S were introduced and discarded after suction as this is generally accepted to represent bronchiolar secretions (Rennard et al. 1990; Montravers et al. 1993a; Rennard et al. 1998). Up to 200 mls of N/S were instilled in 4 x 50 mls aliquots.

2.4 Processing of BAL fluid (BALF)

From the BALF retrieved, an aliquot was sent to Respiratory Microbiology at the Royal Infirmary of Edinburgh for microbiological analysis, including culture for mycobacteria and *Legionella spp.* A further aliquot (when available) was sent to Medical Microbiology at the University of Edinburgh Medical School for detection and characterisation of anaerobes (Dr I. Poxton's laboratory).

The remaining volume was processed as described below:

- 1 20 µl were used to estimate total cell count using a haemocytometer
- 2 1ml was used for microbiological analysis:
 - Serial dilutions were made of 1/10² and 1/10⁴ in phosphate buffered saline (PBS) before plating 100 µl by the spread plate method on a variety of agar plates in duplicate.
 - Agar plates included: blood agar, chocolate agar + bacitracin, MacConkey agar without salt, malt agar and *Pseudomonas spp.* isolation agar (PIA).
 - Agar plates were incubated for 48 hours and viable counts were calculated at 24 and 48 hours.
 - Concentration of respiratory pathogens $\geq 10^4$ cfu/ml of BALF was taken to be diagnostic of VAP (Baselski and Wunderink 1994).
- 3 The remaining BALF was centrifuged at 500g for 10 min. The supernatant was immediately stored in aliquots at -80°C for future analysis.
- 4 Based on the total cell count cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) at a concentration of 1×10^6 /ml.
- 5 Up to 24 cytopsin slides were produced by adding 80 µl of the adjusted cell suspension to cytopsin chambers.

Cells were then spun at 25g for 3 min and were allowed to dry in preparation for staining. Diff-Quick and Gram stain were performed immediately for differential cell count and for diagnostic microbiological purposes.

- The remaining cells were used for further experiments listed below (A layout of all experiments is presented in Figure 3).

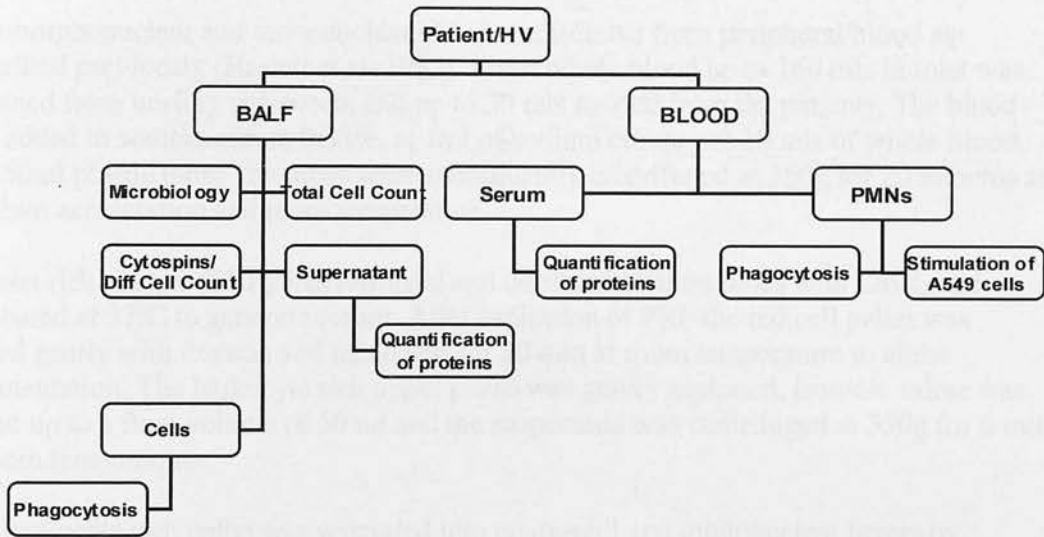


Figure 3: Experimental layout.

2.5 Retrieval and processing of ETA

An endotracheal aspirate from ventilated patients was collected prior to bronchoscopy. The sample was obtained by the nursing staff attending the patient by catheter suction through the ETT. This was used only for microbiological quantitative analysis. Serial dilutions were made of $1/10^2$ and $1/10^4$ in PBS and 100 μ l were spread onto blood agar, chocolate agar + bacitracin, MacConkey agar without salt, malt agar and PIA in duplicate. Agar plates were incubated for up to 48 hours and viable counts were calculated at 24 and 48 hours.

2.6 Bacterial identification

Microbiological identification of pathogens was performed using the API identification system (a combination of biochemical strips and database) by Biomérieux, according to manufacturer's instructions.

System API 20 E was used for identification of Gram (-) bacteria and API 20NE was used for identification of non-enteric Gram (-) bacteria.

2.7 Preparation of serum and human neutrophils

Polymorphonuclear and mononuclear cells were isolated from peripheral blood as described previously (Haslett et al. 1985). Fresh whole blood up to 160 mls in total was obtained from healthy volunteers and up to 30 mls in total from the patients. The blood was added to sodium citrate 0.38%, at 1ml of sodium citrate per 10 mls of whole blood, in a 50ml plastic tube. The tubes were subsequently centrifuged at 350g for 20 minutes at medium acceleration and room temperature.

Platelet rich plasma (PRP) was retrieved and decanted in glass tubes with CaCl_2 and incubated at 37°C to generate serum. After aspiration of PRP the red cell pellet was mixed gently with dextran and incubated for 30 min at room temperature to allow sedimentation. The leukocyte rich upper phase was gently aspirated, isotonic saline was added up to a final volume of 50 ml and the suspension was centrifuged at 350g for 6 min at room temperature.

The leukocyte rich pellet was separated into neutrophil and mononuclear layers by Percoll gradient centrifugation. The gradients were prepared using an initial solution of 90 % Percoll in 10x PBS. The three different gradients (55%, 68% and 80%) were made by diluting Percoll in PBS. 2.5 ml of the 68 % Percoll were layered onto 2.5 ml of the 80% Percoll. The leukocyte rich pellet was resuspended in 2.5 ml of the 55% Percoll and was gently added onto the 68% Percoll layer.

The tubes were centrifuged at 720g for 20 minutes at room temperature.

After centrifugation the polymorphonuclear rich band was separated at the interface between the 68% and the 80% gradient, whilst the mononuclear cell band was separated at the interface of the 55% and 68% gradient. Each band was gently aspirated and resuspended in 50 ml of PBS followed by centrifugation at 230g for 6 minutes at room temperature. Each pellet was washed once more in PBS and resuspended in IMDM at a concentration of $10\text{-}20 \times 10^6$ /ml.

A small aliquot was used for estimation of the total cell count using a haemocytometer and for differential cell count by cytopins stained with Diff-Quick stain. A percentage of neutrophils > 95% was considered satisfactory for subsequent experiments. Autologous serum was used for all experiments and the remaining volume was stored at -80 ° C for further analysis.

2.8 Phagocytosis assay

2.8.1 Phagocytic capacity of peripheral polymorphonuclear cells (PMNs)

This assay used zymosan which is an insoluble cell wall polysaccharide of yeast derived from *Saccharomyces cerevisiae*.

Zymosan particles were incubated with serum for 2 hours at 37°C. After the period of incubation zymosan was centrifuged at 350g for 5 min, washed with PBS and resuspended at a concentration of 0.02mg/ml in IMDM containing 1% autologous serum.

Freshly prepared PMNs were suspended in 10% autologous serum containing IMDM at a concentration of 500,000 cells/ml in a 24-well plate. The cells were used after 60-90 minutes, when adherence was confirmed by light microscopy.

The cells were twice washed gently with PBS aiming for a confluent monolayer after each wash and replenished using 1ml of IMDM containing 1% autologous serum or by 1ml zymosan suspension at a concentration of 0.02mg/ml in IMDM containing 1% autologous serum.

Cells were incubated for 60 minutes at 37 °C and 5% CO₂ atmosphere and washed with PBS twice to remove excess zymosan particles. The wells were allowed to air dry in preparation for staining with Diff-Quick.

The plate was inspected under light microscopy and phagocytic capacity was quantified by counting number of PMNs with ≥ 2 phagocytosed zymosan particles (as "positive") and number of PMNS with < 2 phagocytosed zymosan particles (as "negative") in 2 different and randomly selected fields and to a total number of cells of > 300 . Phagocytic capacity was expressed as the percentage of "positive" cells versus total number of cells from the average percentage of 2 fields.

2.8.2 Phagocytic capacity of alveolar phagocytes

Similar experiment was performed using phagocytes from BALF. This time the cell population in the group of patients was mixed, comprising neutrophils and alveolar macrophages at different percentages whilst in the group of healthy volunteers the cell population was more uniform, consisting predominantly of alveolar macrophages.

Freshly isolated cells as described earlier (section 1.4, p69) were resuspended in IMDM enriched by 10% autologous serum at a concentration of 500,000 cells/ml in a 24 well plate and allowed to adhere for 60-90 minutes. Phagocytic capacity was assessed using the protocol described above (section 1.8.1).

2.9 *In vitro* experiments with A549 cells

2.9.1 Culture and preparation of A549 cells

A549 cells are derived from alveolar cell carcinoma and display morphological features characteristic of type II alveolar epithelial cells (Giard et al. 1973).

A549 cells were suspended in Dulbecco's Modified Eagles Medium (DMEM) enriched with 10% foetal calf serum (FCS), Penicillin G (final concentration 100U/ml), streptomycin sulfate (final concentration 100 µg/ml) and L-glutamine (final concentration 2 µg/ml). Cell viability was assessed by staining with trypan blue and assessing the proportion of viable cells using a haemocytometer. Cells were grown in 24-well tissue culture plates at a concentration of 50,000/ml at 37 °C and 5 % CO₂. They were used in experiments when confluent.

2.9.2 Stimulation of A549 cells with PMNs and LPS

Freshly isolated peripheral polymorphonuclear cells, prepared as described in section 1.7 p70, were resuspended in IMDM containing 1% autologous serum, at a final concentration of 500,000 cells/ml.

LPS (*E. coli*: Serotype 0127:B8) was diluted to a final concentration of 100ng/ml and added directly to the relevant wells.

A549 cells grown to confluence in 24-well plate were washed with PBS and were incubated with:

- IMDM containing 1% autologous serum (as a control) or,
- PMNs at 500,000 cells/ml or,
- LPS at 100 ng/ml or,
- PMNs and LPS (PMNs at 500,000 cells/ml and LPS at 100 ng/ml).

In all groups the final volume of medium was 1ml and the final concentration of serum (derived from the same patient/volunteer as supplied neutrophils) was 1%.

The cells were incubated overnight at 37 °C and 5 % CO₂ for an average of 20-24 hours. The supernatants were retrieved the next day, centrifuged at 225g for 5 minutes to remove cell debris and stored at -80 °C for further analysis. The plate was sealed and frozen for later analysis and estimation of total protein (Bradford technique), as representative of the total number of cells.

2.10 Measurement of elastase inhibitory activity (EIA)

The inhibitory capacity of BALF was examined following incubation with human neutrophil elastase (HNE).

The assay was performed on a 96-well plate. All samples were serially diluted in assay buffer (50 mM Tris, 0.5 M NaCl and 0.1% Triton X-100, pH 8.0).

10 μ l of each dilution was added to 10ng of HNE in 10 μ l of assay buffer (HNE at a final concentration 1ng/ml) and the final volume of each well was made up to 50 μ l with assay buffer. "Blank" wells consisted of assay buffer and "positive control" wells consisted of 10 μ l of HNE and 40 μ l of assay buffer.

The plate was incubated for 30 min at 37 °C before addition of the elastase-specific chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide.

Absorbance was measured spectrophotometrically at 405 nm and expressed as a function of time. Change of absorbance (expressed as changes in optical density (OD), was measured within the linear part of the curve and plotted against volume (in μ l) of the sample. An example of the curve produced by a patient's sample is shown in Figure 4.

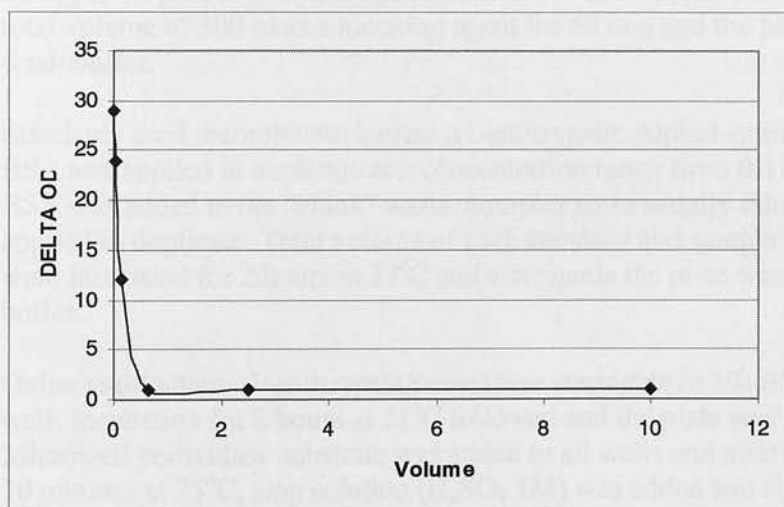


Figure 4: Changes of absorbance with time against volume of BALF.

Extrapolation of the curve to the abscissa indicated the volume of the sample that completely inhibited the known concentration of HNE (1 ng/ μ l) (Bieth 1980), and the molar concentration of elastase inhibitors in the sample (EIA) was calculated, based on the assumption that all inhibitors of HNE form a 1:1 ratio with the enzyme.

2.11 Quantification of lactate dehydrogenase (LDH)

Quantification of LDH release from damaged cells was performed using the LDH-cytotoxicity assay kit (Biovision) according to manufacturer's instructions.

2.12 Quantification of total protein and urea

Total protein was measured using a commercial bicinchoninic acid (BCA) assay (Sigma-Aldrich), and urea was measured by Urea Colorimetric assay kit, Quantichrom (Bioassay systems) according to manufacturers` instructions.

2.13 Quantification of human cytokines and protease inhibitors

2.13.1 Alpha1-antitrypsin ELISA

ELISA was performed on 96-well plates.

All wells were coated with rabbit anti-human α 1-antitrypsin in buffer (0.05M sodium bicarbonate solution, pH 9.6), and incubated overnight at 4°C. "Blank" wells were incubated with buffer alone. The plate was washed with wash buffer (0.05% Tween-20 in PBS, pH 7.4). BSA at a final concentration 5% in PBS, pH 7.4 was added to all wells to a total volume of 300 μ l as a blocking agent for 60 min and the plate was washed with wash buffer.

Standards used recombinant human α 1-antitrypsin. Alpha1-antitrypsin was diluted in 1% BSA and applied in duplicate at a concentration range from 0-112 ng/ml while only 1% BSA was added to the "blank" wells. Samples were serially diluted in 1% BSA and applied in duplicate. Total volume of each standard and sample well was 100 μ l. All wells were incubated for 2 hours at 37°C and afterwards the plate was washed with wash buffer.

Ovine anti-human α 1-antitrypsin/peroxidase conjugate in 1% BSA was added to each well. Incubation for 2 hours at 21°C followed and the plate was washed with wash buffer. Microwell peroxidase substrate was added to all wells and after incubation for a further 10 minutes at 25°C, stop solution (H₂SO₄ 1M) was added and absorbance was read at 450 nm.

Absorbance was plotted against α 1-antitrypsin concentration to create standard curves and α 1-antitrypsin concentration was determined by extrapolation from the curve.

2.13.2 Elafin ELISA

Quantification was performed by Human Elafin kit by Hycult Biotechnology. This is a ready-to-use ELISA based on a sandwich principle using a monoclonal mouse anti-human elafin IgG antibody. The lower detection limit was 156pg/ml. There was no cross reactivity with other molecules (information provided by personal correspondence with the manufacturers).

2.13.3 HNE ELISA

Quantification was performed by HNE kit by Hycult Biotechnology.

This is a ready-to-use ELISA based on a sandwich principle using a monoclonal anti-human HNE IgG antibody. The lower detection limit was 0.4ng/ml. There was no cross reactivity with other molecules (information provided by personal correspondence with the manufacturers).

2.13.4 IL-1 β , IL-6, CXCL8, IL-10, IL-12p70, TNF- α .

Quantification was performed using the Cytokine Bead Assay (CBA) system from BD Bioscience.

Commercially available enzyme-linked immunosorbent assay (ELISA) kits are used to measure levels of cytokines in biological samples. However as most of these kits require a two-fold diluted sample volume of 100 μ l, in order to examine a number of different classes of cytokines, volumes of more than a few hundred μ ls should be available, otherwise dilutions need to be made.

Under these conditions, the process of dilution could result in values that are below the detectable standard. The microbead-based flow cytometry system (Luminex) enables detection of cytokines in small volume (50 μ l) samples of human biological material and this was the main reason that this technique was employed here.

CBA has been used to measure several cytokines in various biological samples. Sensitivity, recovery and linearity of the assay appear comparable to commercial ELISA assays, providing simultaneously the benefit of wider detection range, and requirement of lesser sample volume (Cook et al. 2001; Tarnok et al. 2003; Morgan et al. 2004).

Lower limits of detection were: 3.6 pg/ml for CXCL8, 7.2 pg/ml for IL-1 β , 2.5 pg/ml for IL-6, 2.3 pg/ml for IL-10, 2.2pg/ml for TNF- α and 1.0 pg/ml for IL-12p70. There was no cross reactivity with other molecules (information provided by personal correspondence with the manufacturers).

In this study, IL-8 levels derived from CBA were comparable to results by a commercial IL-8 ELISA kit (R&D).

2.13.5 MCP-1/CCL2 ELISA (Duoset)

ELISA was performed on 96-well plates. All wells were coated with monoclonal anti-MCP-1/CCL2 IgG in buffer (PBS, pH 9.6) (final concentration IgG 0.05 μ g/ml) and incubated overnight at 4 $^{\circ}$ C. "Blank" wells were incubated with buffer alone. The plate was washed with wash buffer (0.05% Tween-20 in PBS).

Bovine serum albumin (BSA) at a final concentration of 1% in PBS, pH 7.4 was added to all wells as a blocking agent for 1 hour and the plate was washed with wash buffer.

Standards used recombinant human MCP-1/CCL2. Human MCP-1/CCL2 standard was diluted in 1% BSA and applied in duplicate at a concentration range from 15-1000 pg/ml while only 1% BSA was added to the "blank" wells. Samples were serially diluted in 1% BSA and applied in duplicate. Total volume of each standard and sample well was 100µl. All wells were incubated for 2 hours at 37°C and afterwards the plate was washed with wash buffer.

Subsequently, all wells were treated with biotinylated anti-human MCP-1/CCL2 antibody in 1% gelatine, (final concentration 0.5 ng/ml) and total volume 100µl, and incubated at 37°C for 2 hours. Following incubation, all wells were washed with wash buffer.

Streptavidin-biotin-horseradish peroxidase (final concentration 0.05 µg/ml in 1% gelatin) was added to each well. Incubation for 20-30 minutes in the dark at 37°C followed and the plate was washed with wash buffer. Microwell peroxidase substrate was added to all wells and after incubation for further 20-30 minutes in the dark at 37°C, stop solution (H₂SO₄ 1M) was added and absorbance was read at 450/570 nm.

Absorbance was plotted against MCP-1/CCL2 concentration to create standard curves and MCP-1/CCL2 concentration was determined by extrapolation from the curve.

2.13.6 SLPI ELISA

Quantification was performed using a Human SLPI ELISA kit (R&D Systems). This is a ready-to-use ELISA based on a sandwich principle using a monoclonal mouse anti-human SLPI IgG antibody. The lower detection limit was 62.5 pg/ml. There was no cross reactivity with other molecules (information provided by personal correspondence with the manufacturers).

2.14 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software CA, USA. Comparisons between two groups were performed using t-test (for normally distributed data) and Mann-Witney test (for non normally distributed data). Normality was tested by Kolmogorov-Smirnov normality test. Comparisons between three groups were performed using One-Way ANOVA test (if data normally distributed) and non-parametric tests (Kruskal-Wallis test) when distribution of data was not Gaussian with post-hoc Bonferroni and Dunn's analysis respectively when more than 2 groups were compared. Nominal data were analysed using the Chi-squared test. A P value of < 0.05 was considered to be statistically significant..

Normally distributed data were expressed as mean values and 95% CI, whilst non-parametric data were expressed as median values and interquartile range (IQR). Correlation was tested by Spearman's rank correlation test.

CHAPTER III: RESULTS AND DISCUSSION

1. DEMOGRAPHIC AND MICROBIOLOGICAL DATA

Data used to test the hypotheses central to this thesis (p 68) can only be interpreted meaningfully if the patient groups to be studied are rigorously defined. The controversies surrounding the diagnosis of VAP have been discussed in detail in the Introduction (p31-36), where I outlined the rationale for confirming VAP based on a pathogen count of $\geq 10^4$ cfu/ml of BALF. Having defined VAP in this way, it is important to determine how similar the VAP and non-VAP groups are in other respects and in terms of potential confounding variables (e.g. age, smoking etc).

The demographics and detailed microbiology of VAP are relatively poorly described. The aim of this chapter was therefore to perform a comprehensive analysis of the microbiology and innate immunity in patients with VAP. In order to achieve this aim the study recruited patients from a general ICU, with clinically suspected VAP. Collection of clinical and microbiological data allowed a comprehensive analysis of the microbiological and demographic spectrum of critically ill patients with VAP and the produced data will be presented in this section of Chapter III.

During the period July 2004 to January 2007, 49 patients with suspected VAP were recruited. VAP was confirmed in 13 patients, based on BALF culture $\geq 10^4$ cfu/ml and these patients constitute the VAP group. The remaining 36 patients had suspected VAP on recruitment but BALF cultures were either negative or grew $< 10^4$ cfu/ml and define the NON VAP group. Within the NON VAP group, 14 patients had suspected VAP and ARDS/ALI. There were no patients with ARDS/ALI and confirmed VAP.

A total of 15 healthy volunteers were also recruited and constitute the Healthy Volunteers (HV) group.

Sections 1.1-1.8 therefore assess whether the study groups had important differences with respect to the potential confounding variables of age, gender distribution, smoking history, distribution of comorbidities, length of stay in hospital prior to enrolment, severity of illness at the time of enrolment, duration of mechanical ventilation prior to enrolment, tolerability of the study procedures, the level of use of antibiotics prior to enrolment, and the anatomical distribution of infections prior to enrolment.

Sections 1.9-1.11 define the microbiological characteristics of the patients as defined by the study procedures.

1.1 Age, gender distribution and smoking history

The mean age of the VAP group was 58 years (95% CI: 49-63) whilst the mean age for the NON VAP group and the HV group was 60 years (54-65) and 62 years (56-48) respectively (Table 2). There was no statistical difference in the mean age between the VAP group, the NON VAP group and the healthy volunteers group (p-value>0.05 by One-way ANOVA test and Bonferroni's post-hoc analysis).

Most of the patients and volunteers recruited were male (77 % for the VAP group and 61 % for the NON VAP group and HV group) (Table 2).

The smoking history was based on information documented in the medical notes. Where there was no information recorded in the notes the smoking history was labeled as unknown. Non-smokers are patients or healthy volunteers who never smoked. Half of the patients in each group (VAP and NON VAP) were smokers with percentages of 54% and 55% respectively. 23% of the VAP patients were ex smokers whilst the remaining patients had an unknown smoking history. In the NON VAP group 17% of patients were ex smokers and 22 % of the patients never smoked. Documentation of smoking history was not available in 23% of the VAP patients and 6% of the NON VAP patients. 21% of the healthy volunteers were smokers and 5% were ex smokers whilst the majority (74%) were non-smokers (Table 2).

		VAP	NON VAP	HV
AGE (mean and 95% CI)		58 (49-63)	60 (54-65)	62 (56-68)
GENDER (% percentage)	MALE	77%	61%	61%
SMOKING HISTORY (% percentage)	SMOKERS	54%	55%	21%
	NON SMOKERS	0%	22%	74%
	EX SMOKERS	23%	17%	5%
	UNKNOWN	23%	6%	0%

Table 2: Demographic data of ICU patients and healthy volunteers.

Data are expressed as percentages of patients and healthy volunteers, (p-value>0.05 by Chi-square test).

1.2 Co-morbidities

The data relating to patients` past medical history were based on the documented information in the patients` medical notes.

Co-morbidities in the critically ill patients are shown in Table 3.

Diabetes mellitus (DM), chronic heart failure, chronic liver disease (CLD) and arterial hypertension were the most prevalent diseases described in the past medical history of patients in the VAP group. Other less common documented diseases were chronic obstructive airways disease (COPD), atrial fibrillation, epilepsy and rheumatoid arthritis.

Ischaemic heart disease (IHD), COPD, arterial hypertension and CLD were documented most commonly in the patients in the NON VAP group. Other diseases included rheumatoid arthritis, ankylosing spondylitis, valvular heart disease and valve replacement, hypothyroidism and psychiatric disorders.

There were 3 patients on immunosuppressant therapy, 1 with rheumatoid arthritis, treated with prednisolone and methotrexate in each group and 1 patient with previous liver transplantation who was maintained on prednisolone and tacrolimus. No patients with known human immunodeficiency virus were included in either group and only 1 patient with previous malignancy was included in the NON VAP group who had undergone previous radical surgery for neck cancer.

The majority of the HV (79%) had an unremarkable past medical history.

CO-MORBIDITIES		VAP	NON VAP	HV
RESPIRATORY	COPD	1 (8%)	6 (17%)	0 (0%)
	Asthma	0 (0%)	3 (8%)	2 (10%)
CARDIOVASCULAR	Ischaemic heart disease	1 (8%)	7 (19%)	1 (5%)
	Chronic heart failure	2 (15%)	0 (0%)	0 (0%)
	Arterial hypertension	2 (15%)	5 (14%)	2 (10%)
	Atrial fibrillation	1 (8%)	1 (3%)	0 (0%)
	Valvular heart disease	0 (0%)	2 (5%)	0 (0%)
	Valve replacement	0 (0%)	1 (3%)	0 (0%)
GASTROINTESTINAL	Chronic liver disease	2 (15%)	4 (11%)	0 (0%)
NEUROLOGICAL	Epilepsy	2 (15%)	1 (3%)	0 (0%)
ENDOCRINE	Diabetes mellitus	3 (23%)	2 (5%)	0 (0%)
	Hypothyroidism	0 (0%)	1 (3%)	0 (0%)
CONNECTIVE TISSUE DISEASE	Rheumatoid arthritis	1 (8%)	1 (3%)	0 (0%)
	Ankylosing spondylitis	0 (0%)	1 (3%)	0 (0%)
OTHER	Psychiatric disorder	0 (0%)	1 (3%)	0 (0%)
	Drug abuse	0 (0%)	1 (3%)	0 (0%)
TRANSPLANT	Liver transplant	0 (0%)	1 (3%)	0 (0%)
PREVIOUS MALIGNANCY	Neck squamous cell carcinoma	0 (0%)	1 (3%)	0 (0%)
	Prednisolone + Methotrexate	1 (8%)	1 (3%)	0 (0%)
IMMUNOSUPPRESSION	Prednisolone + Tacrolimus	0 (0%)	1 (3%)	0 (0%)
NONE		3 (23%)	12 (33%)	15 (79%)

Table 3: Co-morbidities in all critically ill patients.

Data are expressed as numbers of patients and percentages.

There were patients in both groups with more than one disease in the medical history as summarised in Table 4. Three patients in the VAP group and 12 patients in the NON VAP group had no co-morbidities known, whilst 6 and 16 patients in the VAP group and the NON VAP group respectively had only one.

There were 3 patients in the VAP group and 4 patients in the NON VAP group with 2 co morbidities and finally 1 patient in the VAP group and 4 patients in the NON VAP group had more than two. In the healthy volunteers group, only one patient had 2 co-morbidities.

CO-MORBIDITIES	VAP	NON VAP	HV
0	3 (23%)	12 (33%)	15 (79%)
1	6 (46%)	16 (45%)	3 (16%)
2	3 (23%)	4 (11%)	1 (11%)
3	1 (8%)	4 (11%)	0 (0%)

Table 4: Co-morbidities in patients and controls.

Data are expressed as numbers of patients and percentages for both groups, (p-value > 0.05 by Chi-square test).

1.3 Diagnosis on admission to ICU

The working diagnosis on admission to ICU is summarised in Table 5.

Most of the patients in the VAP group were surgical patients (62% versus 38 % medical admissions) admitted postoperatively following trauma surgery (trauma contributed 23 % of all VAP cases), emergency abdominal surgery for intraabdominal sepsis or perforated viscus (contributing 23%) and emergency vascular surgery for ruptured abdominal aortic aneurysms (contributing 15 %).

Most of the medical patients in the VAP group were admitted with severe CAP (15 %) or complications of CLD i.e. hepatic encephalopathy following sepsis or upper gastrointestinal haemorrhage (15%). Severe sepsis with multiple organ dysfunction was found in 1 VAP patient.

In the NON VAP group, there was a slight predominance of medical patients with a percentage of 53 % versus 47 % for surgical admissions although the difference was not significant. Most of the medical patients were admitted with severe community acquired pneumonia (contributing 22 %). 11% of the patients were admitted with chronic liver disease-related complications, 6 % following severe sepsis with multiple organ dysfunction and 14 % with other medical diagnoses including 2 patients with acute respiratory failure following infective exacerbation of COPD, 1 patient with coma following drug overdose, 1 patient with upper gastrointestinal haemorrhage (without CLD) and 1 patient with hospital-acquired pneumonia.

Most of the surgical patients were admitted following emergency abdominal surgery (31%) whilst equal numbers of patients were admitted following emergency vascular surgery and trauma surgery (8%).

DIAGNOSIS ON ADMISSION		VAP N (%)	NON VAP N (%)
SURGICAL	Trauma	3 (23.1%)	3 (8.3%)
	Emergency abdominal surgery	3 (23.1%)	11 (30.6%)
	Emergency vascular surgery	2 (15.4%)	3 (8.3%)
MEDICAL	Community acquired pneumonia	2 (15.4%)	8 (22.2%)
	Chronic liver disease	2 (15.4%)	4 (11.1%)
	Sepsis	1 (7.6%)	2 (5.6%)
	Other medical	0 (0.0%)	5 (13.9%)

Table 5: Diagnosis on admission to the ICU.

Data expressed as numbers and percentage, (p-value > 0.05 by Chi-square test for surgical v medical diagnosis).

1.4 Days in hospital prior to admission to ICU

On average, most patients were admitted to the ICU within 24 hours from presentation to the hospital (median value for the VAP group: 0.00 days, IQR: 0.0-1.0 and 0.00, IQR: 0.0-4.0 for the NON VAP group respectively) (Figure 5).

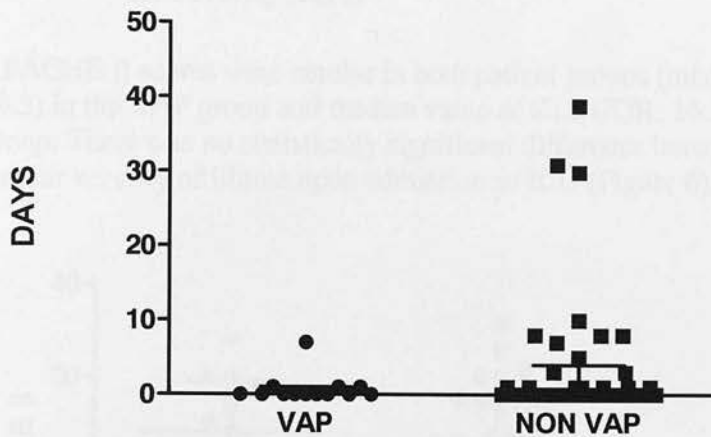


Figure 5: Length of in hospital stay prior to admission to the ICU.

Data are expressed as median values and IQR for both groups, (p-value >0.05 by Mann-Whitney test).

Looking at subgroups of patients in the VAP group, all medical patients were admitted directly to the ICU as direct admissions upon presentation to the Emergency Department (ED) or Combined Admissions Assessment Area (CAAA) whilst the surgical patients were admitted to the ICU on average approximately 12 hours (median value 0.5 days, IQR: 0.0-1.0) after presentation to the hospital (Table 6).

In the NON VAP group, medical patients were on average admitted directly to the ICU from CAAA or ED (median value 0.00, IQR: 0.0-6.0), whilst surgical patients were admitted to the unit within one day (median value 1.00, IQR: 0.0-4.0) post presentation to the hospital (Table 6).

DAYS IN HOSPITAL PRIOR TO ADMISSION TO ICU	VAP	NON VAP
SURGICAL PATIENTS	0.5 (0.0-1.0)	1.0 (0.0-4.0)
MEDICAL PATIENTS	0.0 (0.0-0.0)	0.0 (0.0-6.0)

Table 6: Length of in hospital stay prior to admission to the ICU for the VAP and the NON VAP group.

Data are expressed as median values and IQR, (p-value >0.05 by Mann-Whitney test).

1.5 Severity scores

APACHE II scores were similar in both patient groups (median value of 24.0 (IQR: 17.0-29.5) in the VAP group and median value of 22.5 (IQR: 16.3-27.0) in the NON VAP group. There was no statistically significant difference between the 2 groups suggesting similar severity of illness upon admission to ICU (Figure 6).

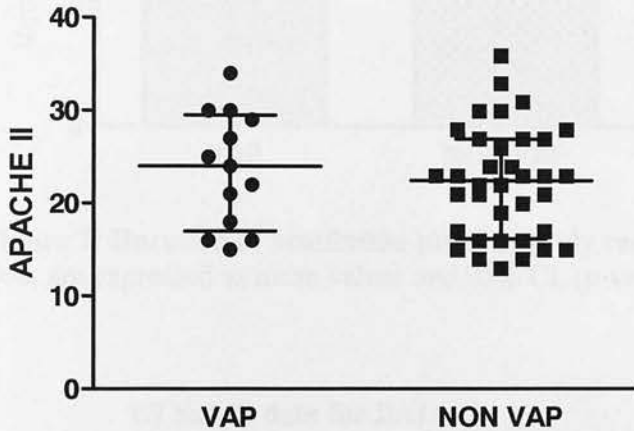


Figure 6: APACHE II scores in ICU groups.

Data are expressed as median values and IQR (p-value >0.05 by Mann-Whitney test).

1.6 Duration of mechanical ventilation

The mean duration of ventilation prior to recruitment in the study was similar for both groups, with a mean value of 9.1 days (95% CI: 6.1-12.0) in the VAP group and 9.3 days (95% CI: 7.2-11.5) for the NON VAP group. There was no statistically significant difference between the mean values of the 2 groups (Figure 7).

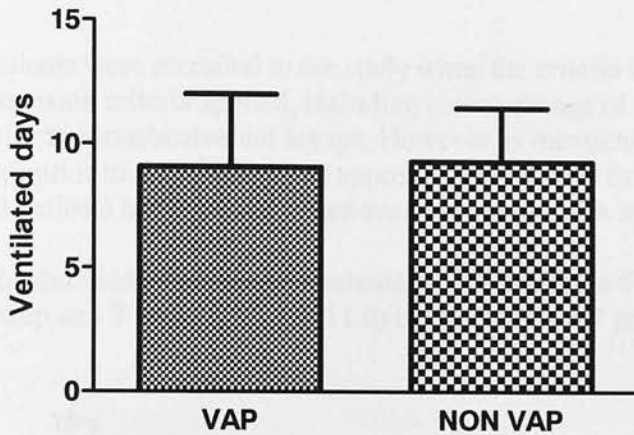


Figure 7: Duration of ventilation prior to study recruitment.

Data are expressed as mean values and 95% CI, (p-value >0.05 by Mann-Whitney test).

1.7 Safety data for BAL

Blood pressure and SaO₂ were recorded pre- and post-BAL for all patients and healthy volunteers. Data for the healthy volunteers are not presented here as the procedure was tolerated well without any complications.

Regarding the 49 patients, the procedure was abandoned in one case due to severe hypoxaemia secondary to pneumothorax.

In the ICU patients, mean systolic blood pressure (SBP) pre-BAL was 130.7 mmHg (95% CI: 124-137) and mean SBP post-BAL was 117.8 mmHg (95% CI: 112-124). Whilst there was a significant difference between pre- and post SBP (p-value<0.0001 with paired t-test), there were no episodes of cardiovascular compromise with sustained hypotension (SBP<90 mmHg).

Mean SaO₂ value pre-BAL was 96.5% (95% CI: 96-97) and post-BAL was 95.6% (95% CI: 94-97). There was no significant difference between pre- and post-BAL values (p-value>0.05 with paired t-test). However, desaturation (SpO₂<90%) was observed in 3 patients post-BAL. In 2 of these 3 patients, desaturation was transient and values returned to normal soon after the end of the procedure. In the remaining one of these patients, the procedure was abandoned due to complications as described earlier.

1.8 Prior antibiotic use

Patients were recruited in the study when the criteria for suspected VAP were met and no exclusion criteria applied, including recent change of antibiotics within the last 72 hours prior to bronchoalveolar lavage. However as mentioned earlier the average stay in the unit prior to recruitment was approximately 9 days for each group and within that period all patients had received broad-spectrum antibiotics at some point during their ICU stay.

The duration of antibiotic treatment was on average 5.0 days (IQR: 5.0-8.5) in the VAP group and 7 days (IQR: 5.8-11.0) in the NON VAP group (Figure 8).

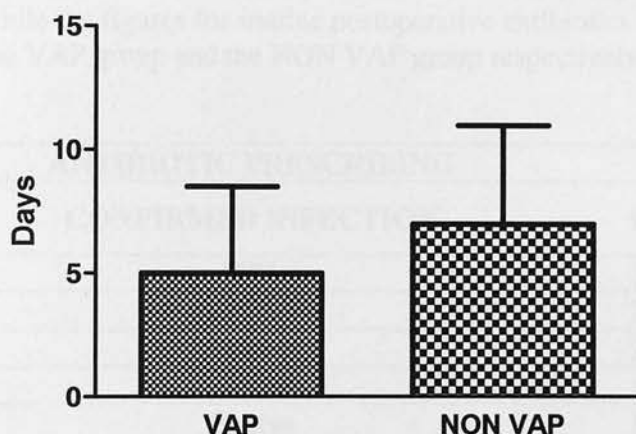


Figure 8: Total number of days on antibiotics.

Data are expressed as median and IQR, (p-value>0.05 by Mann-Whitney test).

Most of the patients in the VAP group received one course of antibiotics, (77% of all VAP patients) whilst 23% of the patients received 2 courses. In the NON VAP group the trend was different as approximately half of the patients (47%) were treated with one course of antibiotics and half of them (53%) had more than one, with 36% of patients being treated with 2 courses, 11% with 3 courses, and 6% with 4 courses (Table 7). However the difference did not reach statistical significance.

COURSES OF ANTIBIOTICS	VAP	NON VAP
1	10 (77%)	17 (47%)
2	3 (23%)	13 (36%)
3	0 (0%)	4 (11%)
4	0 (0%)	2 (6%)

Table 7: Number of courses of antibiotics per patient per group.

Data are expressed as numbers and percentages, (p-value>0.05 by Fisher's exact test comparing patients with 1 and 2 courses between the two groups).

The choice of antibiotics was dictated by positive culture results based on isolates from various sites i.e. ETA, blood, catheter specimen urine (CSU) or fluid from abscesses or intra-abdominal fluid collections, or it was based on routine postoperative cover for abdominal surgery or empirical treatment targeting sepsis of known origin i.e. severe CAP or suspected but unconfirmed source. Most commonly prescribed antibiotics were piperacillin/tazobactam, meropenem, vancomycin, ciprofloxacin, ceftriaxone and clarithromycin.

In the VAP group 56% of courses of antibiotics or episodes prescribed were based on positive results while the percentage of courses of antibiotics based on confirmed infection in the NON VAP group was 35%. Empirical antibiotic treatment was prescribed in 31% of all courses of antibiotics in the VAP group and 49% in the NON VAP group while the figures for routine postoperative antibiotics were 23% and 16% of all courses in the VAP group and the NON VAP group respectively (Table 8).

ANTIBIOTIC PRESCRIBING	VAP	NON VAP
CONFIRMED INFECTION	9 (56%)	22 (35%)
ETA	3 (34%)	8 (36%)
BALF	2 (22%)	5 (22%)
Blood	2 (22%)	5 (22%)
Urine	0 (0%)	2 (10%)
Faeces	1 (11%)	0 (0%)
Wound/Skin ulcers	0 (0%)	2 (10%)
Abscess	1 (11%)	0 (0%)
EMPIRICAL TREATMENT	4 (31%)	31 (49%)
ROUTINE POST OPERATIVE	3 (23%)	10 (16%)

Table 8: Factors influencing prescribing of antibiotics.

Data are expressed as numbers and percentages, (p-value >0.05 by Chi-square test).

In approximately half of the confirmed infection episodes in each group, pathogens were isolated from the respiratory tract either by ETA or previous bronchoalveolar lavage. 22% of all cultures in both groups were positive blood cultures while other sources of infection included faeces in 11% of all positive cultures in the VAP group, urinary tract (specimens taken from urinary catheter) in 10% in the NON VAP group, and abscesses or skin wounds/ulcers in 11% in the VAP group and 10% of all cultures in the NON VAP group (Table 8).

A subgroup of patients in the NON VAP group had pathogens isolated from more than one site during their stay in the unit.

Indeed in 29% of patients in the NON VAP group positive cultures had grown pathogens from two different sites (e.g. blood and BALF), not always simultaneously. In the VAP group there were no patients with isolates from >1 site at any time (Table 9).

Similarly there were patients who had more than one pathogen isolated from the same site i.e. two pathogens mainly from ETA or BALF. 67% of patients in the VAP group versus 78% of the patients in the NON VAP group had cultures that grew a single pathogen while 33% and 22% of patients in the VAP and NON VAP group respectively had two different pathogens isolated from the same site (Table 9).

NUMBER OF PATIENTS	VAP	NON VAP
CULTURES FROM 1 SITE	9 (100%)	12 (71%)
CULTURES FROM 2 SITES	0 (0%)	5 (29%)
CULTURES OF SINGLE PATHOGEN FROM ONE SITE	6 (67%)	14 (78%)
CULTURES OF 2 PATHOGENS FROM ONE SITE	3 (33%)	4 (22%)

Table 9: Number of patients who had positive cultures from ≥ 1 site and number of patients who had more than one pathogen isolated from the same site. .

Data are expressed as numbers and percentages, (p-value>0.05 by Fisher`s exact test).

Finally the most common pathogens isolated from all sites in critically ill patients are listed in Table 10.

	PATHOGENS	VAP	NON VAP
GRAM- POSITIVE	<i>Streptococcus pneumoniae</i>	1	2
	<i>Streptococcus morbillorum</i>	1	0
	<i>Streptococcus pyogenes</i>	0	1
	<i>Enterococcus</i>	1	2
	<i>Staphylococcus aureus</i>	0	1
	<i>Methicillin resistant S. aureus</i>	1	0
GRAM- NEGATIVE	<i>Haemophilus influenzae</i>	2	3
	<i>Escherichia coli</i>	1	3
	<i>Proteus mirabilis</i>	0	1
	<i>Citrobacter freundii</i>	1	1
	<i>Citrobacter koseri</i>	0	1
	<i>Enterobacter aerogenes</i>	0	1
	<i>Enterobacter cloacae</i>	0	1
	<i>Pseudomonas aeruginosa</i>	0	3
	<i>Stenotrophomonas maltophilia</i>	0	1
	<i>Serratia marcescens</i>	0	1
<i>Clostridium difficile</i>	1	0	
FUNGI	<i>Candida albicans</i>	3	5

Table 10. Most common pathogens isolated from all sites in all critically ill patients prior to bronchoalveolar lavage.

Fungi have been included as pathogens, as positive cultures have been included in previous data (tables 8-10) when clinicians initiated treatment with antifungal antibiotics, namely fluconazole.

1.9 Microbiology of BALF

BALF was available in 48 of the patients recruited, as bronchoalveolar lavage was abandoned in 1 case due to desaturation. From the 48 patients, 13 had BALF cultures $\geq 10^4$ cfu/ml defining the incidence of VAP in this study at 27%.

In 38% of patients, VAP was attributed to Gram-negative cocci or bacilli, in 31% of the patients to Gram-positive cocci and in the remaining 31% to other pathogens namely fungi or anaerobes. *H. influenzae* and *E. coli* accounted for 15% of cases each (40% of all Gram-negative pathogens), followed by *E. cloacae* isolated in one case (20% of all Gram-negative bacteria). MRSA was isolated in 15% of cases (50% of all Gram-positive pathogens), with MSSA and coagulase negative *Staphylococci* in one case each (25% of all Gram-positive bacteria). The pathogens in the remaining cases in the VAP group were fungi, namely *C. albicans*, isolated in 23% of all cases and anaerobes isolated in one patient (Table 11).

	Pathogens	Number of patients (%)
Gram-positive	<i>MSSA</i>	1 (8%)
	<i>MRSA</i>	2 (15%)
	<i>Coagulase negative Staphylococci</i>	1 (8%)
Gram-negative	<i>H. influenzae</i>	2 (15%)
	<i>E. coli</i>	2 (15%)
	<i>E. cloacae</i>	1 (8%)
Fungi	<i>C. albicans</i>	3 (23%)
Other	<i>Anaerobes</i>	1 (8%)

Table 11: Microbiological epidemiology of VAP in this study.

In the NON VAP group, 23 patients (66%) had sterile BALF culture and in the remaining 12 patients (34%), pathogens were isolated in concentrations $< 10^4$ cfu/ml. In 9 of these 12 patients a single pathogen was identified while cultures from the BALF of the other 3 patients identified 2 different pathogens, raising the number of cultures $< 10^4$ cfu/ml to a total number of 15 (Figure 9).

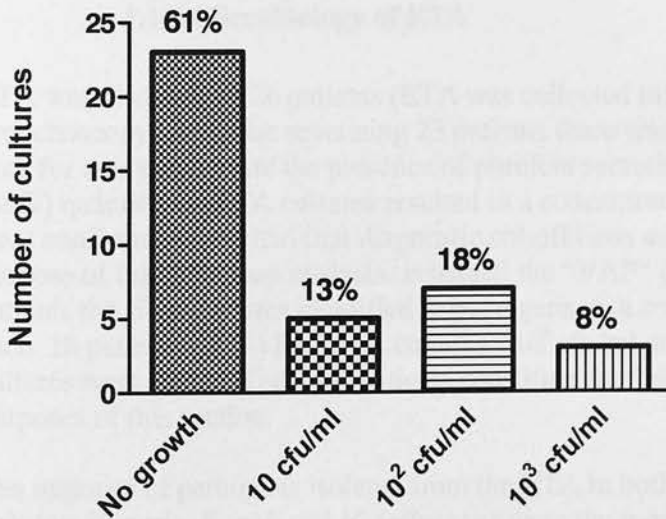


Figure 9: Quantitative results of all BALF cultures from all NON VAP patients. Data are expressed in numbers and % of the total.

Most common pathogens isolated from the BALF of the NON VAP group were Gram-negative bacteria, isolated in 60% of positive cultures. Among them *P. aeruginosa*, *K. oxytoca* and *E. cloacae* were identified in 13% of cultures each and *Citrobacter koseri*, *Serratia liquifaciens* and *H.influenzae* in 7% of cultures each.

Gram-positive cocci were identified in 35% of cultures with a majority identified as *s. aureus* (MRSA in 27% of cases and MSSA in 7% of cases). Finally fungi, namely *Aspergillus fumigatus*, were isolated in 7% of the cases (Table 12).

	PATHOGENS	NUMBER OF POSITIVE CULTURES (%)
GRAM (+)	MSSA	1 (7%)
	MRSA	4 (27%)
GRAM (-)	<i>P. aeruginosa</i>	2 (13%)
	<i>K. oxytoca</i>	2 (13%)
	<i>E. cloacae</i>	2 (13%)
	<i>C. koseri</i>	1 (7%)
	<i>S. liquifaciens</i>	1 (7%)
	<i>H. influenzae</i>	1 (7%)
FUNGI	<i>A. fumigatus</i>	1 (7%)

Table 12: Pathogens isolated from the BALF of the NON VAP patients.

1.10 Microbiology of ETA

ETA was available in 26 patients (ETA was collected immediately prior to the bronchoscopy and in the remaining 23 patients there was no sample available at that time for analysis despite the presence of purulent secretions). In 12 of these patients, (46%) quantitative ETA cultures resulted in a concentration $\geq 10^6$ cfu/ml and would have confirmed VAP, had that diagnostic cut off been employed. This cohort, for the purpose of this subgroup analysis, is named the “VAP” group. In 2 out of these 12 patients the ETA cultures identified 2 pathogens at a concentration $>10^6$ cfu/ml each. 10 patients (39%) had ETA cultures $<10^6$ cfu/ml and in 4 patients (15%), ETA cultures were sterile. These 14 patients constitute the “NON VAP” group for the purposes of this section.

The majority of pathogens isolated from the ETA in both subgroups were Gram-negative bacteria. *E. coli* and *H. influenzae* were the most common Gram-negative pathogens isolated in the “VAP” group in 14% of patients each, followed by *E. cloacae*, *Enterobacter aerogenes*, *C. koseri*, *Stenotrophomonas maltophilia* and *P.aeruginosa* collectively accounting for 35% of cases.

In the “NON VAP” group the most common Gram-negative pathogen was *K. pneumoniae*, identified in 30% of cases followed by *E. cloacae*, *Citrobacter freundii* and *P.aeruginosa* in 1 case each.

Gram-positive cocci were identified in 37% of patients in the “VAP” group with MRSA the most common (30% of cases) pathogen followed by MSSA (7%).

In the “NON VAP” group Gram-positive cocci were isolated in only one case and *C. albicans* in the remaining 30% of the patients in this group (Table 13).

	ETA	≥10 ⁶ cfu/ml	<10 ⁶ cfu/ml
GRAM (+)	<i>MRSA</i>	4 (30%)	0 (0%)
	<i>MSSA</i>	1 (7%)	0 (0%)
	<i>Coagulase negative staphylococci</i>	0 (0%)	1 (10%)
	<i>E.coli</i>	2 (14%)	0 (0%)
GRAM (-)	<i>H.influenzae</i>	2 (14%)	0 (0%)
	<i>E. cloacae</i>	1 (7%)	1 (10%)
	<i>E. aerogenes</i>	1 (7%)	0 (0%)
	<i>C. koseri</i>	1 (7%)	0 (0%)
	<i>C. freundii</i>	0 (0%)	1 (10%)
	<i>K. pneumoniae</i>	0 (0%)	3 (30%)
	<i>S. maltophilia</i>	1 (7%)	0 (0%)
	<i>P.aeruginosa</i>	1 (7%)	1 (10%)
	<i>C. albicans</i>	0 (0%)	3 (30%)

Table 13: Pathogens isolated from the ETA.

1.11 Comparison of ETA and BALF microbiology

Direct comparison between ETA and BALF as diagnostic tools was possible for the 26 patients who had both ETA and BALF samples. ETA was available for 9 of the 13 cases of confirmed VAP and in 17 of the 36 NON VAP cases (Table 14).

	ETA RESULTS	BALF RESULTS	ETA	BALF
1	<i>E. cloacae</i> >10 ⁶ cfu/ml	<i>E. cloacae</i> >10 ⁴ cfu/ml	Positive	Positive
2	<i>MSSA</i> >10 ⁶ cfu/ml	<i>MSSA</i> >10 ⁴ cfu/ml	Positive	Positive
3	<i>P. aeruginosa</i> >10 ⁶ cfu/ml	<i>P. aeruginosa</i> <10 ⁴ cfu/ml	Positive	Negative
4	Coag (-) <i>Staph</i> <10 ⁶ cfu/ml	Anaerobes >10⁴ cfu/ml Coagulase negative <i>Staphylococci</i> <10 ⁴ cfu/ml	Negative	Positive
5	<i>E. coli</i> >10 ⁶ cfu/ml	<i>E. coli</i> >10 ⁴ cfu/ml	Positive	Positive
6	<i>C. koseri</i> >10 ⁶ cfu/ml <i>H. influenzae</i> >10 ⁶ cfu/ml	<i>C. koseri</i> <10 ⁴ cfu/ml <i>H. influenzae</i> <10 ⁴ cfu/ml	Positive	Negative
7	<i>C. freundii</i> <10 ⁶ cfu/ml	No growth	Negative	Negative
8	<i>K. pneumoniae</i> <10 ⁶ cfu/ml	No growth	Negative	Negative
9	No growth	No growth	Negative	Negative
10	<i>P. aeruginosa</i> <10 ⁶ cfu/ml	<i>P. aeruginosa</i> <10 ⁴ cfu/ml	Negative	Negative
11	<i>E. cloacae</i> <10 ⁶ cfu/ml	<i>K. pneumoniae</i> <10 ⁴ cfu/ml	Negative	Negative
12	<i>K. pneumoniae</i> <10 ⁶ cfu/ml	<i>K. pneumoniae</i> <10 ⁴ cfu/ml	Negative	Negative
13	<i>E. coli</i> >10 ⁶ cfu/ml	<i>E. coli</i> >10 ⁴ cfu/ml	Positive	Positive
14	<i>E. aerogenes</i> >10 ⁶ cfu/ml <i>S. maltophilia</i> >10 ⁶ cfu/ml	No growth	Positive	Negative
15	No growth	No growth	Negative	Negative
16	<i>MRSA</i> >10 ⁶ cfu/ml	<i>MRSA</i> >10 ⁴ cfu/ml	Positive	Positive
17	No growth	No growth	Negative	Negative
18	<i>MRSA</i> >10 ⁶ cfu/ml	<i>MRSA</i> <10 ⁴ cfu/ml	Positive	Negative
19	<i>C. albicans</i> <10 ⁶ cfu/ml	<i>C. albicans</i> >10 ⁴ cfu/ml	Negative	Positive
20	<i>MRSA</i> >10 ⁶ cfu/ml	No growth	Positive	Negative
21	<i>H. influenzae</i> >10 ⁶ cfu/ml <i>MRSA</i> <10 ⁶ cfu/ml	<i>H. influenzae</i> >10 ⁴ cfu/ml	Positive	Positive
22	<i>C. albicans</i> <10 ⁶ cfu/ml	No growth	Negative	Negative
23	<i>C. albicans</i> <10 ⁶ cfu/ml	No growth	Negative	Negative
24	<i>K. pneumoniae</i> <10 ⁶ cfu/ml	No growth	Negative	Negative
25	No growth	<i>MRSA</i> >10 ⁴ cfu/ml	Negative	Positive
26	<i>A. fumigatus</i> <10 ⁶ cfu/ml <i>MRSA</i> >10 ⁶ cfu/ml	<i>A. fumigatus</i> <10 ⁴ cfu/ml <i>MRSA</i> <10 ⁴ cfu/ml	Positive	Negative

Table 14: Results of ETA and BALF in the 26 patients for whom both samples were available.

Positive BALF cultures (in bold) are cultures at concentration >10⁴ cfu/ml and negative are all cultures <10⁴ cfu/ml. Similarly in the ETA group, positive results (in bold) are cultures >10⁶ cfu/ml and negative are cultures <10⁶ cfu/ml.

ETA cultures were positive in 6 of the 9 cases in the VAP group assigning a sensitivity of the test of 67%. However in the NON VAP group there were 6 false positive results lowering the specificity of ETA to 65%.

The positive predictive value (PPV) of the test was 50% while the negative predictive value (NPV) was calculated at 79% (Table 15).

ETA v BALF		BALF		
		POSITIVE	NEGATIVE	TOTAL
ETA	POSITIVE	6	6	12
	NEGATIVE	3	11	14
	TOTAL	9	17	26

Table 15: Calculation of accuracy for ETA samples compared with BALF in the diagnosis of VAP.

1.12 Discussion

All patients in the study were recruited in a single ICU with a mixed cohort of general surgical and medical patients excluding neurosurgical and cardiothoracic patients (who were treated in different specialist units). Patients were recruited upon clinical suspicion of VAP according to criteria in keeping with existing literature (Chastre and Fagon 2002), and among all 49 suspected cases, 13 patients had confirmed microbiologically VAP (BALF culture $\geq 10^4$ cfu/ml).

14 patients with ARDS were also recruited as they fulfilled the criteria for suspected VAP and recruitment to the study. None of these 14 patients had evidence for VAP at BAL. As such the NON VAP group contains a sub-group of patients with ARDS. Because of this, subgroup analyses will appear later in this thesis and will include the ARDS subgroup and the remaining 23 patients of the NON VAP group (which will be called the NEITHER subgroup on the basis that these patients have neither VAP nor ARDS).

The inclusion of ARDS patients is not surprising as clinically the features of non-infective inflammation and infection are not discriminatory. Indeed the clinical criteria for diagnosing VAP (fever, leukocytosis and purulent tracheal secretions) are non-specific and 'new' infective radiological changes may be difficult to detect in ARDS patients where diffuse bilateral infiltrates on chest radiographs are present. The diagnosis of VAP in these patients can be very difficult without recourse to formal BAL, although it is well recognised that VAP and ARDS can co exist in 4-70% of patients (Andrews et al. 1981; Bell et al. 1983; Seidenfeld et al. 1986; Sutherland et al. 1995; Delclaux et al. 1997; Chastre et al. 1998; Meduri et al. 1998; Markowicz et al. 2000).

The variation in incidence is explained once more by the diagnostic technique used by different investigators and also by the timing of sampling in association with the introduction of antibiotics. In one study most of the patients underwent bronchoscopy and PSB and BAL at predetermined times during the course of ARDS rather than when pneumonia was suspected, increasing the probability of sampling after the introduction of new antibiotics and raising the possibility of false-negative results (Seidenfeld et al. 1986). Others used quantitative cultures of blind protected catheter specimens via the endotracheal tube, sampled every 48 to 72 hours after the onset of ARDS (Delclaux et al. 1997b).

Within this study there were no patients with ARDS where VAP coexisted and therefore there is no overlap between these two clinical entities although I acknowledge the possibility that 'patchy', regional infection could potentially have existed in some of the ARDS patients, thus skewing results by introducing 'false negatives'. It is also worth mentioning that the ARDS subgroup itself is a rather heterogeneous group which was recruited and investigated at a different stage of the natural history of lung injury. This inclusion of the ARDS subgroup in the study raises the possibility of confounding factors that may influence the data analysis. A subgroup analysis (presented mainly as text) has been performed and will be available in the following sections. In the vast majority of the data the subgroup analysis of VAP, ARDS, NEITHER and HV group did not differ from the initial analysis between the VAP, NON VAP and HV group that will be described in more detail.

Healthy volunteers were used as controls in this study and this model has limitations as the control group is constituted by non-ventilated individuals. However the definition and recruitment of the "ideal" control group poses considerable difficulties. Recruitment of routine postoperative patients prior to extubation would have served as an alternative, although limitations would also arise from:

a) the recruitment as controls of only elective surgical patients with the exclusion of medical patients and b) the heterogeneity of the group and the induced inflammatory response related to the type and the duration of the procedure (i.e. an elective orthopaedic procedure for hip replacement versus an elective thoracoabdominal oesophagectomy for oesophageal carcinoma) and perhaps the intra-operative complications that may have occurred. Similar arguments apply for the recruitment of ventilated patients with a predicted duration of ventilation of less than 48 hours as the majority of the cases would have included primarily elective post-operative patients.

Ultimately I included ambulant age- and sex-matched volunteers principally to serve as a group that would identify the physiological 'average' of the Scottish population. This group serves more as a reference point rather than a control group – the "control" group for VAP is clearly the NON VAP group.

VAP patients were on average 58 years old and the mean age was similar to the NON VAP group and the controls. There was a predominance of male patients in the VAP group compared with the NON VAP group and the healthy volunteers although the difference was not significant. These results are in keeping with the literature.

The largest of its kind retrospective study in epidemiology in VAP with data from a large US database of more than 9,000 patients reported an average age of VAP patients of 61.7 years old versus 64.6 years old for patients without VAP and a predominance of male gender for patients with VAP (Rello et al. 2002).

Half of the patients in each patient group were smokers and an approximately similar percentage in each group were ex smokers. The absence or the presence of co-morbidities did not differ significantly between the groups and no conclusions can be drawn that relate pre-admission medical history to development of VAP. However the study did not aim to look at risk factor analysis. The most common diseases documented in both groups were cardiovascular (arterial hypertension, IHD and heart failure) followed by CLD, COPD, DM and connective tissue disorders in both groups.

Regarding source of admission, patients were admitted to the unit directly from theatre post-elective or emergency surgery, from the ED and from other medical or surgical wards in the hospital including the high dependency unit (HDU) and CAAA.

Looking at diagnosis on admission to the ICU for all patients, percentages were equally shared between surgical (51% of all cases) and medical admissions (49% of all cases) representing a balance of mixed cases in a general medical/surgical ICU. The majority of surgical patients had undergone emergency abdominal surgery followed by trauma surgery and emergency vascular surgery in keeping with admissions to a tertiary referral and trauma centre. Most of the medical patients were admitted to the ICU with severe CAP followed by patients with complications of CLD, sepsis and other medical diagnoses including exacerbation of COPD and coma following drug overdose.

Surgical patients were admitted to the ICU within 24 hours from presentation to the hospital, a time interval that reflects time from presentation to diagnosis and surgery and mainly the length of surgery. Medical patients in this group were admitted to the ICU on the same day as their admission to the hospital via the ED or CAAA or after a short stay in HDU. The main diagnosis and the mean interval between hospital admission and ICU admission is again similar to that reported by Rello and colleagues (Rello et al 2002), underlying the severity of illness on presentation and the early need for either ventilatory or more than single organ support.

Both groups displayed similar severity scores on admission to the ICU, as shown by APACHE II scores calculated within the first 24 hours of admission. However one can speculate that patients who developed VAP were more seriously ill on admission to the hospital and required admission to the ICU within the first 24 hours, whilst patients in the NON VAP group on admission were mostly deemed fit for transfer to a medical/surgical ward. Once in ICU, both groups were ventilated for the same length prior to developing suspected VAP.

The above data suggest that the cohort of patients recruited in the study was consistent with a mixed population of surgical and medical patients.

The case mix of patients was representative of a population admitted to a large general ICU of a tertiary referral and trauma centre.

All patients in this study had received broad-spectrum antibiotics prior to recruitment, although they had to be on the same antibiotic prescription for 72 hours prior to enrolment in the study and bronchoalveolar lavage.

This can be partially explained by looking at the diagnosis on admission. Approximately two thirds of the patients in the VAP and half of the patients in the NON VAP group had undergone emergency abdominal surgery for intraabdominal sepsis followed by trauma and emergency vascular surgery. Medical cases accounted for 38% of patients in the VAP group and 53% of patients in the NON VAP group. Severe CAP and CLD-related complications constituted the majority of medical cases in both groups followed by sepsis in the VAP group and other diagnoses such as COPD in the NON VAP group.

These patients would have been treated with empirical broad-spectrum antibiotics, while other surgical or medical patients with decompensated liver disease patients would have received a course of prophylactic antibiotics post surgery or post gastrointestinal haemorrhage respectively.

More antibiotic days were measured in the NON VAP group compared with the VAP group although the difference was not significant. This is also reflected in the number of courses of antibiotics that patients received in each group. 77% of patients with VAP had been prescribed one course of antibiotics but that figure dropped to 47% of patients in the NON VAP group. The majority of patients in the NON VAP group received more than one course of antibiotics (36% of patients received 2 courses, 11% of patients received 3 courses and 6% of patients received 4 courses of antibiotics).

There are various possible explanations for the above findings. Firstly whilst the average length of ICU stay before recruitment was similar, with an average of 9 ventilated days before recruitment to the study and the average stay in hospital prior to admission to ICU was not significantly longer in the NON VAP group, there were patients with longer stay in the NON VAP group (where IQR was higher). This implies that some patients were admitted to the ICU following deterioration of their clinical condition or development of complications despite initial treatment that would have included antibiotics given the nature of the diagnosis.

Secondly, looking at the factors influencing prescription of antibiotics, 56% of courses of antibiotics prescribed in patients in the VAP group were dictated by positive cultures from various sites whilst only 35% of courses of antibiotics in the NON VAP group were guided by positive results. Approximately half of the courses of antibiotics prescribed in the NON VAP group were empirical. This suggests that there might have been a trend in the clinicians' decision regarding antibiotic therapy towards empirical treatment and perhaps repeated courses of antibiotics when no isolates were found in patients with clinical signs of suspected infection.

Thirdly, a subgroup of patients with ARDS was included in the NON VAP group. As mentioned earlier the diagnosis of pulmonary infection in patients with ARDS can be very difficult. It is therefore likely that these patients may have been treated empirically with a course of antibiotics without microbiological confirmation of infection. In addition depending on the severity of lung injury it is possible that patients had been treated empirically initially as bronchoalveolar lavage may have not been deemed safe at the point that antibiotics were considered.

Regarding previous sources of infection, cultures isolated pathogens from various sites i.e. ETA, blood, CSU or fluid from abscesses or intrabdominal fluid collections. In half of the patients in each group, pathogens were isolated from the respiratory tract either by ETA or previous bronchoalveolar lavage. It is also of note that one third (29%) of patients in the NON VAP group had grown pathogens from two different sites (e.g. blood and BALF), not always simultaneously.

In contrast none of the patients in the VAP group grew pathogens from 2 sites. Similarly one third (33%) of patients in the VAP group and one quarter (22%) in the NON VAP group had polymicrobial infections with more than one pathogen isolated from the same site i.e. two pathogens mainly from ETA or BALF.

It is worth mentioning however that all positive cultures from the respiratory tract mentioned above (i.e. outwith and prior to enrollment to the study) were qualitative and not quantitative. It is known that qualitative cultures carry a high false positive rate due to colonisation and it is likely that a subset of patients in both groups may have been treated for pulmonary infection based on qualitative diagnostic tools available at the time of the decision making.

Moving on to the microbiology of BALF in this study a few interesting points emerge:

1. The incidence of VAP in this study was 27%, in keeping with the literature that suggests an incidence varying from 8-28% depending on the population studied (Rello et al. 2001; Chastre and Fagon 2002).

2. It is recognised that the incidence of VAP in mechanically ventilated patients increases with duration of ventilation and is estimated to be approximately 3.3% per day for the first 5 days. Conversely, as most cases of mechanical ventilation are of short duration, approximately half of all episodes of VAP are estimated to occur within 4 days of mechanical ventilation (Cook et al. 1998). In this study the average duration of ventilation in patients with VAP was 9 days suggestive more of late-onset VAP in this cohort of patients.

3. 38% of pathogens responsible for VAP were Gram-negative, followed by Gram-positive bacteria occurring in 31% of the cases.

The predominance of Gram-negative pathogens is in keeping with previously reported studies where Gram-negative bacteria were responsible in up to 58 % of cases and Gram-positive cocci in up to 35 % of the cases (Chastre and Fagon 2002).

However in other studies, *P.aeruginosa* accounted for half of Gram-negative isolates in VAP with an incidence that approached 24 % of all isolates, whilst Gram-positive bacteria and in particular *S. aureus* (including *MRSA* and *MSSA*) were involved in 20% of the cases. In this study, the most common Gram-negative pathogens were *H.influenzae* and *E. coli* followed by *E. cloacae*. In contrast *P.aeruginosa* was not isolated in a single case of VAP in our population. Among the Gram-positive pathogens, *MRSA* was the most common isolate, in 15% of cases followed by *MSSA* and coagulase negative *Staphylococci*, in keeping with the literature suggestive of an increasingly recognised number of VAP cases due to *MRSA* (Kollef et al. 2005).

These results are of particular importance as in this study VAP was of late onset. In the literature this has been consistently associated with multi-resistant pathogens. Our data also emphasise the differences in the microbiological epidemiology of VAP that can be explained by the local, unit-based microbiological surveillance and also the cohort of patients included which varies between institutions.

Finally in this study, *C. albicans* was isolated in 23% of all cases. *C. albicans* is an opportunistic pathogen frequently found in the normal flora of the human body and isolation of *Candida* from the respiratory tract of mechanically ventilated patients without risk factors for immunosuppression is common (Vincent et al. 1995). Although the significance of these cultures remains under discussion among investigators, it has been repeatedly reported in various studies among VAP cases (Chastre and Fagon 2002; Weber et al. 2007). Interestingly, recent studies have shown that *Candida* colonisation of the respiratory tract of patients receiving mechanical ventilation for > 2 days is associated with an increased risk of *Pseudomonas* VAP, increased hospital morbidity with prolonged ICU and hospital stays (Azoulay et al. 2006), and more recently with worse clinical outcomes, as it is independently associated with increased hospital mortality (Delisle et al. 2008).

4. 34% of patients without VAP had BALF cultures < 10^4 cfu/ml. The equivalent figure for the ETA was approximately the same i.e. 39% of patients for whom ETA samples were available had cultures < 10^6 cfu/ml.

While the presence of pathogens in the ETA suggests colonisation, it is of interest that among the mechanically ventilated patients there is a subgroup of patients with presence of bacteria in the alveolar space at low concentrations. It is unclear if the presence of pathogens per se promotes a persistent "low-grade" inflammation and if there is an association or interaction between the presence of bacteria and their products, leukocyte activation and inflammation and mechanical ventilation that could theoretically enhance ventilator induced lung injury. However on intention to treat analysis these 12 patients were not analysed separately.

5. The discussion on the best diagnostic tool in the diagnosis of VAP is ongoing among investigators. The two main tools under continuous scrutiny are quantitative ETA cultures compared with quantitative BALF cultures with advantages and disadvantages advocated by protagonists of both groups. ETA was available in 53% of all patients recruited in this study and analysis of the data allows 3 important conclusions to be drawn:

- a. 46% of the patients for whom ETA was available had quantitative ETA cultures at concentration $>10^6$ cfu/ml. This indicates that the incidence of VAP would have been almost 2-fold higher if this diagnostic tool had been employed.
- b. The microbiology would have appeared similar as Gram-negative pathogens predominated, however more multi-resistant pathogens would have emerged i.e. *E. cloacae*, *E. aerogenes*, *C. koseri*, *S. maltophilia* and *P. aeruginosa*.
- c. While the above can shed some light on the difficulties and the discrepancies posed in VAP studies that use different diagnostic tools, the comparison of the two tools in our setting confirms only 50% agreement in the diagnosis of VAP.

Therefore if ETA were employed as a diagnostic tool it would have led to inappropriate use of antibiotics in one third of patients without pneumonia. Inappropriate use of antibiotics in critically ill patients has been recognised as a risk factor for the development of multi-resistant pathogens and targeted use of antibiotics has provided an argument towards BAL as a diagnostic tool when resources and skills are available.

In summary, this chapter sought to identify whether the patient groups studied in this thesis were discordant in terms of biological variables that might bias interpretation of results. In general the data suggest that the VAP and NON VAP groups are well matched in terms of age, sex, smoking history, comorbidities, duration of mechanical ventilation prior to enrolment, prior use of antibiotics, and severity of illness at the time of enrolment. Notwithstanding these encouraging findings, it is important to recognise that the numbers of patients in each group are small, and that the NON VAP group contains a subgroup of patients with ARDS. These factors need to be kept in mind in interpreting data in later chapters.

The chapter also sought to characterise the microbiological characteristics of the VAP cohort and to assess the relative utility of BAL and ETA in the diagnosis of VAP. The group is similar to VAP populations described elsewhere in several respects, though the relative absence of *P. aeruginosa* in this study must be borne in mind. The study supports the widely held view that ETA is an inaccurate tool for the diagnosis of VAP.

2. BALF

The central hypotheses of this thesis (p68) are that VAP is associated with deficient antiprotease production, impaired neutrophil phagocytosis and enhanced neutrophil activation. In this model, the consequence would be increased inflammation in the alveolar space. BALF allows interrogation of inflammatory mediators in the alveolar space and the purpose of this chapter was to assess the cellular composition of inflammatory mediators in the lungs of patients with VAP and NON VAP. As such BAL was performed in these patients and both total and differential cell counts were measured to assess the degree of neutrophilia. BALF supernatants were stored for analysis, results of which will be presented in the following sections.

BALF was available for initial analysis from 48 of the 49 patients recruited. 4 of the 48 samples were excluded from the final analysis as they were not cellular and therefore thought not to represent alveolar sampling, adjusting the total number of samples included in the analysis to 44. Serum was available for all 49 recruited patients. BALF and serum were also available from 15 healthy volunteers.

A number of patients with ALI/ARDS (n=14) have been included in the NON VAP group which in the subgroup analysis comprise the ALI/ARDS group while the remaining 22 patients of the NON VAP group will comprise the NEITHER group. Data relating to total and differential cell count/ml and comparisons between VAP, ALI/ARDS and NEITHER groups have been presented mainly as text.

2.1 Volume

The mean volume of BALF retrieved in the VAP group was 32.2mls, 16% of the volume instilled (95%CI: 17.1-47.4). In the NON VAP group the volume retrieved was 33.8mls, 17% of the volume instilled (95%CI: 25.9-41.7) and in the HV group it was 75.0mls, 38% of the instillant (95%CI: 59.9-90.2).

The difference between the VAP group and the HV group and the difference between the NON VAP group and the HV was statistically significant. There was no difference between the volume of the fluid retrieved between the VAP and the NON VAP group (Figure 10).

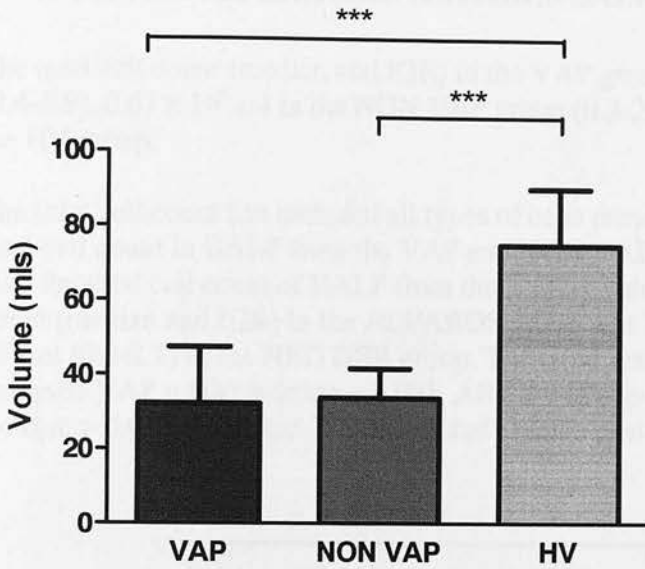


Figure 10: Volume of BALF retrieved in the three groups.

Data are expressed as mean values and 95% CI, (p-value = 0.07 by One-Way ANOVA test), *** = p<0.0001 by Bonferroni's post-hoc analysis).

The difference between the HV and the critically ill patients perhaps reflects the airway pressures, as all ICU patients were ventilated in a pressure control mode of ventilation with a median positive end expiratory pressure (PEEP) of 6.0 cmH₂O (IQR: 5.0-8.0) and 8 cmH₂O (IQR: 5.0-10.0) in the VAP group and the NON VAP group respectively. Median values in the 2 groups did not differ significantly (Figure 11).

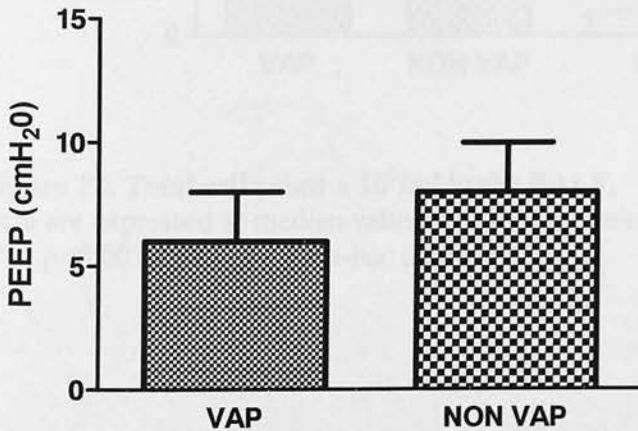


Figure 11: PEEP in ventilated patients.

Data are expressed as median values and IQR, (p-value =0.08 by Mann-Whitney U test). However, within the group of critically ill patients, there was no correlation between the volume of BALF retrieved and the level of PEEP in ventilated patients (p-value > 0.05 by Spearman r correlation).

2.2 Total and differential cell count in BALF

The total cell count (median and IQR) in the VAP group was $1.72 \times 10^6/\text{ml}$ (0.4-3.9), $0.67 \times 10^6/\text{ml}$ in the NON VAP group (0.3-2.5) and $0.25 \times 10^6/\text{ml}$ (0.1-0.2) in the HV group.

The total cell count has included all types of cells present in BALF except red cells. The total cell count in BALF from the VAP and NON VAP group was significantly higher than the total cell count of BALF from the healthy volunteers (Figure 12). The total cell count (median and IQR) in the ALI/ARDS group was $1.23 \times 10^6/\text{ml}$ (0.4-2.9) and $0.60 \times 10^6/\text{ml}$ (0.2-2.1) in the NEITHER group. The trend was the same in the subgroup analysis: VAP v HV: p-value < 0.001, ARDS v HV: p-value < 0.001 and all other groups: p-value > 0.05 by Kruskal-Wallis test and Dunn's post-hoc analysis (Figure 13).

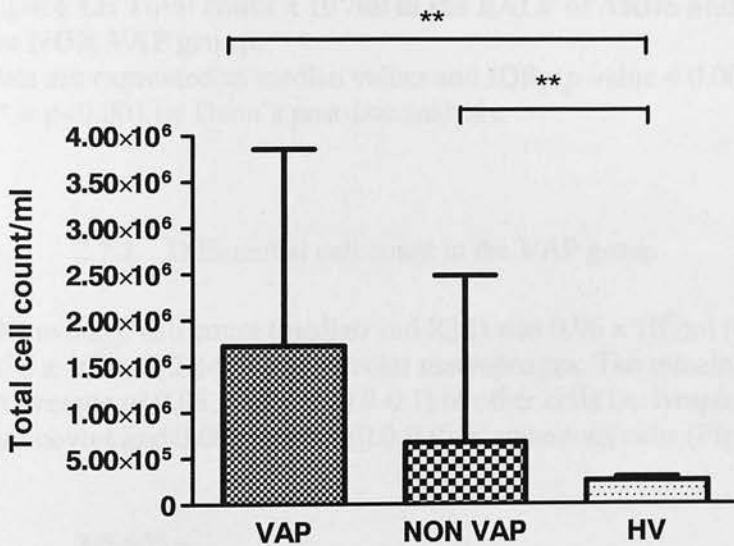


Figure 12: Total cell count $\times 10^6/\text{ml}$ in the BALF.

Data are expressed as median values and IQR, (p-value = 0.0001 by Kruskal-Wallis test),

** = p < 0.001 by Dunn's post-hoc analysis.

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The total cell count has included all types of cells present in BALF except red cells. The total cell count in BALF from the VAP and NON VAP group was significantly higher than the total cell count of BALF from the healthy volunteers (Figure 12). The total cell count (median and IQR) in the ALI/ARDS group was $1.23 \times 10^6/\text{ml}$ (0.4-2.9) and $0.60 \times 10^6/\text{ml}$ (0.2-2.1) in the NEITHER group. The trend was the same in the subgroup analysis: VAP v HV: p-value < 0.001, ARDS v HV: p-value < 0.001 and all other groups: p-value > 0.05 by Kruskal-Wallis test and Dunn's post-hoc analysis (Figure 13).

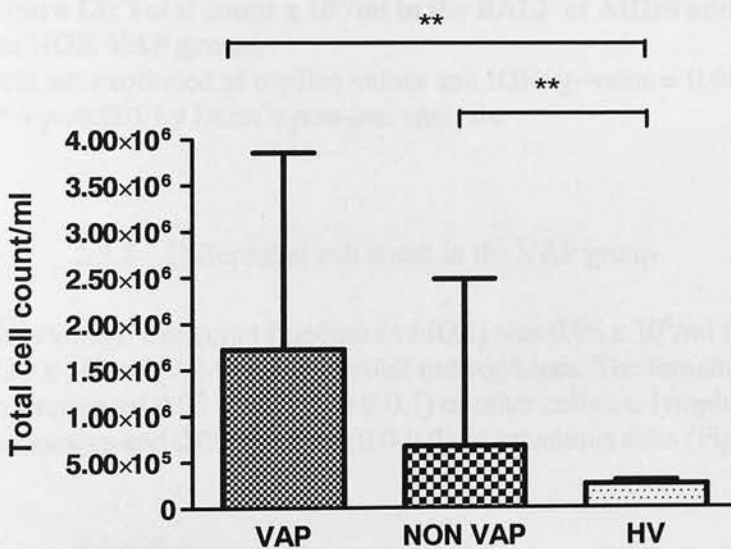


Figure 12: Total cell count $\times 10^6/\text{ml}$ in the BALF.

Data are expressed as median values and IQR, (p-value = 0.0001 by Kruskal-Wallis test),

** = p < 0.001 by Dunn's post-hoc analysis.

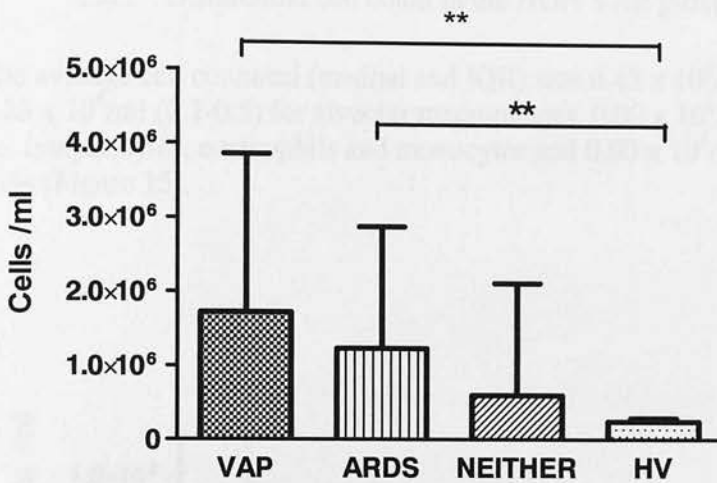


Figure 13: Total count x 10⁶/ml in the BALF of ARDS and NEITHER subgroup of the NON VAP group.

Data are expressed as median values and IQR, (p-value = 0.0001 by Kruskal-Wallis test), ** = p<0.001 by Dunn`s post-hoc analysis.

2.2.1 Differential cell count in the VAP group

The average cell count (median and IQR) was 0.96 x 10⁶/ml (0.0- 2.7) for neutrophils and 0.36 x 10⁶/ml (0.2-0.5) for alveolar macrophages. The remaining cell population included an average of 0.08 x 10⁶/ml (0.0-0.1) of other cells i.e. lymphocytes, eosinophils and monocytes and 0.00 x 10⁶/ml (0.0-0.0) of squamous cells (Figure 14).

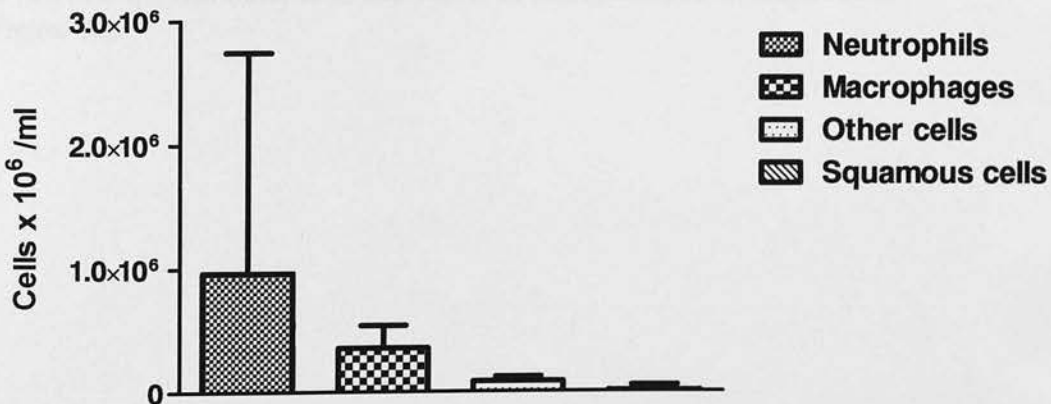


Figure 14: Differential cell count x 10⁶/ml in the BALF of VAP patients.

Data are expressed as median values and IQR.

2.2.2 Differential cell count in the NON VAP group

The average cell count/ml (median and IQR) was 0.43×10^6 /ml (0.1-1.7) for neutrophils, 0.23×10^6 /ml (0.1-0.5) for alveolar macrophages, 0.02×10^6 /ml (0.0-0.1) for other cells i.e. lymphocytes, eosinophils and monocytes and 0.00×10^6 /ml (0.0-0.7) of squamous cells (Figure 15).

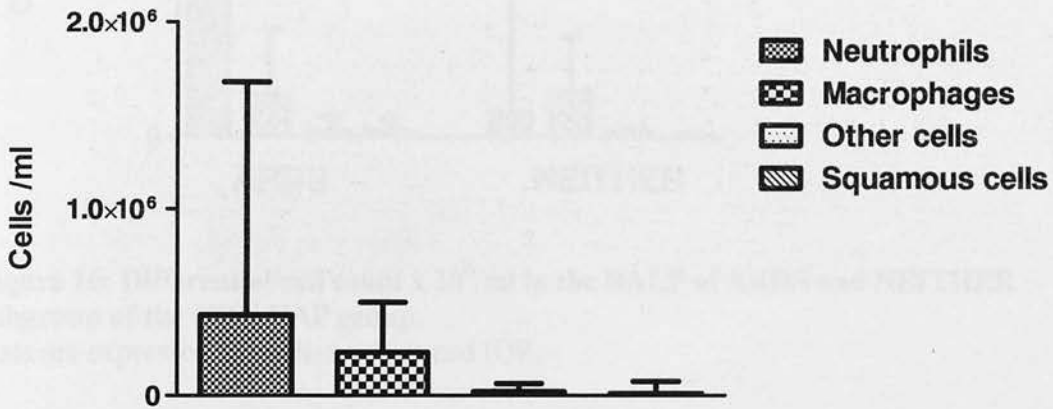


Figure 15: Differential cell count $\times 10^6$ /ml in the BALF of NON VAP patients. Data are expressed as median values and IQR.

The average cell count/ml (median and IQR) in the ALI/ARDS group was 0.9×10^6 /ml (0.2-1.9) for neutrophils, was 0.22×10^6 /ml (0.1-0.6) for alveolar macrophages, 0.03×10^6 /ml (0.0-0.1) for other cells and 0.03×10^6 /ml (0.0-0.1) for squamous cells. Finally the average cell count/ml (median and IQR) in the NEITHER group was 0.14×10^6 /ml (0.0-1.0) for neutrophils, was 0.25×10^6 /ml (0.1-0.6) for alveolar macrophages, 0.02×10^6 /ml (0.0-0.1) for other cells and 0.00×10^6 /ml (0.0-0.) for squamous cells (Figure 16).

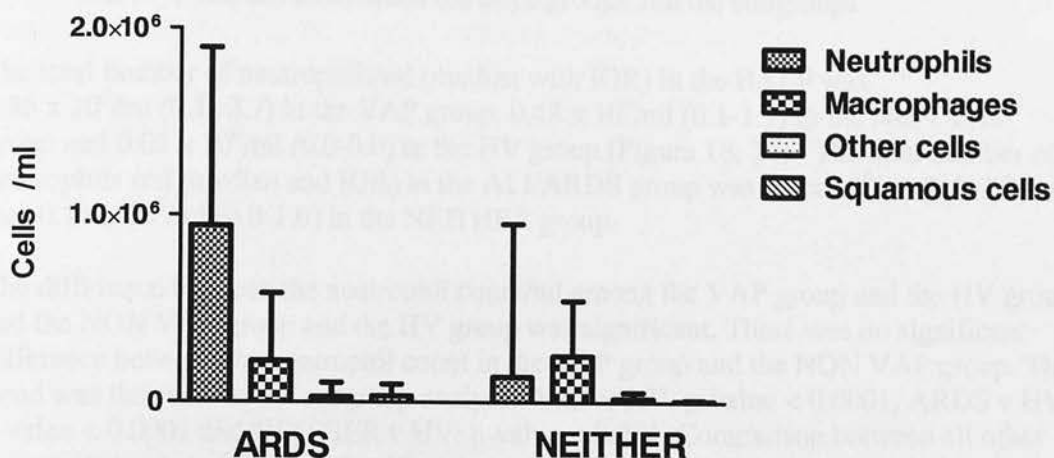


Figure 16: Differential cell count x 10⁶/ml in the BALF of ARDS and NEITHER subgroup of the NON VAP group.

Data are expressed as median values and IQR.

2.2.3 Differential cell count in the HV group

The average cell count/ml (median and IQR) was 0.03 x 10⁵/ml (0.0-0.0) for neutrophils, 2.2 x 10⁵/ml (1.3-2.7) for alveolar macrophages, 0.2 x 10⁵/ml (0.0-0.3) for other leukocytes and 0.03 x 10⁵/ml (0.0-0.1) for squamous cells (Figure 17).

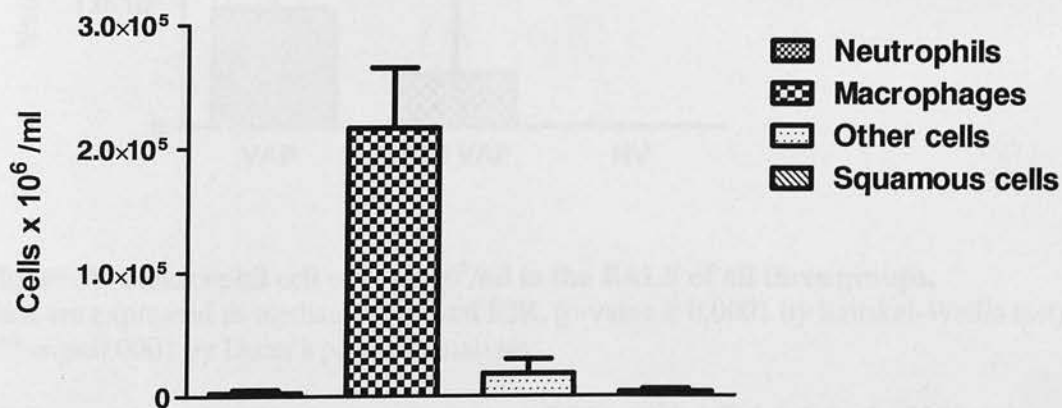


Figure 17: Differential cell count x 10⁵/ml in the BALF of healthy volunteers.

Data are expressed as median values and IQR.

2.2.4 Comparison between the three groups and the subgroups

The total number of neutrophils/ml (median with IQR) in the BALF was 0.96×10^6 /ml (0.1- 2.7) in the VAP group, 0.43×10^6 /ml (0.1-1.7) in the NON VAP group and 0.03×10^6 /ml (0.0-0.0) in the HV group (Figure 18, 20). The total number of neutrophils /ml (median and IQR) in the ALI/ARDS group was 0.9×10^6 /ml (0.2-1.9) and was 0.14×10^6 /ml (0.0-1.0) in the NEITHER group.

The difference between the neutrophil count/ml among the VAP group and the HV group and the NON VAP group and the HV group was significant. There was no significant difference between the neutrophil count in the VAP group and the NON VAP group. The trend was the same in the subgroup analysis: VAP v HV: p-value < 0.0001, ARDS v HV: p-value < 0.0001 and NEITHER v HV: p-value < 0.001. Comparison between all other groups showed no statistical significance: p-value > 0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis (Figure 21).

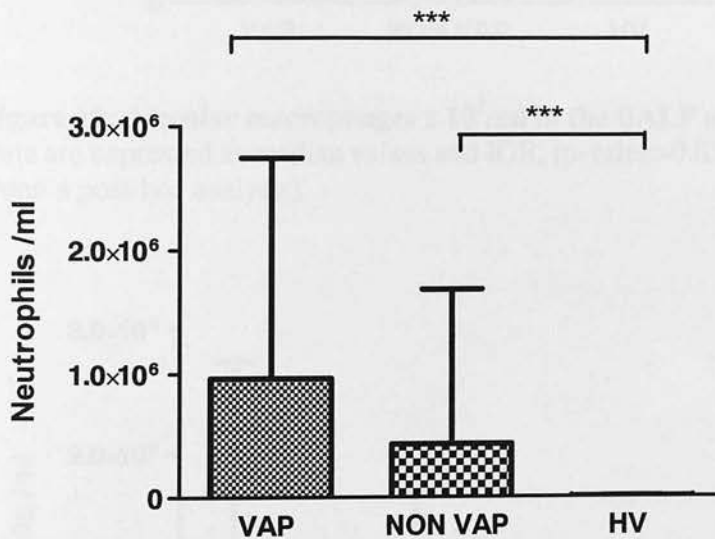


Figure 18: Neutrophil cell count $\times 10^6$ /ml in the BALF of all three groups.

Data are expressed as median values and IQR, (p-value = 0.0001 by Kruskal-Wallis test), *** = p<0.0001 by Dunn`s post-hoc analysis.

Median value (and IQR) of macrophages was 3.56×10^5 /ml (2.1-5.4) in the VAP group, 2.32×10^5 /ml (1.4-5.0) in the NON VAP group and 2.17×10^5 /ml (1.3-2.7) in the HV group (Figure 19, 20). Median value (and IQR) of macrophages/ml was 2.22×10^5 /ml (1.4-5.8) in the ALI/ARDS group and 2.50×10^5 /ml (0.9-5.5) in the NEITHER group.

The average number of alveolar macrophages/ml was higher in the VAP group compared with the NON VAP group and the healthy volunteers although the difference was not significant.

The trend was the same in the subgroup analysis: comparison between all other groups showed no statistical significance, p-value > 0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis (Figure 21).

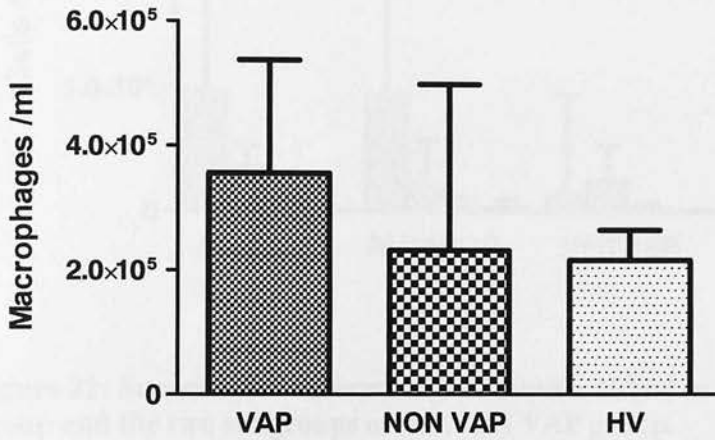


Figure 19: Alveolar macrophages x 10⁵/ml in the BALF of all three groups. Data are expressed as median values and IQR, (p-value>0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

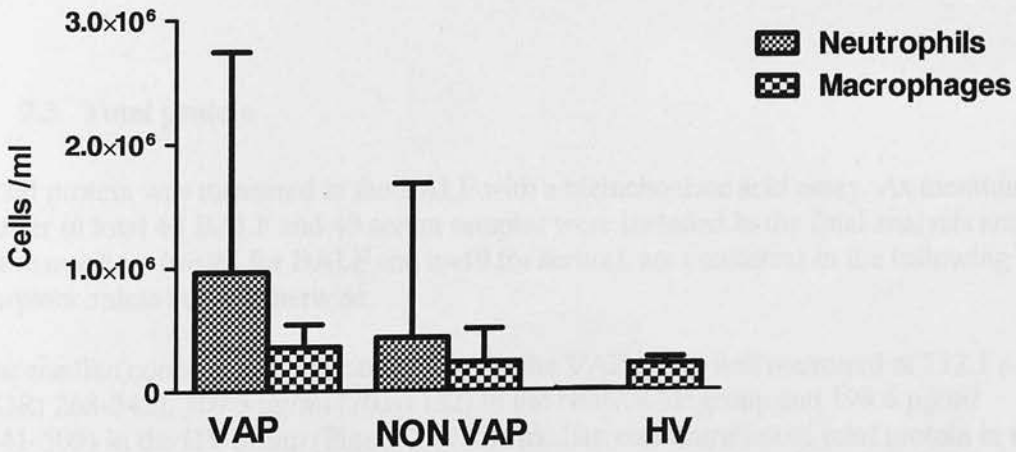


Figure 20: Leukocytes (neutrophils and alveolar macrophages) x 10⁶/ml in the BALF of all three groups. Data are expressed as median values and IQR.

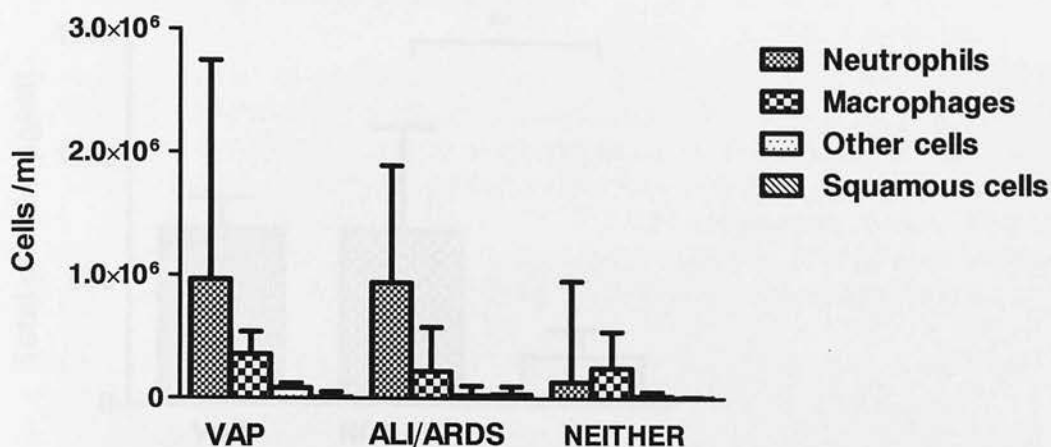


Figure 21: Summary of differential cell count $\times 10^6/\text{ml}$ in the BALF of the VAP group and the two subgroups of the NON VAP group.

Data are expressed as median values and IQR, (p -value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis comparing neutrophil count and macrophage count of the VAP group and the 2 subgroups (ALI/ARDS and NEITHER) of the NON VAP group).

Finally there were no differences between the median concentration of other cells and squamous cells between the three groups (VAP, NON VAP and HV) and in subgroup analysis (p -value > 0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

2.3 Total protein

Total protein was measured in the BALF with a bicinchoninic acid assay. As mentioned earlier in total 44 BALF and 49 serum samples were included in the final analysis and these numbers ($n= 44$ for BALF and $n=49$ for serum), are consistent in the following chapters unless stated otherwise.

The median concentration of total protein in the VAP group was measured at $712.1 \mu\text{g}/\text{ml}$ (IQR: 268-842), $709.5 \mu\text{g}/\text{ml}$ (203-1132) in the NON VAP group and $199.6 \mu\text{g}/\text{ml}$ (141-309) in the HV group (Figure 22). The median concentration of total protein in the ARDS subgroup was $813 \mu\text{g}/\text{ml}$ (641-1307) and in the NEITHER group was $591 \mu\text{g}/\text{ml}$ (133-1068).

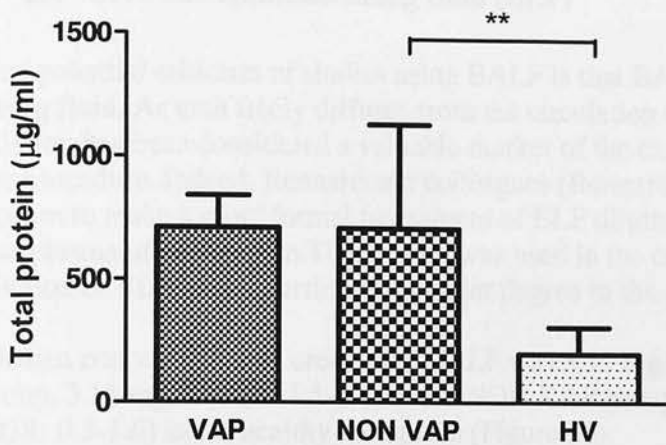


Figure 22: Concentration of total protein in the BALF of the three groups. Data are expressed as median values and IQR, (p -value = 0.002 by Kruskal-Wallis test), ** = $p < 0.001$ by Dunn's post-hoc analysis.

The total protein in the VAP and the NON VAP group was 3-times higher than the average total protein measured in the BALF of the healthy volunteers although the difference was only significant between the NON VAP group and the healthy volunteers group. A high total protein concentration was also observed in the ALI/ARDS and the NEITHER group which did not reach statistical significance in subgroup analysis (Figure 23).

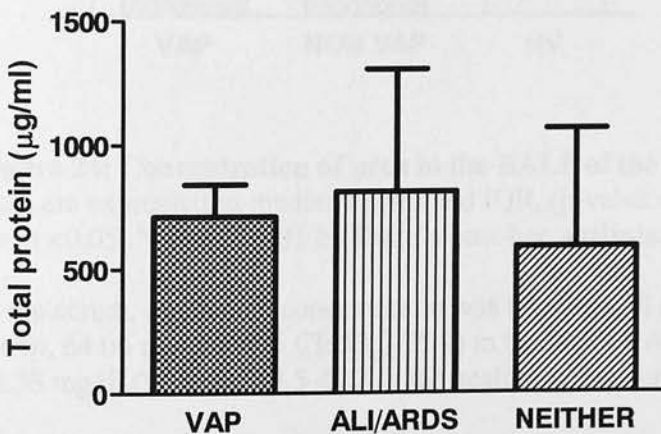


Figure 23: Concentration of total protein (in the BALF of the VAP group and the two subgroups of the NON VAP group). Data are expressed as median values and IQR, (p -value > 0.05 by Kruskal-Wallis test and post-hoc Dunn's analysis).

2.4 Urea and epithelial lining fluid (ELF)

One potential criticism of studies using BALF is that BAL variably dilutes epithelial lining fluid. As urea freely diffuses from the circulation to epithelial lining fluid, urea dilution has been considered a valuable marker of the extent of ELF dilution conferred by the procedure. Indeed, Rennard and colleagues (Rennard et al. 1986) have utilised this system to make a more formal assessment of ELF dilution, if the concentration of BALF and plasma urea is known. This system was used in the current work to estimate whether dilution of ELF was occurring to a similar degree in the groups of patients studied.

Median concentration of urea in the BALF was 3.17 mg/dl (IQR: 1.1-6.6) in the VAP group, 3.14 mg/dl (IQR: 1.1-8.4) in the NON VAP group and 0.78 mg/dl (IQR: 0.3-1.6) in the healthy volunteers (Figure 24).

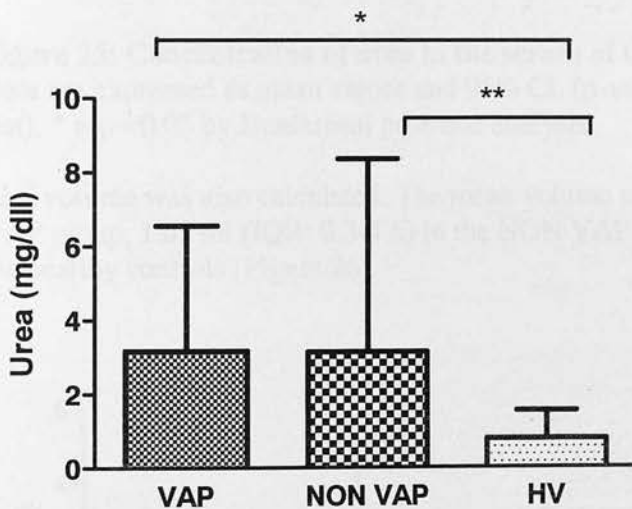


Figure 24: Concentration of urea in the BALF of the three groups.

Data are expressed as median values and IQR, (p -value = 0.003 by Kruskal-Wallis test), * = $p < 0.05$, ** = $p < 0.001$ by Dunn's post-hoc analysis.

In the serum, mean urea concentration was 63.69 mg/dl (95% CI: 43.9-83.4) in the VAP group, 64.06 mg/dl (95% CI: 53.2-75.9) in the NON VAP group and 41.38 mg/dl (95% CI: 33.5-49.2) in the healthy volunteers (Figure 25).

Mean concentration of urea in the BALF and the serum was elevated in both groups of critically ill patients and the difference was statistically significant between the NON VAP group and the healthy controls.

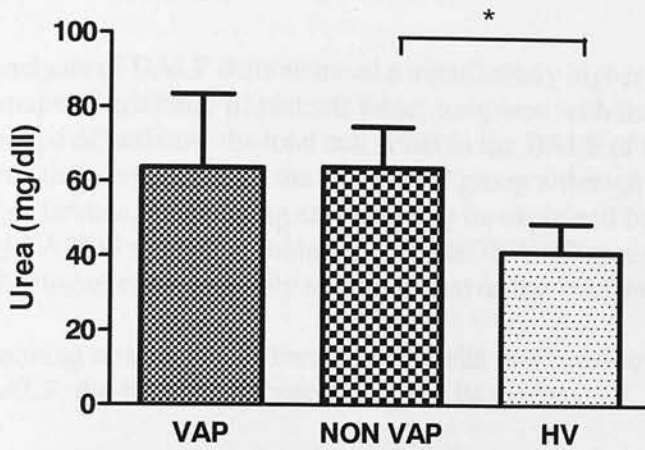


Figure 25: Concentration of urea in the serum of the three groups.

Data are expressed as mean values and 95% CI, (p-value = 0.02 by One-Way ANOVA test), * = $p < 0.05$ by Bonferroni post-hoc analysis.

ELF volume was also calculated. The mean volume was 2.05 ml (IQR: 0.5-3.6) in the VAP group, 1.65 ml (IQR: 0.3-4.6) in the NON VAP group and 0.89 ml (IQR: 0.3-1.5) in the healthy controls (Figure 26).

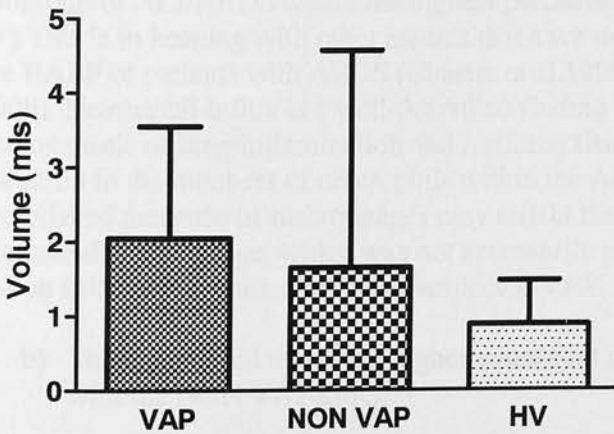


Figure 26: Volume of ELF in all groups.

Data are expressed as median values and IQR, (p-value > 0.05 by Kruskal-Wallis test and Dunn's post-hoc analysis).

2.5 Discussion

Analysis of BALF demonstrated a significantly higher total cell count in BALF in both groups of critically ill patients when compared with healthy volunteers. Between the two groups of patients, the total cell count in the BALF of the VAP group was 2-fold higher than the average one in the NON VAP group although the difference did not reach significance. The finding may partially be explained by the number of patients with ALI/ARDS who were included in the NON VAP group and had an average total number of cells/ml approximately similar to that of the VAP group.

Looking next at the different type of cells that constitute the cellular component of the BALF, the following observations can be made:

- a) The majority of the cells in the VAP group were neutrophils.

The average neutrophil cell count in the VAP group was approximately 3-fold higher than the alveolar macrophages. In the NON VAP group, the average neutrophil cell count was approximately 1.5-fold higher than the macrophage count. Both groups had significantly higher counts of neutrophils compared with the healthy volunteers.

This neutrophil influx in the setting of ventilator-associated pneumonia may reflect the inflammatory process driven by the presence of significant numbers of pathogens in the lung and mediated by a variety of cytokines and chemoattractants.

The number of neutrophils noted in the NON VAP group is strongly influenced by the subgroup of ALI/ARDS where the highest percentage of neutrophils was counted (Figure 21). This is in keeping with other studies that have demonstrated excess of neutrophils in the BALF of patients with ARDS (Chastre et al. 1998 (Marshall et al. 1998; Kambas et al. 2008). Neutrophil influx is a well-described finding that also has been implicated in the pathogenesis of lung inflammation and resulting tissue injury. In this study there was a variation in the numbers of neutrophils within the ALI/ARDS group that along with the established presence of macrophages may reflect the difference in the timing of the bronchoalveolar lavage, which was not necessarily performed at the onset of ALI/ARDS but on fulfillment of the criteria for suspected VAP.

- b) There is a trend towards a higher neutrophil count in the VAP group compared with the NON VAP group.

The neutrophil cell count was 2-fold higher in the VAP group compared with the neutrophil cell count in the NON VAP group although the difference was not significant. The trend documented in this study is in keeping with other studies that have demonstrated significant neutrophilia in BALF samples of patients with pulmonary infections. Kirtland et al. found that the percentage of neutrophils in the lavage of histologically confirmed pneumonia was significantly higher (mean of $75 \pm 17\%$) than in the lavage of patients without histologically confirmed pneumonia (mean of $46 \pm 28\%$).

They also demonstrated that a value of less than 50% PMNs in BALF had a 100% negative predictive value for pneumonia (Kirtland et al. 1997).

Other studies have found increased number of neutrophils in patients with VAP compared with patients without pneumonia ($87 \pm 13\%$ versus $49 \pm 32\%$ respectively) (Marquette et al. 1995) and significantly higher (absolute as well as percentages) number of neutrophils in BALF samples with pulmonary infection in comparison with non infectious BALF (Drent et al. 1996; Cobben et al. 1999;).

In this study there was lack of statistical significance between the VAP and the NON VAP group. That may partly reflect the small numbers of patients recruited in the study that may have not been sufficient to demonstrate statistical significance. In addition, the neutrophil count of the ALI/ARDS group was similar to that of the VAP group (both VAP and the ALI/ARDS group displayed an approximately 7-fold higher –although not significant– neutrophil count than the average one in the NEITHER group) and it is possible that this may have contributed to the weakness of the statistical comparison.

While the lack of statistical significance between the VAP and the NON VAP group is acknowledged, it is of interest that the cell population in the VAP group resembles that of the ARDS group suggesting that the development of VAP seemed to make the inflammatory profile more ‘ARDS-like’ in many respects.

- c) Alveolar macrophages are present at similar concentrations in the BALF of all groups.

In the healthy volunteers group, alveolar macrophages are by far the most predominant cell type as expected in normal conditions (Figures 17, 19). The average macrophage count was 1.5-fold higher in the VAP group compared with the NON VAP, ALI/ARDS, NEITHER and healthy volunteers group although the difference was not significant. This trend is perhaps not surprising as macrophages are recruited in the lung following neutrophil recruitment at the site of the infection, where they will contribute not only to bacterial killing but also to the resolution of the inflammatory process, promoting clearance of apoptotic neutrophils.

- d) Squamous cells (as an index of quality of BAL) appeared at similar concentration in all groups of patients.

No differences were noted among the squamous cells in the BALF in the VAP group, the NON VAP group and the healthy volunteers group. The percentage of squamous cells is a quality measure of the bronchoalveolar lavage as they represent bronchial rather than alveolar sample. Most studies will exclude samples with a percentage $>5\%$ (Meduri and Chastre 1992). In this study there were no BAL samples with a percentage of squamous cells $> 5\%$.

Total protein was found to be 3-times higher in the BALF of the VAP and the NON VAP group when compared with the healthy volunteers.

The difference between the NON VAP group and the healthy volunteers was statistically significant and the higher concentration of total protein in the NON VAP group may reflect in part capillary leak due to increased alveolar-capillary membrane permeability in patients with ALI/ARDS (Figure 23).

Finally to assess the dilution factor of BALF, urea was measured in both BAL and serum.

Urea is freely diffused through most body compartments including the lung (Taylor et al. 1965; Reynolds et al. 1975; Theodore et al. 1975) and has been used to estimate the amount of epithelial lining fluid (ELF).

The major advantages in using urea to quantify the amount of ELF recovered by bronchoalveolar lavage are:

- a. urea is an endogenous marker of dilution i.e. it is not necessary to administer a tracer molecule to estimate the dilution of the recovered ELF.
- b. since urea has a low molecular mass and it diffuses across the alveolar wall and lung, urea will be in equilibrium with plasma urea, thus the urea concentration in ELF is assumed to be equal to the urea concentration in plasma.
- c. urea does not have a net charge at physiologic pH and is not consumed or produced by lung cells.
- d. since the concentration of urea in plasma (and therefore ELF) is relatively high, even after dilution by bronchoalveolar lavage fluid, it is technically easy to measure the concentration of urea in the recovered lavage fluid. Thus the dilution of a small volume of ELF by a large volume of saline results in a large decrease in the concentration of urea, a change that can be determined accurately (Rennard et al. 1986).

In this study BAL and serum urea were similar in the VAP and the NON VAP group which is reassuring. Although the HV BAL appeared slightly more dilute there were no significant differences in the dilution factor of the BALF among the mechanically ventilated patients and the healthy controls. Therefore in the following sections, results have been expressed as concentrations/ml rather than concentration/ml of ELF.

In summary this chapter sought to assess the cellular composition of the BALF and the degree of neutrophilia that may be present in the BALF of patients with VAP compared with the NON VAP patients. The results suggest a trend toward a higher neutrophil count in patients with pneumonia as compared with patients without pneumonia which did not reach statistical significance perhaps due to small numbers of patients recruited and the inclusion of ARDS patients in the NON VAP group.

These two groups (VAP and ARDS) displayed a similar profile which is of particular relevance to the key issue addressed by this thesis that relates to the function and activation of these neutrophils and to the effect of bacterial infection on inflammatory cytokines – these issues will be discussed in more detail in the next section.

3 CYTOKINES / CHEMOKINES IN THE BALF AND SERUM

The initial hypothesis was that VAP is characterised by poorly phagocytic but over-activated neutrophils recruited to the lung by pathogens, collectively resulting in an exaggerated pro-inflammatory response in the alveoli. On this basis, the purpose of the current chapter was to quantify acute inflammatory cytokines in VAP. This was tested with a detailed quantification of inflammatory markers namely CXCL8, IL-1 β , TNF- α , IL-6, IL-10 and IL-12p70, in the lung compartment and in the systemic circulation using cytokine bead array kits. MCP-1/CCL2 concentrations were also measured in BALF and serum and will also be discussed at this section.

3.1 CXCL8

Median concentration of CXCL8 in BALF from the VAP group was 2908 pg/ml (IQR: 869-16096), 211 pg/ml (IQR: 100-826) in the NON VAP group and 51 pg/ml (IQR: 29-277) in the healthy volunteer group.

Levels of CXCL8 were significantly higher in the VAP group when compared with the NON VAP group and the healthy volunteers. Significant differences were also seen comparing levels of CXCL8 between the NON VAP group and the healthy volunteers (Figure 27).

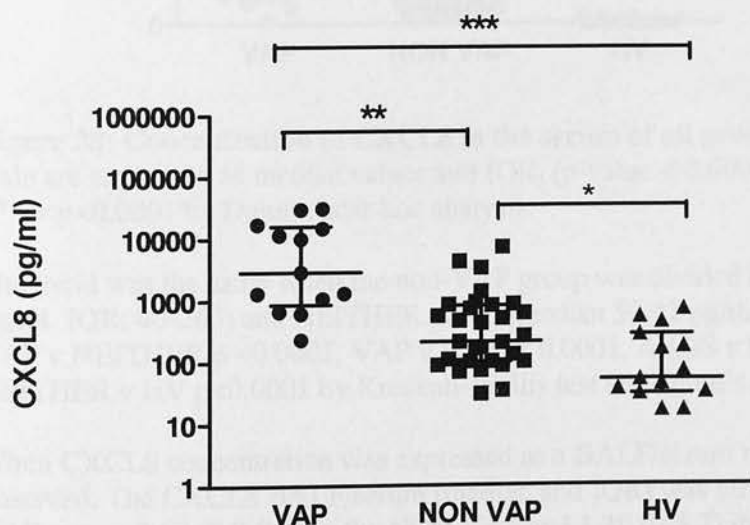


Figure 27: Concentration of CXCL8 in BALF of all groups.

Data are expressed as median values and IQR, (p-value < 0.0001 by Kruskal-Wallis test),

* = p < 0.05, ** = p < 0.001 and *** = p < 0.0001 by Dunn's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (median 469 pg/ml, IQR: 196-726) and NEITHER group (median 110 pg/ml, IQR: 80-901): VAP v NEITHER $p < 0.05$ and VAP v HV $p < 0.0001$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

In the serum, median concentration of CXCL8 in the VAP group was 69.85 pg/ml (IQR: 54-271), 64.37 pg/ml (IQR: 35-228) in the NON VAP group and 3.12 pg/ml (IQR: 0-12) in the healthy volunteer group.

Levels of CXCL8 were significantly elevated in the serum of both groups of critically ill patients when compared with the healthy volunteers (Figure 28).

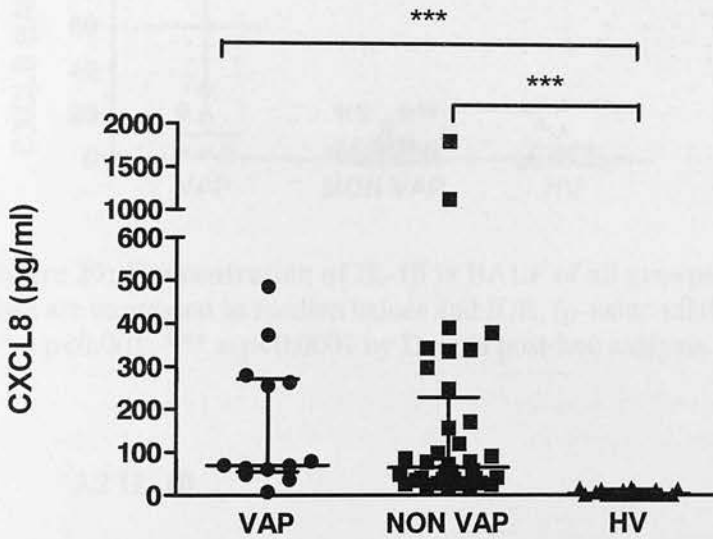


Figure 28: Concentration of CXCL8 in the serum of all groups.

Data are expressed as median values and IQR, (p -value < 0.0001 by Kruskal-Wallis test), *** = $p < 0.0001$ by Dunn's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (median 77.28 pg/ml, IQR: 40-260) and NEITHER group (median 50.42 pg/ml, IQR: 27-212): VAP v NEITHER $p < 0.0001$, VAP v HV $p < 0.0001$, ARDS v HV $p < 0.0001$ and NEITHER v HV $p < 0.0001$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

When CXCL8 concentration was expressed as a BALF/serum ratio, similar results were observed. The CXCL8 BALF/serum (median and IQR) was 60.24 (10.9-160.6) in the VAP group, 3.63 (1.2-8.1) in the NON VAP and 1.39 (0-5.7) in the healthy volunteers group. The CXCL8 BALF/serum ratio was significantly higher in the VAP group when compared with the NON VAP group and the healthy volunteers (Figure 29).

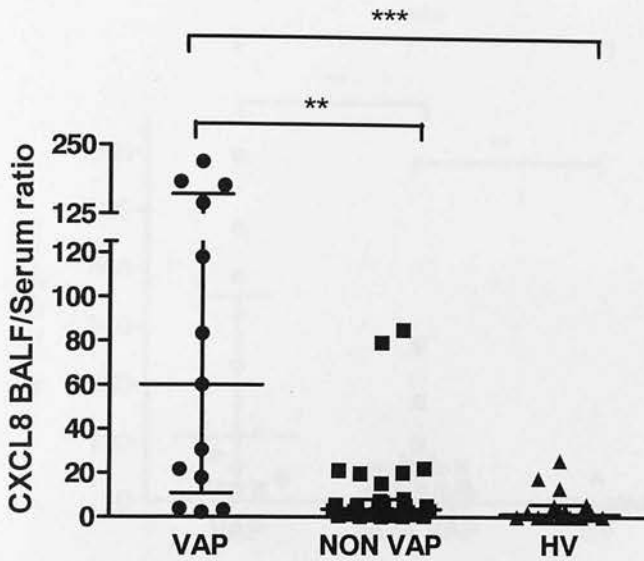


Figure 29: Concentration of IL-1 β in BALF of all groups.

Data are expressed as median values and IQR, (p-value <0.0001 by Kruskal-Wallis test), ** = p<0.001, *** = p<0.0001 by Dunn`s post-hoc analysis.

3.2 IL-1 β

Median IL-1 β concentration in the BALF of the VAP group was 112.3 pg/ml (IQR: 30-352), 17.5 pg/ml (IQR: 0-57) in the NON VAP group and 0.0 pg/ml (IQR: 0-0) in the healthy volunteers group. IL-1 β levels were significantly elevated in the BALF of VAP patients when compared with the NON VAP group and healthy controls. IL-1 β levels were also significantly higher in the NON VAP group when compared with controls (Figure 30).

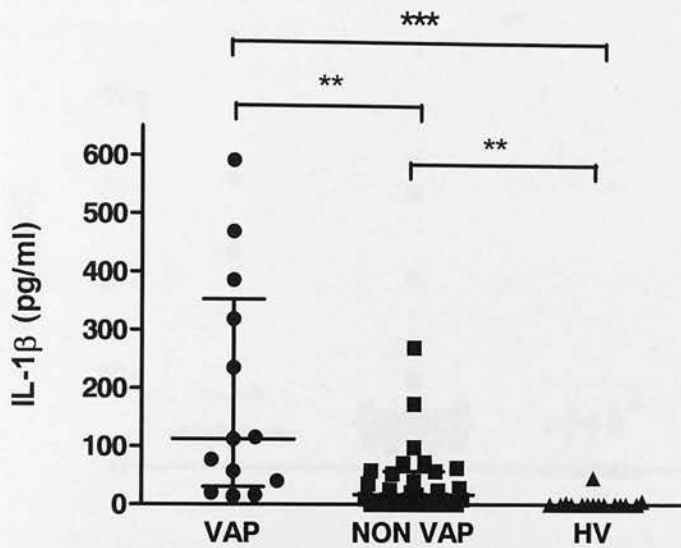


Figure 30: Concentration of IL-1 β in BALF of all groups.

Data are expressed as median values and IQR, (p-value <0.0001 by Kruskal-Wallis test), ** = p<0.001, *** = p<0.0001 by Dunn`s post-hoc analysis.

The trend was similar when the non-VAP group was divided into ARDS (median 22.2 pg/ml, IQR: 5-55) and NEITHER group (median 9.37 pg/ml, IQR: 0-60): VAP v NEITHER p <0.05, VAP v HV p < 0.0001, ARDS v HV p<0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

In the serum, median concentration of IL-1 β in the VAP group was 3.75 pg/ml (IQR: 3-8), 4.88 pg/ml (IQR: 3-6) in the NON VAP group and 0.0 pg/ml (IQR: 0-5) in the healthy volunteers group.

Serum levels of IL-1 β were significantly higher in the NON VAP group when compared with the healthy controls (Figure 31).

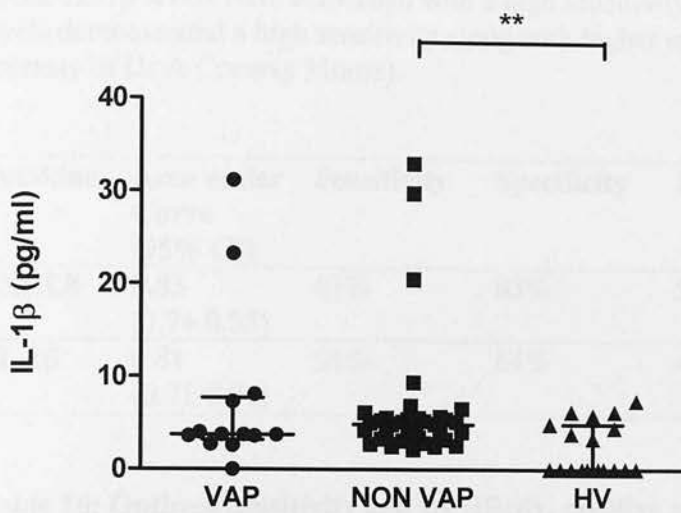


Figure 31: Concentration of IL-1 β in the serum of all groups.

Data are expressed as median values and IQR, (p -value = 0.009 by Kruskal-Wallis test), ** = $p < 0.001$ by Dunn's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (median 4.59 pg/ml, IQR: 3-6) and NEITHER group (median 4.95 pg/ml, IQR: 3-6): NEITHER v HV $p < 0.05$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

BALF CXCL8 and IL-1 β levels were consistently and significantly higher in the VAP patients when compared with NON VAP patients upon completion of the study, $n=17$ VAP, $n=55$ NON VAP (personal correspondence and data kindly provided by Dr A Conway Morris). The capacity for CXCL8 and IL-1 β to distinguish VAP was tested by constructing receiver operator characteristic (ROC) curves and results are shown in Figure 32.

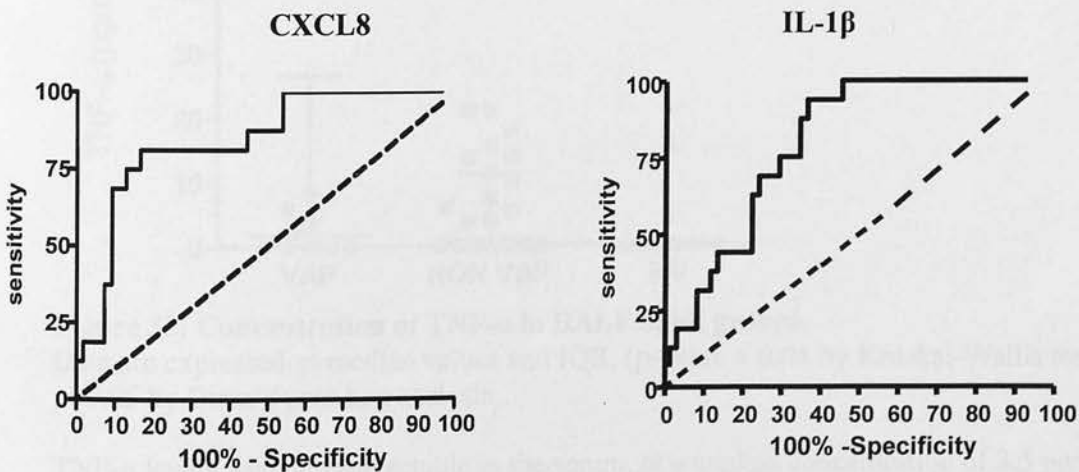


Figure 32: ROC curves for CXCL8 and IL-1 β in BALF.

BALF IL-1 β levels were associated with a high sensitivity but lower specificity. CXCL8 levels demonstrated a high sensitivity along with higher specificity (Table 16, data courtesy of Dr A Conway Morris).

Cytokine	Area under Curve (95% CI)	Sensitivity	Specificity	PPV	NNV	+LR	-LR
CXCL8	0.83 (0.74-0.95)	81%	83%	59%	94%	4.7	0.22
IL-1 β	0.81 (0.71-0.91)	94%	64%	43%	97%	2.4	0.09

Table 16: Optimal sensitivity and specificity, positive predictive value (PPV), negative predictive value (NPV), positive and negative likelihood ratio (LR) for CXCL8 and IL-1 β in BALF

3.3 TNF- α

TNF- α levels were detected in the BALF of critically ill patients at a median concentration of 1.79 pg/ml (IQR: 0-26) in the VAP group, 0.0 pg/ml (IQR: 0-11) in the NON VAP group and in negligible levels of 0.0 pg/ml (IQR: 0-0) in the healthy controls (Figure 33).

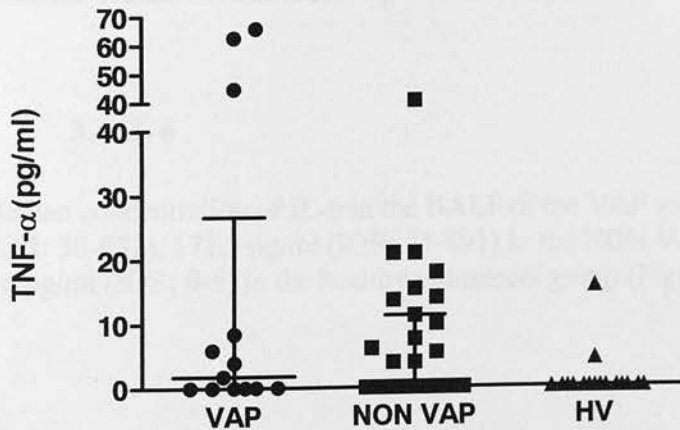


Figure 33: Concentration of TNF- α in BALF of all groups.

Data are expressed as median values and IQR, (p-value = 0.04 by Kruskal-Wallis test), p>0.05 by Dunn's post-hoc analysis.

TNF- α levels were also detectable in the serum, at a median concentration of 3.5 pg/ml (IQR: 2-5) in the VAP group, 4.2 pg/ml (IQR: 3-5) in the NON VAP group and 0.0 pg/ml (IQR: 0-5) in the healthy volunteers (Figure 34).

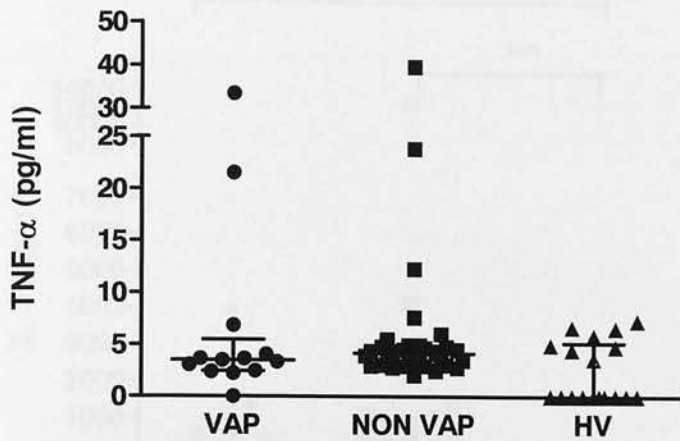


Figure 34: Concentration of TNF- α in the serum of all groups.

Data are expressed as median values and IQR, (p -value = 0.09 by Kruskal-Wallis test), $p > 0.05$ by Dunn's post-hoc analysis.

There was no significant difference in the average levels of TNF- α in BALF or serum between the three groups.

Similar trends were noted when the non-VAP group was divided into ARDS (median BALF concentration 1.97 pg/ml, IQR: 0-14 and serum concentration 4.2 pg/ml, IQR: 3-5) and NEITHER group (median BALF concentration 0.00 pg/ml, IQR: 0-8 and serum concentration 4.2 pg/ml, IQR: 3-5): $p > 0.05$ in comparison of all pairs of groups by Kruskal-Wallis test and Dunn's post-hoc analysis.

3.4 IL-6

Median concentration of IL-6 in the BALF of the VAP group was 201.7 pg/ml (IQR: 58-952), 171.3 pg/ml (IQR: 21-891) in the NON VAP group and 1.6 pg/ml (IQR: 0-5) in the healthy volunteers group (Figure 35).

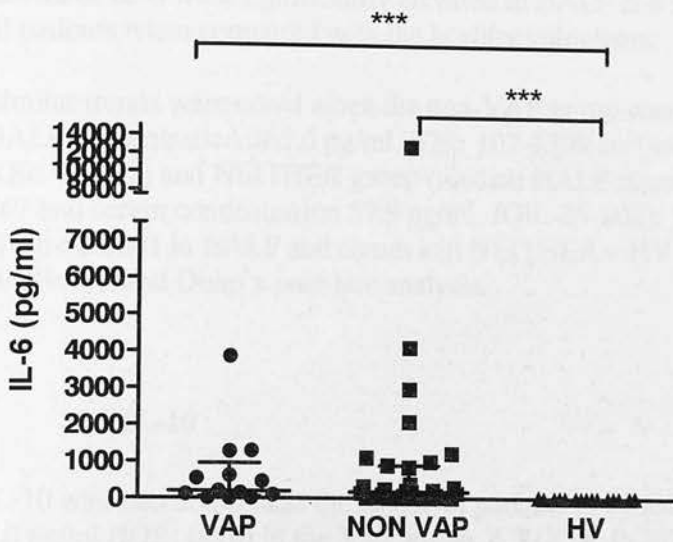


Figure 35: Concentration of IL-6 in BALF of all groups.

Data are expressed as median values and IQR, (p -value < 0.0001 by Kruskal-Wallis test), *** = $p < 0.0001$ by Dunn`s post-hoc analysis.

In the serum, median concentration of IL-6 in the VAP group was 106.5 pg/ml (IQR: 55-259), 69.9 pg/ml (IQR: 28-259) in the NON VAP group and 2.8 pg/ml (IQR: 0-3) in the healthy volunteers group (Figure 36).

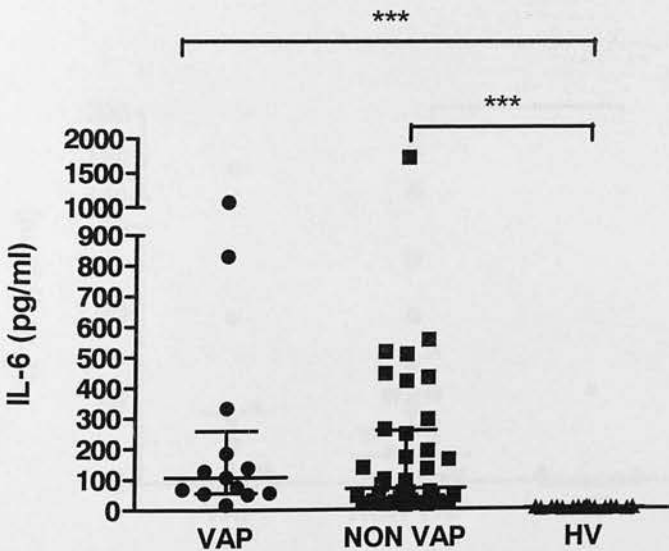


Figure 36: Concentration of IL-6 in the serum of all groups.

Data are expressed as median values and IQR, (p -value < 0.0001 by Kruskal-Wallis test), *** = $p < 0.0001$ by Dunn`s post-hoc analysis.

In the serum, median concentration of IL-10 in the VAP group was 7.7 pg/ml (IQR: 5-13), 8.8 pg/ml (IQR: 5-19) in the NON VAP group and 2.9 pg/ml (IQR: 0-4) in the healthy volunteer group. Levels were significantly elevated in the serum of critically ill patients compared with the healthy controls (Figure 38).

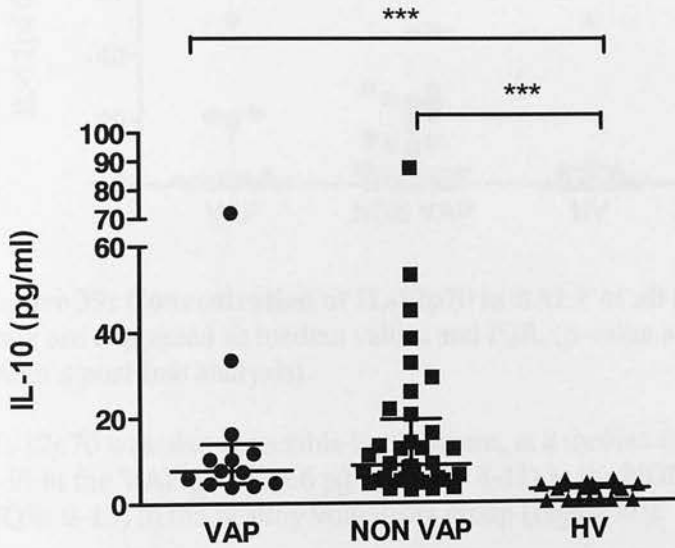


Figure 38: Concentration of IL-10 in the serum of all groups.

Data are expressed as median values and IQR, (p -value < 0.0001 by Kruskal-Wallis test), *** = p < 0.0001 by Dunn`s post-hoc analysis.

Similar trends were noted when the non-VAP group was divided into ARDS (median 9.7 pg/ml, IQR: 5-30) and NEITHER groups (median 8.5 pg/ml, IQR: 5-15): VAP v HV p < 0.0001, ARDS v HV p < 0.0001 and NEITHER v HV p < 0.0001 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

3.6 IL-12p70

Median concentration of IL-12p70 in the BALF of VAP group was 1.9 pg/ml (IQR: 0-22), 4.4 pg/ml (IQR: 0-25) in the NON VAP group and 0.0 pg/ml (IQR: 0-5) in the healthy volunteer group (Figure 39).

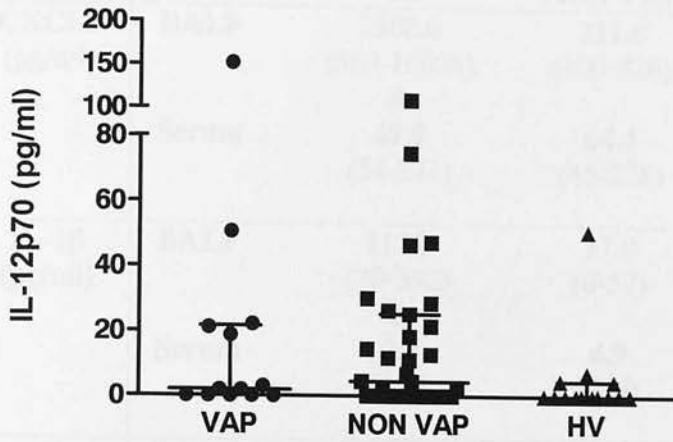


Figure 39: Concentration of IL-12p70 in BALF of all groups.

Data are expressed as median values and IQR, (p-value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

IL-12p70 was also detectable in the serum, at a median concentration of 6.2 pg/ml (IQR: 4-9) in the VAP group, 6.6 pg/ml (IQR: 4-11) in the NON VAP group and 4.3 pg/ml (IQR: 0-13) in the healthy volunteers group (Figure 40).

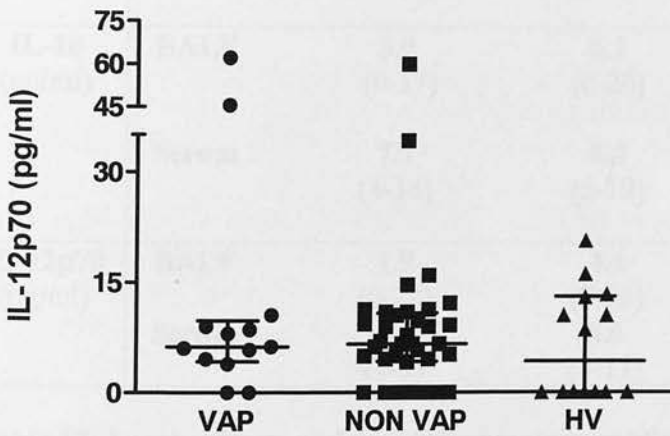


Figure 40: Concentration of IL-12p70 in the serum of all groups.

Data are expressed as median values and IQR, (p-value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

The average levels of IL-12p70 in BALF and serum did not differ significantly between the three groups. Similar trends were noted when the non-VAP group was divided into ARDS (median BALF concentration 3.4 pg/ml, IQR: 0- 26 and serum concentration 6.2 pg/ml, IQR: 0-10) and NEITHER groups (median BALF concentration 4.4 pg/ml, IQR: 0-26 and serum concentration 7.2 pg/ml, IQR: 4-11); p >0.05 in comparison of all pairs of groups by Kruskal-Wallis test and Dunn`s post-hoc analysis. All results from this section are summarised in Table 17.

		VAP	NON VAP	HV
CXCL8 (pg/ml)	BALF	2908.0 (869-16096)	211.8 (100-826)	51.5 (29-277)
	Serum	69.9 (54-271)	64.4 (45-228)	3.1 (0-12)
		&		\$\$
IL-1β (pg/ml)	BALF	112.3 (30-352)	17.0 (0-57)	0.0 (0-0)
	Serum	3.7 (3-8)	4.9 (3-6)	0.0 (0-5)
				\$\$
TNF-α (pg/ml)	BALF	1.8 (0-26)	0.0 (0-11)	0.0 (0-0)
	Serum	3.5 (2-5)	4.2 (3-5)	0.0 (0-3)
IL-6 (pg/ml)	BALF	201.7 (58-952)	171.3 (21-891)	1.6 (0-8)
	Serum	106.5 (55-259)	69.9 (28-259)	2.9 (0-3)
				\$\$
IL-10 (pg/ml)	BALF	0.0 (0-17)	6.3 (0-20)	0.0 (0-0)
	Serum	7.7 (4-14)	8.8 (5-19)	2.9 (0-4)
				\$\$
IL-12p70 (pg/ml)	BALF	1.9 (0-22)	4.4 (0-25)	0.0 (0-5)
	Serum	6.2 (4-9)	6.6 (4-11)	0.0 (4-13)

Table 17: Levels of pro- and anti-inflammatory mediators in BALF and serum.

Data expressed as median and IQR.

& = significant difference comparing VAP and NON VAP, Kruskal-Wallis test with Dunn`s post-hoc analysis,

\$=significant difference comparing HV and VAP, Kruskal-Wallis test with Dunn`s post-hoc analysis,

§ = significant difference comparing HV and NON VAP, Kruskal-Wallis test with Dunn`s post-hoc analysis).

3.7 MCP-1/CCL2

MCP-1/CCL2 was measured in BALF and serum of patients and HV. In BALF, median concentration of MCP-1 was 1186pg/ml (IQR: 53-1929 pg/ml) in the VAP group, 2168pg/ml (IQR: 175-4034 pg/ml) in the NON VAP group and 27 pg/ml (95%CI: 0-35 pg/ml) in the HV group (Figure 41).

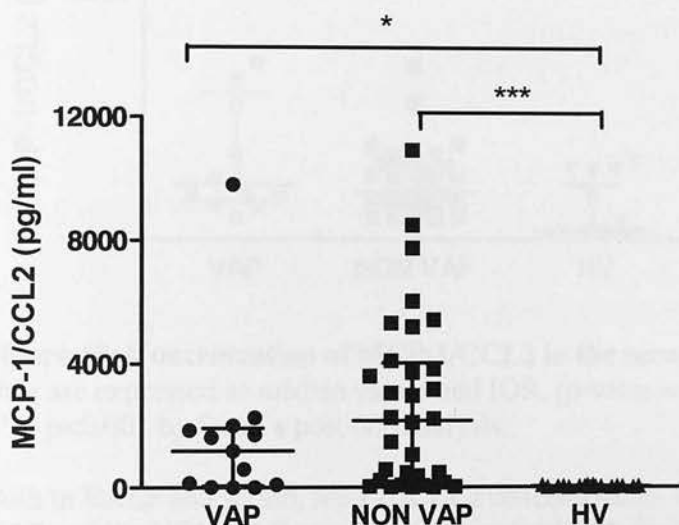


Figure 41: Concentration of MCP-1/CCL2 in BALF of all groups.

Data are expressed as median values and IQR, (p-value < 0.0001 by Kruskal-Wallis test), * = p < 0.05 and *** = p < 0.0001 by Dunn's post-hoc analysis.

In the serum median concentration of MCP-1/CCL2 was 443 pg/ml (IQR: 310-1168 pg/ml) in the VAP group, 377 pg/ml (95%CI: 264-606 pg/ml) in the NON VAP group and 51 pg/ml (IQR: 42-435 pg/ml) in the HV group (Figure 42).

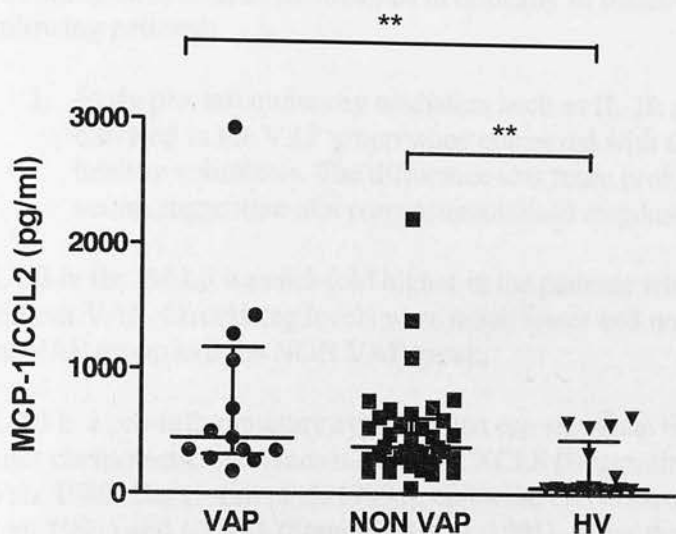


Figure 42: Concentration of MCP-1/CCL2 in the serum of all groups.

Data are expressed as median values and IQR, (p -value = 0.0007 by Kruskal-Wallis test), ** = $p < 0.001$ by Dunn's post-hoc analysis.

Both in BALF and serum, MCP-1/CCL2 concentrations were significantly higher in the VAP and the NON VAP group when compared with the healthy volunteers.

Similar trends were noted when the non-VAP group was divided into ARDS (median BALF concentration 3968 pg/ml, IQR: 2198-6464) and NEITHER groups (median BALF concentration 425 pg/ml, IQR: 32-2463): VAP v HV $p < 0.05$, ARDS v HV $p < 0.0001$ and ARDS v NEITHER $p < 0.05$ by Kruskal-Wallis test and Dunn's post-hoc analysis. Median MCP-1/CCL2 serum concentration in the ARDS group was 502 pg/ml (IQR: 306-652) and 393 pg/ml (IQR: 218-548) in the NEITHER group with statistical significance between VAP v HV $p < 0.001$ and ARDS v HV $p < 0.001$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

3.8 Discussion

It is well recognised that regulation of the inflammatory response depends upon a complex interaction between immune cells and inflammatory cytokines. Cytokines produced by a variety of cells play a central role in the inflammatory process and among them TNF- α , IL-1 β , IL-6 and CXCL8 are described as early response mediators. However inflammatory stimuli also activate production of specific cytokine neutralising molecules such as soluble TNF receptors, and counter-inflammatory cytokines such as IL-10 and IL-1ra, that can downregulate the host inflammatory response, and may have a key role to play in controlling the pro-inflammatory cytokine cascade.

The study of cytokines/chemokines in critically ill patients in this study revealed the following patterns:

1. Early pro-inflammatory mediators such as IL-1 β and CXCL8 were significantly elevated in the VAP group when compared with the NON VAP group and the healthy volunteers. The difference was more profound in the BALF than the serum suggestive of a compartmentalised response in the lung.

IL-1 β in the BALF was 6.5-fold higher in the patients with VAP compared with patients without VAP. Circulating levels were much lower and not significantly different between the VAP group and the NON VAP group.

IL-1 β is a pro-inflammatory cytokine that can stimulate the production of a variety of other chemotactic cytokines including CXCL8 (Baggiolini et al. 1989b; Baggiolini and Walz 1989; Baggiolini et al. 1989a), epithelial cell neutrophil activator (ENA-78) (Walz et al. 1991) and MCP-1 (Standiford et al. 1991). It has therefore gained a prominent position at the head of the inflammatory cytokine cascade and has been studied in patients with ARDS and pneumonia.

Several investigators identified the presence of IL-1 β from BALF in ARDS (Siler et al. 1989; Suter et al. 1992; Goodman et al. 1996; Park et al. 2001) and also demonstrated strong correlation between the IL-1 β system and clinical lung injury severity and outcome in patients with ARDS (Park et al. 2001).

It is of note that its naturally occurring antagonist IL-1ra has also been detected in BALF from patients with ARDS (Donnelly et al. 1996; Goodman et al. 1996). IL-1ra competitively inhibits binding of IL-1 β to its primary cell surface signaling receptor IL-1RI in 1:1 stoichiometry (Arend et al. 1989; Arend 1993). However the IL-1 β /IL-1ra ratio has not been consistent in the ARDS studies where it has been found to be as high as 10:1 in some (Goodman et al. 1996) and as low as 1:10 in others (Park et al. 2001) depending on the immunoassay used. In addition another IL-1 receptor antagonist, soluble IL-1 receptor II (sIL-1RII), is detectable in BALF (Park et al. 2001) and can enhance the inhibitory activity of IL-1ra while IL-1RI can be shed and bind IL-1ra hindering its inhibitory activity (Burger et al. 1995).

In the present study BALF IL-1 β levels were significantly higher in VAP patients and this is in agreement with other studies. IL-1 β is stimulated by bacterial products (Brazel et al. 1991) and the expression of IL-1 β at the early stages of the inflammatory cascade assigns IL-1 β an important role as early response mediator in regulating cellular function.

Similar findings have been shown in BALF from ventilated patients with severe community-acquired pneumonia (Monton et al. 1999; Nys et al. 2002; Nys et al. 2003; Wu et al. 2003) and patients with VAP (Nys et al. 2002; Millo et al. 2004). Levels correlated with the bacterial burden only in patients with community-acquired pneumonia (Wu et al. 2003).

Other studies have measured the net biological activity of IL-1 β in ARDS and pneumonia BALF and demonstrated a net pro-inflammatory activity (Pugin et al. 1996; Pugin et al. 1999; Park et al. 2001). This has been supported by evidence from studies studying BALF from ARDS and pneumonia patients, which induced NF-kB activity, this correlating well with levels of IL-1 β (Nys et al. 2002).

The above studies suggest that despite the complexities listed above, IL-1 β appears to play an important role at the early stages of the inflammatory cascade while exaggerated release can facilitate and/or perpetuate a persistent inflammatory response. Furthermore although IL-1 β (and TNF- α) are both lacking direct chemoattractant properties, they are potent inducers of CXCL8 production, which has been identified as the major neutrophil chemotactic factor in the lung.

BALF levels of CXCL8 were markedly elevated in patients with VAP compared with patients without VAP. CXCL8 concentration was 14-fold higher in BALF from patients with VAP compared with critically ill patients without VAP. Both groups had significantly elevated levels of CXCL8 when compared with healthy controls.

Also of note, BALF CXCL8 levels in the VAP group were 40-fold higher than the circulating CXCL8 levels suggesting a profound response that is confined in the lung. The difference is likely to be even higher as levels in the BALF are diluted. In the NON VAP group BALF CXCL8 levels were 3-fold higher as compared with circulating levels.

BALF CXCL8 levels have been studied more extensively in patients with ARDS. Previous studies have consistently demonstrated increased BALF CXCL8 concentrations in patients with established ARDS (Jorens et al. 1992; Chollet-Martin et al. 1993; Torre et al. 1993) and, importantly in patients at risk for developing ARDS (Donnelly et al. 1993; Nys et al. 2002).

In the context of infection, BALF CXCL8 levels were elevated in the affected lung -but not in serum- in human studies of community-acquired pneumonia (Dehoux et al. 1994; Boutten et al. 1996; Schutte et al. 1996; Bohnet et al. 1997; Wu et al. 2003) and of human volunteers after intratracheal LPS administration (Boujoukos et al. 1993). Nys et al. measured BALF cytokine levels in VAP patients and showed significantly higher CXCL8 concentrations in the pneumonia group as compared with the two other groups (ARDS and ARDS/Infection free) (Nys et al. 2002). The results of the present study are in line with the above findings.

Attempts have also been made by various investigators to link the intensity of the inflammatory response with the lung bacterial burden. Monton et al. showed no clear relationship between the local lung bacterial burden and the intensity of the inflammatory response in ventilated patients with pneumonia. In this study, which was a study of ventilated patients with severe community-acquired pneumonia and not a study of VAP, cytokine expression appeared independent from the bacterial burden in the presence of antibiotic treatment (Monton et al. 1999).

The opposite was confirmed in non ventilated patients with community-acquired pneumonia where higher CXCL8 concentrations in BAL were found in patients with positive microbiological results (Bohnet et al. 1997). In addition, BAL CXCL8 levels were positively correlated with bacterial load in cystic fibrosis patients (Muhlebach et al. 1999).

Finally elevated serum CXCL8 levels have also been documented in sepsis and multiorgan failure (Friedland et al. 1992; Hack et al. 1992; Marty et al. 1994).

Summarising the above, 3 important points can be drawn from the results of this study so far:

- I. In this study BALF levels of IL-1 β and CXCL8 were significantly elevated in patients with VAP compared with patients without VAP and controls.

Elevated BALF IL-1 β and CXCL8 levels have been demonstrated in previous studies of patients with pneumonia and/or ARDS and our results are in agreement with previous observations. The results of the current study suggest that IL-1 β and CXCL8 are up-regulated in the lungs of patients of VAP, in keeping with the hypothesis that inflammatory signals are increased in VAP.

IL-1 β is stimulated by bacterial products and (along with LPS and TNF- α) will result in activation of NF- κ B (Christman et al. 2000) and will lead to cytokine and chemokine gene expression, including CXCL8. CXCL8 is synthesised not only by alveolar macrophages but also by epithelial and endothelial cells (Becker et al. 1991b; Smith et al. 1991c; Miller et al. 1992;). The maintenance of an CXCL8 dependent chemotactic gradient by both the immune and non-immune cellular constituents of the alveolar capillary membrane will provide a chemotactic signal for an amplified recruitment of neutrophils into the alveolar space. In addition CXCL8 will not only drive neutrophil recruitment but will also upregulate neutrophil adhesion through upregulation of CD11a/CD 18 complex (Constantin et al. 2000) and augment neutrophil activation through enhancement of phagocytosis, superoxide generation and granule release (Baggiolini et al. 1994a).

No inferences can be made regarding the net biological activity of IL-1 β and CXCL8 from this study although it is known that CXCL8 maintains its biological activity in the presence of significant changes in pH and resists mild proteolytic degradation compared with other known chemotactic factors (Strieter et al. 1993). Therefore the above findings are suggestive of a pro-inflammatory response in patients with VAP that aims to stimulate recruitment of phagocytes and phagocyte activation in the battle of host defense against bacterial invasion while if unregulated, can lead to neutrophil-mediated tissue injury through release of proteases.

In addition the significant difference demonstrated between levels in the VAP group compared with the NON VAP group in the present study, is suggestive of a potential discriminative role of IL-1 β and CXCL8 in the diagnosis of VAP which has been repeatedly difficult to diagnose accurately on clinical grounds (Koenig 2006). BALF IL-1 β appeared to be powerful in excluding VAP leading to an approximately 10-fold reduction in post test probability although it was less useful as a positive discriminator. A high BALF CXCL8 increased the post-test probability of VAP being present 5 times while a low concentration decreased the post-test probability 5 times. The above suggests that CXCL8 can increase the likelihood of a correct diagnosis 5-fold and a low IL-1 β can decrease the likelihood by 10 times.

In this study patients with clinically suspected VAP were recruited and a standardised directed BAL technique was used to confirm or exclude VAP. While the rigorous evaluation and standardisation of patient recruitment and BAL procedure constitute strengths of this study, a few important caveats have to be considered with regard to general applicability of the above data:

- a) These data represent sampling from a variety of mixed surgical and medical cases with the exclusion of neurosurgical and cardiothoracic cases. Therefore the above results have to be evaluated in a larger and a non-exclusive cohort of patients. The small number of patients in this study also precluded any meaningful correlation of cytokines with clinical or bacteriological data that may emerge in a larger population study.
- b) These data reflect the state of the pro-inflammatory mediators at the time of the first clinical suspicion. Therefore as inflammation is a dynamic process the discriminative role of IL-1 β and CXCL8 values may be further strengthened or weakened at different time points.
- c) These results were generated using an invasive technique which may be contraindicated in some patients and involves time and expertise that may not be generally available.

- II. BALF levels of IL-1 β and CXCL8 were not only significantly elevated in VAP patients when compared with the NON VAP patients and healthy volunteers but levels in the BALF were much higher than levels in the serum.

IL-1 β is produced early and mainly by leukocytes i.e. monocytes, activated macrophages and peripheral granulocytes but also epithelial, endothelial and vascular smooth cells following stimulation by other cytokines, endotoxins, viruses and antigens (di Giovine and Duff 1990; Brazel et al. 1991). Alveolar macrophages appear to be the major cellular source of CXCL8 in the lungs (Becker et al. 1991; Kunkel et al. 1991; Donnelly et al. 1993), although studies have demonstrated that endothelial cells (Miller et al. 1992), fibroblasts, (Strieter et al. 1989; Takashiba et al. 1992), type II-like epithelial cells, (Smith et al. 1991b) and bronchial epithelial cells (Hack et al. 1992) produce CXCL8. In addition human neutrophils have been shown to produce CXCL8 (Bazzoni et al. 1991; Cassatella et al. 1992).

It is more likely, given low circulating levels of the relevant cytokines, that the source is related to the alveolar epithelium and/or macrophages rather than shedding from the pulmonary circulation. The VAP group appears therefore to display a florid inflammatory response confined to the lung.

- III. BALF IL-1 β and CXCL8 levels were also significantly elevated in the NON VAP group when compared with healthy volunteers.

As mentioned in the previous chapter the NON VAP group of patients demonstrates a high leukocyte count in BALF which is shared between neutrophils and macrophages. This finding is likely to reflect the presence of an inflammatory process at different time points, namely ARDS, and is in keeping with previous studies showing elevated BALF IL-1 β and CXCL8 levels.

2. Other early proinflammatory cytokines namely IL-6 were significantly elevated in BALF and serum in both groups of critically ill patients when compared with the healthy volunteers.

Like CXCL8, IL-6 is produced upon stimulation mainly by TNF- α and IL-1 β and its main sources are activated monocytes, fibroblasts and endothelial cells but also osteoclasts, T-cells and B-lymphocytes, smooth muscle cells, eosinophils, mast cells and glial cells. IL-6 is one of the major physiological mediators of the acute phase response, inducing the synthesis of acute phase proteins by the liver (Akira et al. 1990).

Unlike TNF- α , levels are sustained over time and IL-6 has been demonstrated in large quantities in the vascular compartment under conditions of sepsis and septic shock (Hack et al. 1989; Waage et al. 1989; Dofferhoff et al. 1992; Friedland et al. 1992; Pinsky et al. 1993; Donnelly et al. 1994).

Persistently elevated serum IL-6 concentrations have been described as a characteristic indicator for the development of multiple system organ failure (Pinsky et al. 1993a) and high levels were found to be associated with a poor prognosis in sepsis and septic shock (Hack et al. 1989; Waage et al. 1989; Friedland et al. 1992; Casey et al. 1993; Patel et al. 1994). It is therefore not surprising that IL-6 serum levels have been used to stratify patients in clinical trials for new therapies in sepsis (Damas et al. 1992; Abraham et al. 1997).

The results of this study are in agreement with findings from previous studies. IL-6 levels have been elevated both in the BALF and in serum in ventilated patients with severe pneumonia, (Monton et al. 1999) similar to patients with VAP (Millo et al. 2004). In the present study BALF IL-6 levels were also significantly elevated in the NON VAP group as compared with the healthy volunteers. Results are likely to be attributed to the elevated BALF IL-6 levels in the ARDS group included in the NON VAP group as shown previously in other ARDS studies (Schutte et al. 1996; Donnelly et al. 1996; Kiehl et al. 1998).

Increased levels of serum IL-6 were also described in other studies looking only at the systemic inflammatory response in ventilated patients with pneumonia and ARDS (Bonten et al. 1997; Headley et al. 1997; Bauer et al. 2000) and in patients with community-acquired pneumonia (Glynn et al. 1999; Antunes et al. 2002).

The significance of the above findings in the pathogenesis of sepsis is not yet clear. IL-6 appears to be both a marker and a mediator of sepsis and it is not clear whether the findings represent a consequence or a causative effect in the context of acute inflammation.

3. Other pro inflammatory cytokines e.g. TNF- α were detectable at very low levels in the VAP group and were undetectable in the NON VAP group. A different trend was observed with BALF and serum levels of the anti-inflammatory mediator IL-10, which were higher in the NON VAP group than the VAP group. Similar results were observed with IL-12p70.

TNF- α is a central mediator of the host's response to inflammation (Carswell et al. 1975; Old 1985). It is produced rapidly (Beutler and Cerami 1987; van Deventer et al. 1990) following either antigen-specific or nonspecific stimulation and has been designated an early response or "alarm" cytokine. It is a potent pro-inflammatory cytokine that is produced by activated monocytes, macrophages, neutrophils, endothelial cells, and other immune cells and displays a very short half-life (14–18 min) (Blick et al. 1987).

Production of TNF- α leads to activation of neutrophils and macrophages that will result in protease release, stimulation of respiratory burst and augmentation of leukocyte phagocytic and microbicidal activity (Oswald et al. 1992; Tan et al. 1995). Although not directly chemotactic, TNF- α can mediate neutrophil influx either by stimulating the expression of chemotactic mediators or by inducing the expression of adhesion molecules on the surface of leukocytes and vascular endothelial cells (Le and Vilcek 1987).

TNF- α has been measured in ARDS and pneumonia BALF and levels in previous studies have not always been consistent. In patients with community-acquired pneumonia (Dehoux et al. 1994; Kiehl et al. 1998) and ARDS, elevated alveolar space TNF- α levels were found in some studies although measured values did not predict clinical outcome (Millar et al. 1989; Hyers et al. 1991; Suter et al. 1992; Park et al. 2001). The findings have not always been reproduced in other studies, where TNF- α was rarely detected in BAL fluid either in ARDS or in severe pneumonia (Schutte et al. 1996).

Elevated levels of circulating TNF- α were also observed in the majority of patients with ARDS and/or pneumonia although concentrations were lower than the BALF levels (Hyers et al. 1991; Roten et al. 1991; Donnelly et al. 1994; Schutte et al. 1996; Millo et al. 2004). Plasma TNF- α levels are elevated in septic patients, and the degree of elevation is associated with disease severity and possibly individual genetic variability (Casey 2000; Stuber 2001).

Based on the evidence that excessive and unregulated TNF- α secretion can be detrimental, it is worth mentioning that clinical trials with anti-TNF- α in patients with severe sepsis were conducted. The results were disappointing and the trials were discontinued due to lack of efficacy based on no survival benefit suggesting (as is now well recognized far more complex mechanisms in cytokine regulation in sepsis (Fisher, Jr. et al. 1994; Opal et al. 1997; Abraham et al. 1998; Reinhart et al. 2001).

As mentioned earlier TNF- α levels in previous studies have varied and discrepancies have been acknowledged by previous investigators (Parsons et al. 1992). In this study TNF- α levels were not significantly elevated in any of the groups of critically ill patients. It is likely that our findings reflect differences in the timing of measurement. This can be a very important factor with respect to the short half-life of this cytokine, a problem acknowledged earlier. This view has been strengthened by studies that found elevated TNF- α mainly within the early phase after onset of disease (Hyers et al. 1991; Suter et al. 1992). Early peaks of TNF- α may have been missed in our study due to the timing of recruitment and bronchoalveolar lavage.

IL-12p70 levels were suggestive of a trend towards elevated levels in the NON VAP group compared with the VAP group and controls. However the difference was not significant.

IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits designated p35 and p40. When co-expressed in the same cell, these subunits form the biologically active p70 heterodimer.

IL-12 is produced by monocytes, macrophages, dendritic cells, neutrophils, and to a lesser extent B cells (Gately et al. 1991; Gubler et al. 1991; Wolf et al. 1991; Trinchieri and Scott 1995). A variety of different pathogenic organisms induce high levels of IL-12p70 production, including Gram-positive and Gram-negative bacteria, parasites, viruses and fungi, and microbial products such as LPS and LTA.

The major actions of IL-12 are on T and natural killer (NK) cells. IL-12 induces proliferation, IFN-gamma production, and increased cytotoxic activity of these cells. Importantly, IL-12 induces the polarisation of CD4+ T cells to the Th1 phenotype that mediates immunity against intracellular pathogens (Chehimi and Trinchieri 1994). IL-12, especially in combination with IL-18, also acts on macrophages and dendritic cells to induce IFN-gamma production (Watford et al. 2003).

In vivo studies have demonstrated that IL-12 represents an integral component of innate immunity in bacterial pneumonia. In an animal model of *K. pneumoniae* pneumonia, passive immunisation of mice with anti-IL-12 antibodies resulted in an increase in *K. pneumoniae* isolated from lung homogenates. The *in vivo* neutralisation of IL-12 significantly decreased short and long-term survival in animals challenged with *K. pneumoniae* (Greenberger et al. 1996a).

Studies in human sepsis have shown a defect in the pulmonary and systemic production of IL-12 in septic patients, suggesting a dysregulation of innate immunity during the course of sepsis (Ethuin et al. 2004). In septic patients, *ex vivo* IL-12p70 production by patients' peripheral monocytes and alveolar macrophages after stimulation by LPS and IFN-gamma was impaired (Ethuin et al. 2003). Similar studies of postoperative patients revealed that monocyte IL-12 production was severely and selectively impaired in patients developing postoperative sepsis compared with patients who had uncomplicated recovery. The suppression of IL-12 preceded the onset of sepsis and the extent of monocyte IL-12 suppression correlated with the severity of postoperative sepsis (Hensler et al. 1998).

The results of this study show that IL-12p70 levels were lower in the VAP group although the difference was not statistically significant. One would expect that IL-12p70 production would be up-regulated in the VAP group perhaps facilitating bacterial clearance, however the opposite was observed in the VAP patients, raising the likelihood of monocyte down-regulation.

The possibility of inflammatory down-regulation in the context of critical illness that might precede the development of pneumonia or other infections is the focus of ongoing research among investigators. The concept of immunoparesis in sepsis received more attention recently after the results of the anti-inflammatory therapies failed to show a survival benefit and directed attention to the role of the compensatory anti-inflammatory response syndrome (Bone 1996). Several studies had shown that anti-inflammatory therapies may have been beneficial in patients with septic shock when given early, while in the subsequent weeks and in less severe group of patients the treatment was associated with increased mortality (Eichacker et al. 2002).

Studies using measurement of HLA-DR expression in peripheral monocytes as a marker of global immune function, demonstrated not only low levels of HLA-DR expression in patients who subsequently developed nosocomial infections but also that low levels of HLA-DR expression were an independent predictor of septic complications (Allen et al. 2002; Muehlstedt et al. 2002; Finck et al. 2003; Le et al. 2004). It has been shown that in patients with sepsis, trauma, or pancreatitis, HLA-DR expression decreases in the first few days after admission to the intensive care unit, a change that does not discriminate survivors and non survivors at this early stage (Hynninen et al. 2003; Lekkou et al. 2004). However persistence of low HLA-DR expression in monocytes at 5 to 7 days after septic shock was associated with increased mortality (Finck et al. 2003). It is also of interest that in survivors, while HLA-DR levels would normalise within a week, recovery of HLA-DR expression appears to be blunted by subsequent nosocomial infections, increasing the risk of further episodes of sepsis (Flohe et al. 2004).

Finally and in keeping with the concept of a compensatory anti-inflammatory response, it has been increasingly recognised that another process characterised by increased synthesis of anti-inflammatory molecules such as IL-10 and IL-1RA coexists along with the upregulation and enhanced cellular production of pro-inflammatory mediators, that aims to modulate the effect of the inflammatory process.

In this study a contrasting trend was noted for BALF IL-10, levels being lower in the VAP group compared with the NON VAP group.

IL-10 is a counter-regulatory cytokine produced by activated T helper cells, B cells, monocytes and macrophages. IL-10 greatly reduces production of TNF- α , IL-1 β , IL-6, and CXCL8 by monocytes while up-regulating production of IL-1ra thus promoting the development of Th2-type immune responses while inhibiting the cell-mediated (Th1-type) immune response (Fiorentino et al. 1991; Howard and O'Garra 1992; Ramani et al. 1993; Cassatella et al. 1994; Van der Poll et al. 1996b; Van der Poll et al. 1996a; Van Der Poll et al. 1996).

A major stimulus for the production of IL-10 is inflammation itself, as IL-1 β and TNF- α can stimulate IL-10 production directly, suggesting the existence of a negative feedback loop, whereby inflammatory processes are self-limited by the endogenous production of IL-10 (Van Der Poll et al. 1994).

Several studies have characterised the IL-10 response in patients with sepsis syndromes (Marchant et al. 1994; Lehmann et al. 1995; Marchant et al. 1995). These studies showed that IL-10 concentrations in the blood, while varying greatly, were higher in patients with septic shock than in patients with sepsis and correlated strongly with circulating TNF- α levels (Lehmann et al. 1995; Marchant et al. 1995). In other studies IL-10 concentrations correlated with APACHE scores and predicted the prevalence and severity of multiple-system organ failure (Friedman et al. 1997). Similarly non-survivors or patients who developed multiple organ failure in a trauma population had higher IL-10 levels than survivors or patients with an uncomplicated stay (Ertel et al. 1997; Neidhardt et al. 1997).

Circulating and/or BALF IL-10 levels have been studied previously in patients with pneumonia and ARDS. In patients with community-acquired pneumonia, circulating levels of IL-10 were higher and correlated strongly with levels of IL-6 (Glynn et al. 1999), similar to studies in paediatric sepsis (Doughty et al. 1996). In another similar study, the highest risk of death in patients with pneumonia was associated with combined high levels of pro-inflammatory IL-6 and anti-inflammatory IL-10 cytokine activity (Kellum et al. 2007).

In ARDS patients circulating levels of IL-10 were significantly higher in non survivors compared with survivors (Parsons et al. 1997), although the opposite was found in studies looking at BALF levels in ARDS where non survivors demonstrated lower levels of IL-10 in the BALF fluid compared with the survivors (Donnelly et al. 1996). However in the latter study there was only one time point as opposed to serial time points of sampling in the former study that may partly explain the discrepancy. Taken together, these findings suggest that the magnitude of the endogenous IL-10 response correlates with both the severity of the inflammatory insult and the plasma concentrations of pro-inflammatory cytokines such as TNF- α .

In this study BALF IL-10 levels were not significantly different between the 2 groups of critically ill patients although a trend towards higher levels in the NON VAP group was noted, arising more likely from the higher BALF IL-10 levels in the ARDS subgroup. Circulating levels of IL-10 were significantly elevated in both groups of critically ill patients when compared with the healthy volunteers. No significant difference was observed between the two groups of critically ill patients, perhaps due to small number of patients included. These results may reflect the attempted host response to self-limit inflammation within or outwith the context of infection by upregulating anti-inflammatory mediators.

4. Finally BALF and circulating MCP-1/CCL2 levels were significantly elevated in both groups of patients when compared with levels in HV.

There was a trend towards higher BALF MCP-1/CCL2 levels in the NON VAP group as compared with the VAP group, although the difference was not significant. A contrasting trend was observed in circulating MCP-1/CCL2 levels with higher (but non-significant) MCP-1/CCL2 levels in the VAP group compared with the NON VAP group.

MCP-1/CCL2 (also named chemokine ligand CCL2), belongs to the CC family of chemokines and is the most thoroughly studied chemokine of the group. The CC group is the largest of the four groups of chemokines and it is named after the first two of four cysteine residues that are adjacent to each other (Charo and Ransohoff 2006).

MCP-1/CCL2 is produced by a variety of cells including alveolar macrophages and remains a potent chemoattractant for monocytes, dendritic cells, basophils, eosinophils and memory T cells suggesting a role in regulation of the inflammatory response (Leonard and Yoshimura 1990; Brieland et al. 1992; Jones et al. 1992b; Daly and Rollins 2003).

During bacterial infection monocytes and macrophages infiltrate into the infection site after neutrophil recruitment. MCP-1/CCL2 is produced during infection and is presumed to have a pivotal role in monocyte/macrophage recruitment and activation (Jansen et al. 1995; Olszyna et al. 2001). Recruited and activated monocytes at the site of infection are expected to enhance further bacterial clearance and resolution of inflammation through clearance of apoptotic neutrophils.

It was therefore hypothesised that MCP-1/CCL2 will confer a protective role. Indeed, *in vivo* studies of pneumonia have shown a protective role of MCP-1/CCL2 that is not however consistent across pneumonia models employing different pathogens. For example, *in vivo* studies of *P. aeruginosa* pneumonia have demonstrated that MCP-1/CCL2 is protective against systemic lethal infection when administered 6 hours prior to the infection (Nakano et al. 1994). Similarly studies of *Listeria monocytogenes* pneumonia demonstrated that mice deficient in CCR2 (MCP-1 receptor) exhibited defective macrophage recruitment and bacterial clearance (Kurihara et al. 1997).

This was not repeated in a pneumococcal pneumonia model where the host response was unchanged in MCP-1/CCL2 deficient mice (Dessing et al. 2006). Therefore it is likely that the protective mechanism demonstrated *in vivo* is pathogen-specific which may be relevant in patients with pneumonia.

Furthermore an anti-inflammatory role of MCP-1/CCL2 has been anticipated. In acute endotoxaemia in mice, i.v. administration of MCP-1/CCL2 reduced plasma levels of TNF- α and improved survival rates in association with increased IL-10 production. In the same studies augmented TNF- α production reduced IL-10 production and decreased survival following blockade of MCP-1/CCL2 suggesting that MCP-1/CCL2 may under special circumstances have anti-inflammatory effects (Zisman et al. 1997). In addition, in an *in vivo* model of *P. aeruginosa* pneumonia, administration of anti-MCP-1/CCL2 antibodies resulted in enhanced lung injury without influencing the clearance of *P. aeruginosa*. In contrast administration of MCP-1/CCL2 increased the number of alveolar macrophages ingesting neutrophils, eventually attenuating lung tissue injury (Amano et al. 2004).

However this anti-inflammatory role has been challenged: in an IgA immune complex-induced lung injury model, blockade of MCP-1/CCL2 significantly reduced the extent of lung tissue injury suggesting that MCP-1/CCL2 may further activate alveolar macrophages (Jones et al. 1992a). In intravenous glucan-induced pulmonary granulomatous vasculitis, anti-MCP-1/CCL2 reduced the inflammatory reaction (Jones and Warren 1992). In IgG immune complex-induced lung injury MCP-1/CCL2 was shown to be up-regulated in lung shortly after the deposition of IgG immune complexes. However administration of anti-MCP-1/CCL2 had no effect on pulmonary neutrophil recruitment or the extent of lung injury (Bless et al. 2000).

The above findings reflect the attention that has recently been directed at the role of MCP-1/CCL2 in the balance between monocyte recruitment and subsequent monocyte activation. It is now recognised that alveolar macrophages are one main source of endogenous ROS and that generation of ROS/reactive nitrogen species (RNS) is involved in many signaling pathways including those regulating cell growth and proliferation, cell survival and inflammation via the NF- κ B pathway (Schreck et al. 1991; Gwinn and Vallyathan 2006).

The current study found increased MCP-1/CCL2 levels in BALF of both groups of critically ill patients with a trend towards higher levels in the NON VAP group, arising more likely from the ARDS subgroup. Only a few investigators have studied MCP-1/CCL2 in septic patients. Goodman et al. showed significantly increased levels of MCP-1/CCL2 in BALF of patients with ARDS, that were directly correlated with lung injury score on Days 7, 14, and 21 (Goodman et al. 1996). Serum MCP-1/CCL2 levels have been found to be elevated in patients with sepsis and septic shock and higher levels were measured in patients with more severe sepsis (Bossink et al. 1995). More recently, it was reported that serum concentrations of MCP-1 in patients with meningococcal sepsis were predictive of mortality and correlated strongly with disease (Vermont et al. 2006).

In another recent study of severe sepsis, circulating MCP-1/CCL2 levels showed good accuracy for predicting early (< 48 hours) and late (at 28 days) mortality. In the same study, in multivariate analysis, MCP-1/CCL2 was independently associated with prognosis (Bozza et al. 2007).

In this study, serum MCP-1/CCL2 levels were elevated in both groups of critically ill patients with a trend towards higher levels among the VAP patients compared with the NON VAP patients. These findings are likely to represent the intensity of the host's response to infection and systemic inflammation in the context of critical illness.

In summary elevated circulating levels of pro-inflammatory cytokines in all ventilated patients support the concept of a major systemic inflammatory response in the context of critical illness and /or mechanical ventilation itself although the pathogenic significance of the above findings remains to be answered.

The elevated levels of the two early pro-inflammatory mediators, IL-1 β and CXCL8, in the BALF of patients with pneumonia may reflect the degree of the inflammatory activity in the lung. These data have implications for both diagnosis and understanding of the biology of the disease. The above findings can potentially be useful as diagnostic discriminators of pneumonia in ventilated patients although they have to be validated in a wider population.

While CXCL8 and IL-1 β levels were elevated in patients without infection reflecting an inflammatory response driven by the nature of the critical illness and perhaps mechanical ventilation, the profound difference between the VAP and the NON VAP group is suggestive of an exaggerated and compartmentalised response that appears to be VAP specific. The above findings in association with a trend towards a higher concentration of neutrophils in the VAP group indicate an inflammatory process that appears to be characterised by a pro-inflammatory reprogramming of the inflammatory cascade in mechanically ventilated patients who develop pneumonia. Inflammation in the setting of pulmonary infection is a complex and dynamic process that requires the balanced expression of both pro-inflammatory and anti-inflammatory mediators.

While this initial response aims to recruit phagocytes in the battle of the host defense against bacterial invasion, it is well recognised that unregulated recruitment can lead to neutrophil-mediated tissue injury through release of proteases.

In addition, results from this study suggest that along with neutrophil recruitment, MCP-1/CCL2 driven monocyte recruitment is enhanced in both groups of critically ill patients. One can also speculate that the trend towards higher MCP-1/CCL2 levels in the BALF of patients in the NON VAP group (and more specifically of patients with ARDS) suggests not only with enhanced monocyte recruitment but also with activation of alveolar macrophages that can perhaps perpetuate the ongoing inflammation and lung injury. Evidence has emerged from models of lung injury that outwith pneumonia models and the protective role of MCP-1/CCL2 in the setting of infection, monocyte activation may have a damaging effect enhancing tissue injury.

Strands of evidence from this chapter and the one preceding it begin to support the idea that the inflammatory response may be exaggerated in the lungs of mechanically ventilated patients who acquire pneumonia. In particular, IL-1 β and CXCL8 are up-regulated in the alveolar space and a non-significant trend towards greater numbers of neutrophils in the lung has been identified. The original hypothesis contended that activated neutrophils in the lung may be damaging in VAP as a consequence of a relative deficiency of inhibitors of HNE. The expression of protease inhibitors as a compensatory mechanism to limit tissue damage will therefore be discussed in the following section.

Neutrophils in the lung in response to either bacterial stimuli, they are probably dependent on increased PMN at the neutrophil surface (metalloproteinase) to target neutrophils, produced by the liver and which is thought to diffuse into the lung passively via the circulation. In addition, the lung's 'natural' capacity to remove HNE was quantified by testing the capacity of the BALF to inhibit HNE activity in CIA assay. Finally, the level of HNE antigens in BALF was measured. The starting hypothesis was that the 'locally' available HNE inhibitors would be down-regulated in VAP.

3.1.1.1. HNE

Median HNE concentration in BALF from patients with VAP was 11.41 ng/ml (IQR: 4.5-15.8), mean concentration in the NON VAP group was 5.25 ng/ml (IQR: 2.6-11.45) and 2.37 ng/ml (IQR: 1.0-3.7) in the BALF of healthy volunteers (Table 3.1).

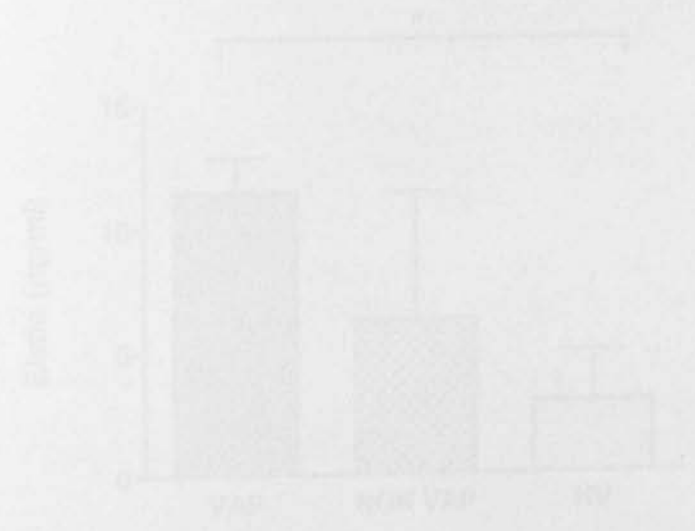


Figure 3.1. Concentration of HNE in BALF of all groups. Data are expressed as mean values and SD. p -value = 0.02 by Kruskal-Wallis test. $p < 0.05$ by Dunnett's post-hoc test.

4 PROTEASE INHIBITORS

In the initial hypothesis it was anticipated that along with the presence of a pro-inflammatory profile in patients with VAP, an impaired local antiprotease/antimicrobial expression will also co-exist, allowing or facilitating neutrophil-mediated tissue injury. This hypothesis was tested with quantification of the major species of endogenous HNE inhibitors in the lung. These broadly comprise elafin and SLPI (low molecular weight cationic inhibitors of HNE which harbour antimicrobial activity and which are produced locally in the lung in response to inflammatory stimuli; they are probably capable of accessing HNE at the neutrophil surface) and alpha1-antitrypsin (a larger molecule produced by the liver and which is thought to diffuse into the lung passively via the circulation). In addition the lung's 'global' capacity to neutralise HNE was quantified by testing the capacity of the BALF to inhibit HNE using an EIA assay. Finally, the level of HNE antigen in BALF was measured. The starting hypothesis was that the 'locally responsive' HNE inhibitors would be down-regulated in VAP.

4.1 Elafin

Median elafin concentration in BALF from patients with VAP was 11.45 ng/ml (IQR: 6.4-12.9), mean concentration in the NON VAP group was 6.25 ng/ml (IQR: 1.6-11.65) and 2.89 ng/ml (IQR: 1.4-4.9) in the BALF of healthy volunteers (Figure 43).

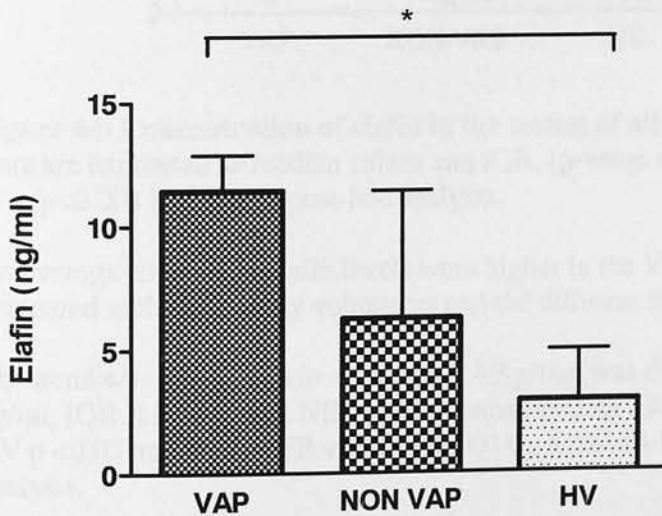


Figure 43: Concentration of elafin in BALF of all groups.

Data are expressed as median values and IQR, (p -value = 0.02 by Kruskal-Wallis test),

* = $p < 0.05$ by Dunn's post-hoc analysis.

Levels of elafin in the BALF of patients with VAP were significantly higher when compared with levels measured in the BALF of healthy volunteers.

The trend was the same when the non-VAP group was divided into ARDS (median 7.1 ng/ml, IQR: 2-12) and NEITHER group (median 3.0 ng/ml, IQR: 1-11): VAP v HV $p < 0.001$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

Elafin levels in the serum were 27.18 pg/ml (IQR: 16.0-113.1) in the VAP group, 34.96 pg/ml (IQR: 16.8-63.6) in the NON VAP group and 12.98 pg/ml (IQR: 9.6-13.8) in the healthy volunteers (Figure 44).

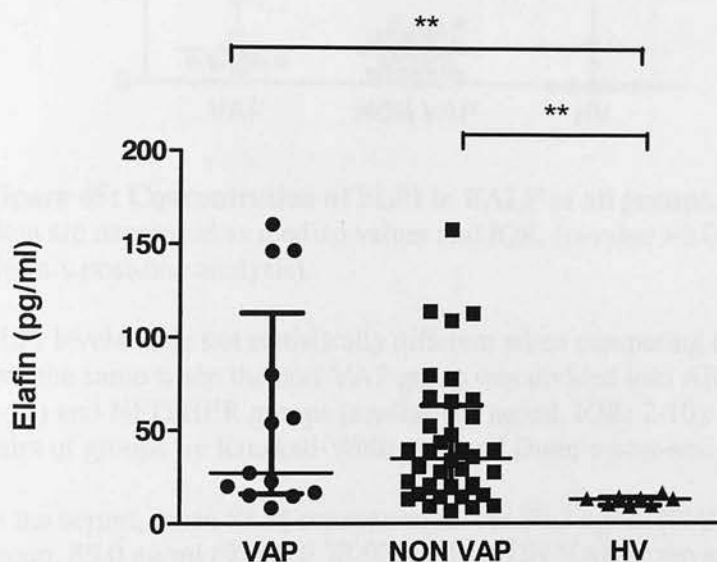


Figure 44: Concentration of elafin in the serum of all groups.

Data are expressed as median values and IQR, (p -value = 0.002 by Kruskal-Wallis test), ** = $p < 0.001$ by Dunn's post-hoc analysis.

On average circulating elafin levels were higher in the VAP and the NON VAP group compared with the healthy volunteers and the differences were statistically significant.

The trend was the same when the non-VAP group was divided into ARDS (median 28.58 ng/ml, IQR: 14.0-50) and NEITHER groups (median 39.58 ng/ml, IQR: 19-70): VAP v HV $p < 0.05$ and NEITHER v HV $p < 0.001$ by Kruskal-Wallis test and Dunn's post-hoc analysis.

4.2 SLPI

SLPI levels (median and IQR) in the BALF of patients with VAP were 7.1 ng/ml (4.3-19.9), whilst SLPI levels in the NON VAP group and the healthy volunteers were 6.5 ng/ml (2.1-11.1) and 8.2 ng/ml (0.0-52.2) respectively (Figure 45).

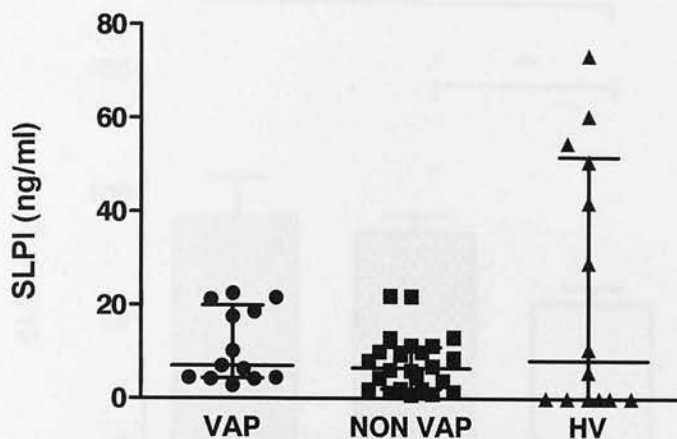


Figure 45: Concentration of SLPI in BALF of all groups.

Data are expressed as median values and IQR, (p-value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

SLPI levels were not statistically different when comparing the study groups. The trend was the same when the non-VAP group was divided into ARDS (median 7.0 ng/ml, IQR: 2-12) and NEITHER groups (median 6.6 ng/ml, IQR: 2-10): p >0.05 in comparison of all pairs of groups by Kruskal-Wallis test and Dunn`s post-hoc analysis.

In the serum, mean SLPI concentration was 90.7 ng/ml (95%CI: 74-107) in the VAP group, 85.0 ng/ml (95%CI: 78-92) in the NON VAP group and 56.7 ng/ml (95%CI: 50-63) in the healthy volunteers (Figure 46).

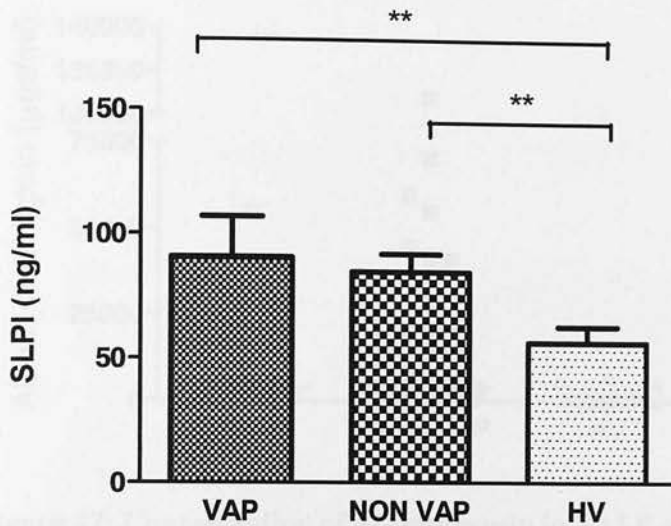


Figure 46: Concentration of SLPI in the serum of all groups.

Data are expressed as mean values and 95%CI, (p-value = 0.001 by One-Way ANOVA test), ** = p<0.001 by Bonferroni's post-hoc analysis.

Circulating SLPI levels were significantly higher in both groups of critically ill patients compared with the healthy volunteer group.

The trend was the same when the non-VAP group was divided into ARDS (median 92.2 ng/ml, IQR: 64-97) and NEITHER group (median 83.3 ng/ml, IQR: 68-98): VAP v HV p <0.001, ARDS v HV p< 0.05 and NEITHER v HV p< 0.001 by Kruskal-Wallis test and Dunn's post-hoc analysis.

4.3 Alpha1-antitrypsin

Alpha1-antitrypsin levels in the BALF of patients with VAP were 3743 µg/ml (IQR: 2042-5067), 3896 µg/ml (IQR: 977-21052) in the NON VAP group and 669 µg/ml (IQR: 362-1903) in the healthy volunteer group (Figure 47).

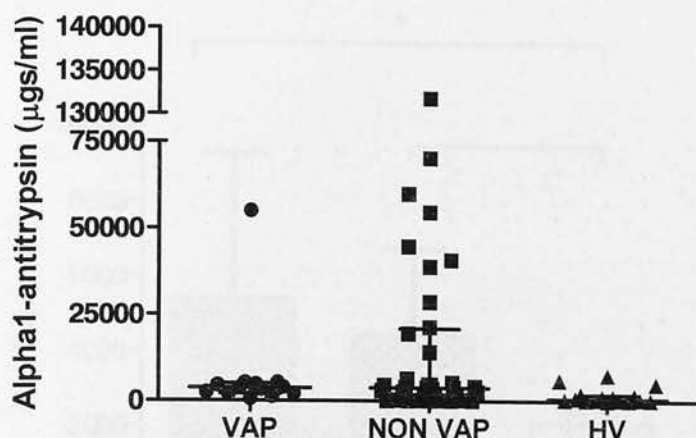


Figure 47: Concentration of α 1-antitrypsin in BALF of all groups.

Data are expressed as median values and IQR, (p-value = 0.05 by Kruskal-Wallis test), p>0.05 by Dunn`s post-hoc analysis.

BALF α 1-antitrypsin levels were higher in the VAP group and in the NON VAP group although this did not reach statistical significance when compared with levels in the healthy volunteers.

When the non-VAP group was divided into ARDS (median 16918 μ g/ml, IQR: 3716-44533) and NEITHER groups (median 1523 μ g/ml, IQR: 151-4989) there was statistical difference between the ARDS group and the NEITHER group (p <0.001) and between the ARDS and HV groups p< 0.0001 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

Alpha1-antitrypsin levels in the serum were 5399 μ g/ml (IQR: 2560-9297) in the VAP group, 4393 μ g/ml (IQR: 3023-6736) in the NON VAP group and 2055 μ g/ml (IQR: 1695-2336) in the healthy volunteers group (Figure 48).

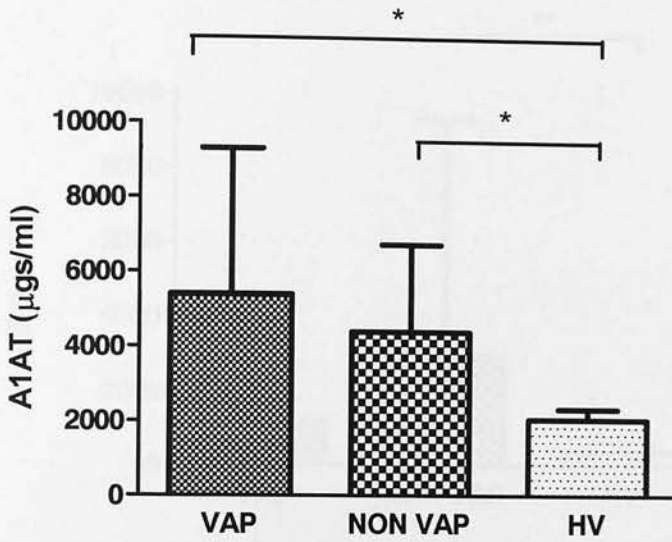


Figure 48: Concentration of α 1-antitrypsin in the serum of all groups.

Data are expressed as median values and IQR, (p-value = 0.01 by Kruskal-Wallis test), * = p<0.05 by Dunn`s post-hoc analysis.

Serum alpha 1-antitrypsin levels were significantly higher in the VAP group and the NON VAP group compared with the healthy volunteers.

The trend was similar when the non-VAP group was divided into ARDS (median 3970 μ g/ml, IQR: 1876-5619) and NEITHER groups (median 4985 μ g/ml, IQR: 3501-7348): VAP v HV p <0.001 and NEITHER v HV p< 0.001 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

4.4 Elastase inhibitory activity (EIA)

The capacity of BALF to inhibit neutrophil elastase was also measured and expressed as EIA in nM. Median EIA in the VAP group was 1221 nM (IQR: 415-1765), 2959 nM (IQR: 1004-9361) in the NON VAP group and 381 nM (IQR: 144-642) in the BALF of healthy volunteers (Figure 49).

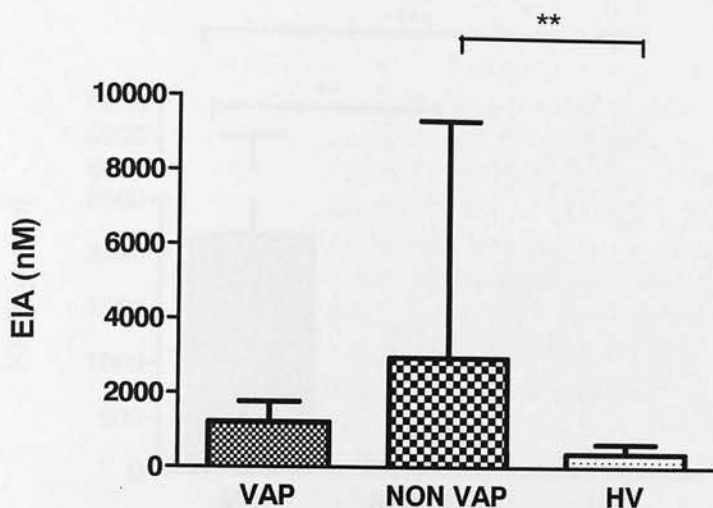


Figure 49: Elastase inhibitory activity in BALF of all groups.

Data are expressed as median values and IQR, (p -value = 0.001 by Kruskal-Wallis test), * $p < 0.001$ by Dunn`s post-hoc analysis.

There was no significant difference between the two groups of critically ill patients but the EIA in the BALF of patients in the NON VAP group was significantly higher when compared with healthy volunteers.

Similar trends were noted when the non-VAP group was divided into ARDS (median 5156 nM, IQR: 2147-15056) and NEITHER groups (median 1160 nM, IQR: 368-4736): ARDS v HV $p < 0.0001$; VAP v ARDS $p < 0.05$ by Kruskal-Wallis test and Dunn`s post-hoc analysis.

4.5 Human Neutrophil Elastase

Median concentration of HNE (and IQR) in BALF was 2180.0 pg/ml (558-6151) in the VAP group, 121.8 pg/ml (43-355) in the NON VAP group and 21.1 pg/ml (2-109) in the healthy volunteers group (Figure 50).

HNE was significantly higher in the BALF of patients with VAP compared with NON VAP patients and healthy volunteers. Similar trends were noted when the non-VAP group was divided into ARDS (median 90.8 pg/ml, IQR: 42-352) and NEITHER group (median 142.7 pg/ml (IQR: 43-637): VAP v ARDS $p < 0.05$ and between VAP and HV $p < 0.001$ by Kruskal-Wallis test and Dunn`s post-hoc analysis.

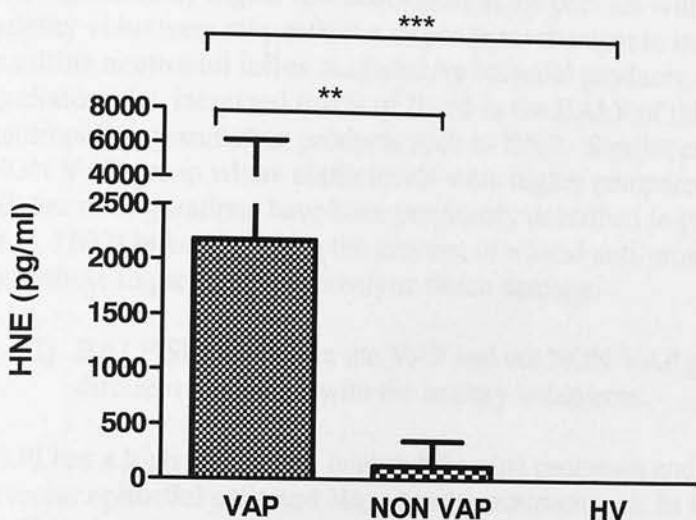


Figure 50: HNE in BALF of all groups.

Data are expressed as median values and IQR, (p-value = 0.001 by Kruskal-Wallis test, ** p< 0.001, *** P<0.0001 by Dunn`s post-hoc analysis), data courtesy of Dr A Conway Morris.

4.6 Discussion

The main observations from the above measurements of anti-elastase antigen and activity are:

- 1) elafin levels were 2-times higher in the BALF of patients with VAP compared with patients without VAP and 3-times higher in the VAP group compared with healthy volunteers. The difference between the VAP group and the healthy volunteers was statistically significant.

It is known that elafin is secreted from alveolar type II epithelial cells and alveolar macrophages (Sallenave et al. 1993; Mihaila and Tremblay 2001) and its expression is up-regulated not only by neutrophil elastase but also in the early phases of the inflammatory response by IL-1 β and TNF- α (Sallenave et al. 1994; Tanaka et al. 2000b). Elafin has a limited spectrum of protease inhibition since its main targets are HNE and neutrophil proteinase 3 (Tremblay et al. 2000), suggesting that the primary function is to protect tissues from neutrophil-mediated injury as a result of neutrophil activation and protease release.

The significantly higher levels of elafin in the patients with VAP compared with the healthy volunteers may reflect a response mechanism in the context of infection and the resulting neutrophil influx mediated by bacterial products, circulating pro-inflammatory mediators (i.e. increased levels of IL-1 β in the BALF of this group of patients) or neutrophil degranulation products such as HNE. Similar explanations may apply for the NON VAP group where elafin levels were higher compared with the healthy volunteers. Higher concentrations have been previously described in patients with ARDS (Sallenave et al. 1997) in keeping with the concept of a local anti-protease shield to which elafin can contribute in preventing proteolytic tissue damage.

- 2) BALF SLPI levels in the VAP and the NON VAP group were not significantly different compared with the healthy volunteers.

SLPI has a high affinity for neutrophil serine proteases and is secreted by bronchial and alveolar epithelial cells and also alveolar macrophages. In keeping with elafin regulation, SLPI is similarly upregulated by neutrophil elastase and alarm signals such as LPS, TNF- α and IL-1 β (Sallenave et al. 1993; Sallenave et al. 1997).

In this study SLPI followed a different pattern from elafin with levels not higher in BALF of both groups of critically ill patients compared with healthy volunteers. In previous studies SLPI levels in the BALF of patients with ARDS were increased compared with healthy controls (Sallenave et al. 1999). Elevated levels were also found in patients with community-acquired pneumonia compared with healthy controls (Greene et al. 2003). The above findings have not been confirmed in this study. Patients in the VAP group were recruited and bronchoalveolar sampling was performed as soon as new radiological changes were identified, whilst in the previous pneumonia study anti-proteases were studied on average at day 6 ± 0.8 . The variation in time of sampling may account partially for the discrepancy.

Down-regulation of SLPI expression and/or secretion could offer another explanation for the trend observed in the mechanically ventilated patients. It has now been demonstrated that SLPI enters cells, and is rapidly localised to the cytoplasm and nucleus where it exerts its anti-inflammatory properties on NF- κ B activation (Taggart et al. 2005). Therefore the lack of higher concentration of SLPI levels in the BALF of critically ill patients may not necessarily represent attenuated expression, but may purely reflect the amount of free SLPI as opposed to intracellular component.

- 3) There was a trend towards higher levels of BALF α 1-antitrypsin in the VAP and the NON VAP group compared with the healthy volunteers. Levels were significantly higher in the ARDS group compared with the NEITHER group and the healthy volunteers.

In the BALF of the healthy volunteers, α 1-antitrypsin is represented with the highest concentration (669 μ g/ml) among the three protease inhibitors, with SLPI second (8.2 ng/ml) and elafin third (2.9 ng/ml). This is in agreement with previous studies of healthy volunteers (Tremblay et al. 1996).

Alpha1-antitrypsin mainly reaches the alveolar airspace by diffusion from the systemic and pulmonary circulation through the alveolar-capillary membrane. During inflammation serum levels can increase 3- to 4-fold and this increase has been attributed to upregulated production by hepatocytes, giving α 1-antitrypsin properties of an “hepatic acute phase reactant”.

Diffusion of α 1-antitrypsin from the systemic circulation can explain the huge amount of α 1-antitrypsin compared with SLPI and elafin found in this study. However it has been shown that SLPI can enter the nucleus of monocytes and macrophages and therefore the differences in SLPI concentrations in the nucleus of these cell populations are likely to influence the SLPI concentration in the surrounding environment (Taggart et al. 2005). The elafin molecule can also anchor to the extracellular matrix through transglutamination via the NH₂ domain (Guyot et al. 2005). It is likely that the free amount of SLPI and elafin as opposed to the huge excess of α 1-antitrypsin represent a percentage of the expressed molecule that is secreted.

Finally the presence α 1-antitrypsin in higher levels in critically ill patients may represent purely increased production by the hepatocytes as a host response to inflammation. However α 1-antitrypsin can be secreted by inflammatory cells (neutrophils, alveolar macrophages and blood monocytes), although in substantially lower contribution compared to the hepatocytes, and further augmentation of local production in the context of the acute inflammatory response may provide another contributing protective mechanism against proteolytic damage.

- 4) HNE was significantly higher in the VAP group compared with the NON VAP group and the healthy volunteers. EIA displayed a trend towards lower NE inhibition in BALF of patients with pneumonia compared with the NON VAP group although the differences were not statistically significant.

The overall activity of the bronchoalveolar fluid against NE appears to be decreased in patients with VAP suggesting that perhaps the inhibitory properties of the protease inhibitors may be compromised. This is in keeping with studies of antiprotease activity in community-acquired pneumonia (Greene et al. 2003) and ARDS (Sallenave et al. 1999).

The presence of free HNE in the BALF poses an important caveat. HNE was found in significantly higher concentration in the BALF of VAP patients compared with NON VAP patients and healthy volunteers. The implication is that neutrophils were more activated in patients with VAP.

While the excess of alpha1-antitrypsin in BALF may potentially be sufficient to neutralise the excess of HNE identified in VAP, it remains distinctly possible that HNE can produce damage at tissue/substrate level, where alpha1-antitrypsin is less capable of neutralising the enzyme (Owen et al. 1999).

Thus it appears that the original hypothesis is partly supported, but may require modification – the data presented suggest that neutrophils may indeed be more activated in VAP (as evidenced by significantly higher levels of extracellular HNE), but local low molecular weight inhibitors of HNE do not appear to be down-regulated. Nevertheless, the net effect of a lower threshold for HNE-mediated tissue damage does appear to exist.

- 5) Circulating elafin, SLPI and α 1-antitrypsin concentrations followed similar pattern with levels in the serum of both critically ill patients groups significantly higher than the levels measured in the serum of healthy individuals.

Alpha 1-antitrypsin production is known to be upregulated by hepatocytes during acute inflammation and this alone could explain the elevated levels in the serum of patients with critical illness compared with healthy controls.

SLPI levels have been found to be elevated in the serum of patients with pneumonia (Kida et al. 1992) but also in patients with sepsis (Grobmyer et al. 2000). In the latter group of patients SLPI levels correlated well with maximal multiple organ dysfunction scores. In the same study, administration of LPS to normal subjects led to an elevation in serum SLPI extending findings already demonstrated by *in vivo* work (Tanaka et al. 2000b). It has been anticipated that increased levels in the serum reflect increased production along with perhaps decreased metabolism (Alkemade et al. 1995).

In this study SLPI levels were elevated in the serum of both groups of critically ill patients suggesting that serum SLPI is not purely related to pulmonary infection but instead appears to be associated with a major systemic inflammatory response activated by diverse triggers ranging from trauma to intra-abdominal sepsis and pneumonia.

While it remains unclear if serum SLPI levels are sepsis-specific, one can however anticipate that high circulating SLPI levels in patients with critical illness result as part of the host response to control the systemic inflammatory process and to contain neutrophil mediated injury. This appears to be more plausible for a molecule with antimicrobial properties along with the ability to limit protease-release damage, than the opposite suggestion that SLPI acts as a purely anti-inflammatory mediator, associated with worsening organ dysfunction and tissue injury.

Circulating elafin levels in humans have been studied more extensively in patients with skin disorders and much less so in human sepsis. In generalised pustular psoriasis, serum elafin levels have been demonstrated to be elevated and possibly dependent on the extent of the involved area (Stephan et al. 2002; Kaufmann et al. 2006). Serum levels of elafin have been proposed as markers of disease activity in severe psoriasis, during treatment (Tomlinson 1993; Miletic and Frank 1995).

More recently circulating elafin levels were measured in ARDS population (at risk and established) and compared with ICU patients (at risk who did not develop ARDS) and healthy controls (Wang et al. 2009). Circulating elafin levels were significantly higher in all groups compared with controls in agreement with the results shown above.

Furthermore Wang et al. demonstrated that at the onset of ARDS, the ARDS patients had significantly higher HNE but lower PI3 resulting in increased HNE/PI3 ratio compared with patients at risk who did not develop ARDS (who had a higher but not statistically significant HNE/PI3 ratio than that of healthy individuals), suggesting that the balance between circulating elafin levels and HNE may be related to the risk of ARDS development.

In this study, serum levels of elafin were significantly elevated in both groups of critically ill patients compared with the healthy volunteers. The exact biological effect of the circulating elafin remains unclear and association between severity of illness or degree of organ dysfunction is not available in the current literature. It is likely that, in keeping with the high levels of circulating SLPI, increased production results in high circulating elafin levels in the setting of the human response to systemic inflammation, although its role remains to be elucidated.

In summary concentration of pulmonary protease inhibitors appears to follow 2 different patterns with the first one demonstrating higher levels of elafin and α 1-antitrypsin in the critically ill patients compared with the healthy volunteers although one can speculate that elafin may be inactivated, and the second one displaying no difference in SLPI levels in patients with critical illness compared with the healthy individuals.

Despite overall increase in anti-proteases (elafin and α 1-antitrypsin), activity against NE is impaired in these patients as a result of a possible multifactorial process. Inactivation of anti-proteases can result in the breakdown of the anti-protease shield raised by the host defence, allowing or facilitating neutrophil mediated pulmonary inflammation and tissue injury. The latter is also supported by the excess HNE present in significantly higher levels in BALF of VAP patients as compared with NON VAP patients and healthy volunteers.

Finally it is worth remembering that antimicrobial and anti-inflammatory activity has been demonstrated for both elafin and SLPI. Both protease inhibitors share antimicrobial activity against Gram-negative and Gram-positive bacteria and are now believed to play an important role in the mucosal host defence system (Simpson et al. 1999; Simpson et al. 2001; Meyer-Hoffert et al. 2003; McMichael et al. 2005). Various studies have also demonstrated that elafin and SLPI display anti-inflammatory properties in disorders such as lung emphysema, atherosclerosis and myocardial infarction via a mechanism that interferes with NF- κ B activation (Tremblay et al. 2002; Henriksen et al. 2004b; Taggart et al. 2005; Butler et al. 2006).

One can therefore speculate that down-regulation and /or inactivation of these molecules, if present, may be relevant in patients with critical illness and/or VAP contributing to an impaired anti-protease and anti-inflammatory shield in the presence of neutrophilia and a florid pro-inflammatory response as described in previous sections.

Results of this chapter did not reveal any VAP-specific differences in antiprotease activity. However, I did find significantly elevated levels of extracellular HNE in BALF, consistent with excessive neutrophil activation.

With this in mind it is particularly important to assess whether neutrophils in VAP are functionally defective. This is the focus of the next chapter.

The phagocytic capacity of peripheral blood neutrophils in VAP was assessed with fluorescent latex particles. The phagocytic capacity of neutrophils was assessed by counting the number of fluorescent latex particles (FLPs) ingested by neutrophils. The phagocytic capacity of peripheral blood neutrophils (PBNe) was studied using the systematic phagocytosis assay [200].

In brief, PBNe prepared in heparinized citrate were incubated with latex particles. Phagocytic capacity was quantified by counting number of FLPs with γ H2A antibody against FLPs (the "positive") and number of FLPs with γ H2B antibody against latex particles (the "negative"). Phagocytic capacity was expressed as the percentage of "positive" cells which ingest one or more latex particles per cent of total neutrophils.

The average phagocytic capacity in the VAP group was 25% (95% CI: 23-46), in the NON VAP group 42% (95% CI: 37-47) and in the HV group 71% (95% CI: 67-74) (Figure 41).



Figure 41: Phagocytic capacity of peripheral blood neutrophils. Data are expressed as mean values and 95% CI, and as percentage of cells with a fluorescent latex particle (positive) and without (negative) (One-Way ANOVA test, $p < 0.001$ VAP vs NON VAP, and $p < 0.001$ NON VAP vs HV).

There was no difference in the phagocytic capacity of neutrophils in VAP (25%, 95% CI: 23-46) and NON VAP (42%, 95% CI: 37-47) (VAP vs NON VAP, $p < 0.001$ by One-Way ANOVA test and $p < 0.001$ by post-hoc analysis).

5 PHAGOCYTOSIS

The starting hypothesis for this work was that VAP may be associated with impaired ability to eliminate pathogens. This hypothesis was tested using a phagocytic assay and quantifying phagocytic capacity of neutrophils. The phagocytic capacity of peripheral blood neutrophils (PMNs) was studied using the zymosan phagocytosis assay [n=43 patients (VAP=12 and NON VAP= 31) and n=15 for the HV group]. In the remaining 6 patients the assay was not deemed satisfactory for analysis due to small and therefore non-representative number of cells remaining in the wells at the final stage of the assay.

In brief freshly prepared PMNs were incubated with zymosan particles. Phagocytic capacity was quantified by counting number of PMNs with ≥ 2 phagocytosed zymosan particles (as "positive") and number of PMNs with < 2 phagocytosed zymosan particles (as "negative"). Phagocytic capacity was expressed as the percentage of "positive" cells versus total number of cells from the average percentage of 2 fields.

The average phagocytic capacity in the VAP group was 35% (95%CI: 25-46), in the NON VAP group 42% (95% CI: 37-47) and in the HV group 71% (95% CI: 67-75) (Figure 51).

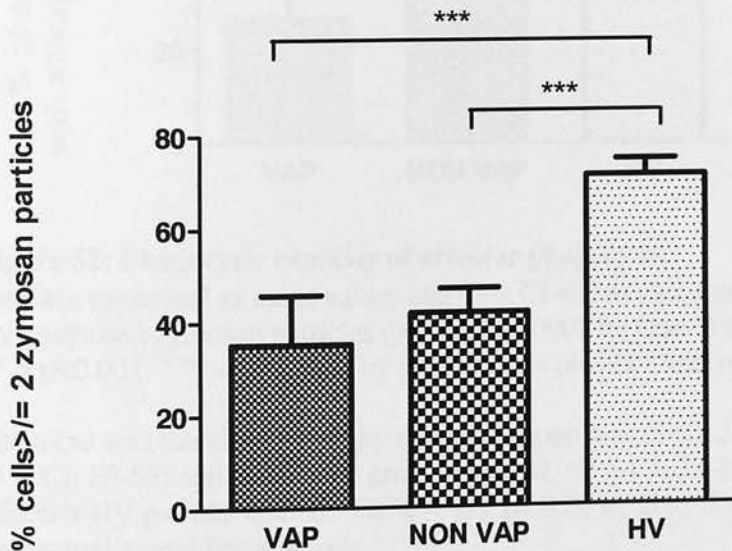


Figure 51: Phagocytic capacity of peripheral blood neutrophils.

Data are expressed as mean values and 95% CI and as percentage of cells with ≥ 2 phagocytosed zymosan particles, (p-value = 0.004 by One-Way ANOVA test), *** = p<0.0001 by Bonferroni's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (mean 39, 95% CI: 28-50) and NEITHER group (mean 42, 95% CI: 36-48): VAP v HV p < 0.0001, ARDS v HV p < 0.0001 and NEITHER v HV p < 0.0001 by One-Way ANOVA test and Bonferroni's post-hoc analysis.

The phagocytic capacity of alveolar cells was also studied. In this experiment the population of phagocytes in the group of patients was mixed, comprising of neutrophils and alveolar macrophages while in the group of healthy volunteers the cell population consisted predominantly of alveolar macrophages [n=22 patients (VAP=6, NON VAP=12) and n=12 for HV, numbers are limited for reasons similar to those mentioned for the blood PMN phagocytosis assay).

The mean phagocytic capacity of alveolar cells in the VAP and the NON VAP group was 27% (95% CI: 20-34) and 39% (95% CI: 32-46) respectively. On average the mean phagocytic capacity of the HV was 59% (95% CI: 48-70) (Figure 52).

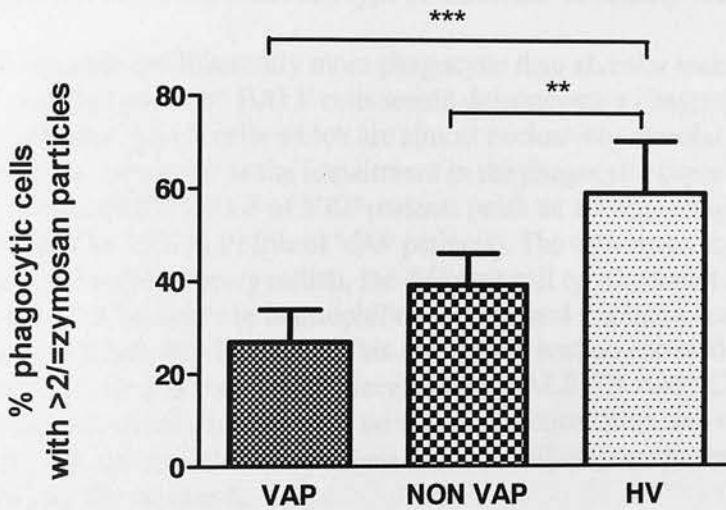


Figure 52: Phagocytic capacity of alveolar phagocytes.

Data are expressed as mean values and 95% CI and as percentage of cells with ≥ 2 phagocytosed zymosan particles (p-value = 0.0001 by One-Way ANOVA test), ** = $p < 0.001$, *** = $p < 0.0001$ by Bonferroni's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (mean 37, 95% CI: 20-53) and NEITHER group (mean 41, 95% CI: 33-49): VAP v HV $p < 0.0001$, ARDS v HV $p < 0.05$ and NEITHER v HV $p < 0.05$ by One-Way ANOVA test and Bonferroni's post-hoc analysis.

5.1 Discussion

The phagocytic capacity of circulating neutrophils was significantly impaired in both groups of critically ill patients compared with the healthy volunteers. The phagocytic capacity of blood PMNs in the VAP and the NON VAP group was approximately 50% lower than in the HV respectively.

Phagocytosis of the alveolar cells was also significantly impaired in all critically ill patients, following a pattern similar to the one demonstrated in the study of blood neutrophils. Overall an impairment of 29% was observed among the VAP patients when compared to the healthy volunteers and 20% in the NON VAP group compared with the healthy volunteers.

The population of cells in the BALF is more heterogeneous and the experiments were performed without cell type sorting. In the VAP group, neutrophils dominated the BALF population at 3-fold higher concentration than the alveolar macrophages. In the NON VAP group, there was less of a neutrophil predominance, with neutrophil cell count approximately 1.5-fold higher than the macrophage count. Alveolar macrophages were by far the most dominant cell type in the BALF of healthy volunteers.

Neutrophils are inherently more phagocytic than alveolar macrophages and one would expect that patients' BALF cells would demonstrate a phagocytic 'advantage' over volunteers' BALF cells which are almost exclusively alveolar macrophages. The opposite trend was observed as the impairment in the phagocytic capacity was more profound in the neutrophilic BALF of VAP patients (with an average phagocytic capacity of 27% as opposed to 35% in PMNs of VAP patients). The difference may reflect the influence of the local inflammatory milieu, the different cell types present in the BALF, or the inherent differences in neutrophilic function, and implies a compartmentalised and multifactorial, mechanism. In this study there was no correlation between the phagocytic impairment and the cytokine levels in the BALF (Dr Andy Conway Morris, personal communication). In particular, no significant correlation was observed for either CXCL8 or IL-1 β , the two BALF cytokines found to differentiate between VAP and non-VAP in Chapter III, section 3.

Neutrophil dysfunction in critical illness has been demonstrated in previous studies and various mechanisms have been proposed to explain these observations (Solomkin et al. 1981). More recently, studies have tried to elucidate the role of complement anaphylatoxin C5a in the regulation of innate immunity in sepsis. It has been demonstrated that high levels of C5a can lead to nonspecific, chemotactic "deactivation" and neutrophil dysfunction (Huber-Lang et al. 2002; Riedemann et al. 2003b; Riedemann et al. 2003a; Guo et al. 2004; Ward 2008). Since the completion of my experimental work, my colleague has extended the findings presented here and demonstrated a clear role for C5a in mediating impairment of neutrophil phagocytosis in critically ill patients (Conway Morris et al. 2009)

Other potential contributory mechanisms responsible for the observed phagocytic impairment could be related to deficient opsonisation, inhibition or down-regulation of phagocytic receptors, impaired ROS production and bacterial killing.

Finally it is clear that the phagocytic capacity of the alveolar phagocytes in the NON VAP group was significantly impaired compared with the healthy volunteers.

As mentioned in the previous chapter half of the phagocytes in this group were alveolar macrophages and the results may perhaps indicate a further defect in the host defence in the setting of critical illness.

Macrophages are one of the main mediators of the immune response along with the neutrophils, although their role is more extended as they are involved in both the innate and adaptive immune responses. They exhibit various functions, such as phagocytosis, secretion of cytokines, chemokines, and several other factors, and may be involved in antigen presentation (Monick and Hunninghake 2002). The macrophages in the lung are compartmentalised in airways, interstitium, and intravascular domains, are derived from pulmonary circulating monocytes and represent dynamic responders to several extrinsic and intrinsic stimuli playing a central role in host defense.

They are activated by interferon-gamma, cytokines, viruses, viral particles, bacteria, airborne pathogens and particles, and changes in calcium (Ma et al. 2003). IL-12 and IL-18 are produced by activated macrophages and are involved in stimulating T-helper 1 (Th 1) responses. Th 1 cells will produce IFN-gamma that will activate macrophages further and provide a positive feedback loop (Monick and Hunninghake 2002). Internalisation of IFN-gamma/interferon-gamma receptor complex as well as uptake of apoptotic cells generating anti-inflammatory signals will result in deactivation (Mayeux 1997).

Alveolar macrophages display a variety of receptors including Fc receptors, G-protein coupled receptors (GPCRs), integrins, CD14, Toll-like receptors (TLRs), cytokine receptors and chemokine receptors (Ryan et al. 2004). CD14 and TLR are both integral parts of the LPS response pathway (Akira 2003) that activates the NF- κ B pathway and induces cytokine production and it has been shown that the strongest response to LPS requires both CD14 and TLR in a complex form (Rao 2000).

Alveolar macrophages are able to discriminate pathogens with the aid of TLRs for efficient killing (Ryan et al 2004) which is accomplished by phagocytosis and the generation of ROS/RNS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (Gwinn and Vallyathan 2006).

The phagocytic capacity of alveolar macrophages has been studied in chronic lung diseases and early reports had linked impaired phagocytosis and airway diseases like asthma (Matusiewicz and Rusiecka-Matusiewicz 1987) and chronic bronchitis (Nielsen and Bonde 1986). Much less is known about the function of alveolar macrophages in critical illness where most studies have focused on monocyte downregulation in the context of sepsis (Payen et al. 2000).

This study raises the likelihood of a further phagocytic defect that affects alveolar macrophages. The defect can arise at various stages in the process including receptor binding, engulfment of pathogens and killing or degradation by ROS.

Alveolar macrophages are not only dynamic responders of innate immunity but their antigen processing and presentation ability adds a specific role in adaptive immunity. Dysfunction or deactivation can therefore further compromise an already impaired host response of the ventilated patient, reinforcing a vicious cycle of susceptibility to sequential infections and ultimately worse outcome (Wunderink 2005).

In summary, this section has described a clear impairment of neutrophil phagocytic capacity in both the blood and alveolar space of critically ill patients with clinically suspected VAP.

The findings presented do not support the original hypothesis that VAP is specifically associated with neutrophil phagocytic dysfunction (though a trend in that direction emerged both here and in the extended dataset examined by Dr Conway Morris (Conway Morris et al, 2009).

6 *IN VITRO* STIMULATION OF A549 CELLS WITH NEUTROPHILS AND/OR LPS

The original hypothesis proposed that neutrophils from patients with VAP may be in an activated and dysfunctional state. The previous chapter demonstrated that neutrophils from patients with critical illness are functionally deficient but that this observation was not VAP-specific. However in Chapter III section 4, I showed that release of extracellular HNE was increased in VAP, suggesting increased and possibly inappropriate activation. The purpose of this section was to assess whether neutrophils from patients with VAP had relatively pro-inflammatory actions when exposed to respiratory epithelium.

This was assessed by measurement of LDH release as an index of 'toxic' release of cellular contents. LDH is an intracellular molecule found in many cell types. It is abundant in alveolar epithelial cells and present (though to a lesser degree) in neutrophils. MCP-1 (CCL2) secretion was also measured in order to assess the pro-inflammatory effect on epithelial cells. MCP-1/CCL2 is released by epithelial cells under inflammatory conditions. MCP-1/CCL2 is highly relevant in evolving lung inflammation as a potent monocyte chemoattractant and has been implicated in the development lung injury. Therefore, the markers described were chosen to reflect toxicity (LDH) and inflammatory secretion/toxicity (MCP-1/CCL2).

The cell line used in the following experiments was the A549 cell. A549 cells share similar morphological features with type II alveolar epithelial cells. For example they produce lamellar bodies and surfactant, characteristic features distinguishing type II alveolar epithelial cells from among mixed populations of lung cells.

In these experiments A549 cells were co-cultured with peripheral blood neutrophils and/or LPS for 24 hours, in order to study the effect of neutrophils on alveolar epithelial cells (number of experiments: n= 39 for patients (VAP=11 and NON VAP =28) and n=10 for healthy volunteers).

In brief freshly isolated neutrophils were resuspended in IMDM containing 1% autologous serum, at a final concentration of 500,000 cells/ml. A549 cells grown to confluence in 24-well plate were washed with PBS and were incubated with:

- IMDM containing 1% autologous serum (as a control) or,
- Neutrophils at 500,000 cells/ml or,
- LPS at 100ng/ml or,
- Neutrophils and LPS (Neutrophils at 500,000cells/ml and LPS at 100ng/ml).

In all groups the final volume of medium was 1ml and the final concentration of autologous serum (derived from the same patient/volunteer as supplied neutrophils) was 1 %.

The cultures were incubated overnight. Supernatants were retrieved the next day, processed and stored at -80°C for analysis of MCP-1/CCL2 and LDH activity.

As autologous serum was present in all incubating media, in a small number of the above experiments (n=4, HV), A549 cells were also incubated with serum-free IMDM as well as conditioned mentioned above, in order to detect differences related to serum proteins.

Mean MCP-1/CCL2 concentration in supernatants from A549 cells was 633 pg/ml (95% CI: 102-1368) in serum-free conditions and 651 pg/ml (95% CI: 108-1410) in serum-containing IMDM (Figure 53). There was no significant difference in the baseline MCP-1/CCL2 secretion from A549 cells between the two groups (serum-free and serum-containing IMDM), and thus all data presented below resulted from supernatants containing 1% autologous serum.

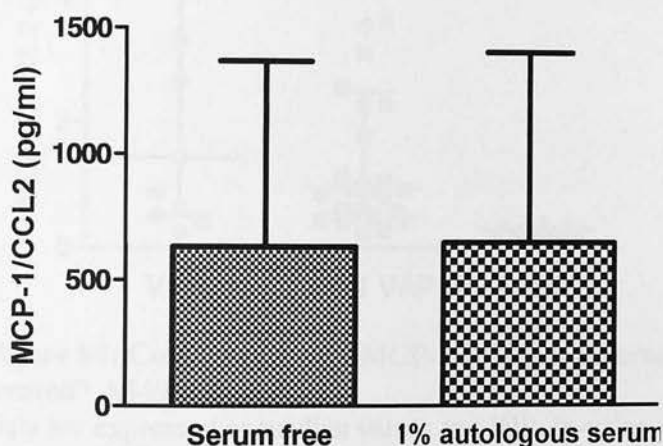


Figure 53: Concentration of MCP-1/CCL2 in supernatants after incubation of A549 cells with serum-free IMDM and IMDM containing 1% autologous serum.

Data are expressed as mean values and 95% CI, (p-value >0.05 with Chi-square test).

The following data are presented as:

- Control (supernatants from A549 cells incubated in IMDM and autologous serum 1%),
- Neutrophils (supernatants from A549 cells co-cultured with patients`/healthy volunteers` neutrophils in IMDM and autologous serum 1%),
- LPS (supernatants from A549 cells incubated with LPS in IMDM and autologous serum 1%) and
- Neutrophils and LPS (supernatants from A549 co-cultured with patients`/healthy volunteers` neutrophils and LPS in IMDM and autologous serum 1%).

6.1 MCP-1/CCL2 concentration

Median concentration of MCP-1/CCL2 in supernatants from the “control-treated” A549 cells was 1.42 ng/ml (IQR: 0.5-4.2) in the VAP group, 0.96 ng/ml (IQR: 0.5-2.5) in the NON VAP group and 0.30 ng/ml (IQR: 0.2-0.3) in the healthy volunteers group (Figure 54).

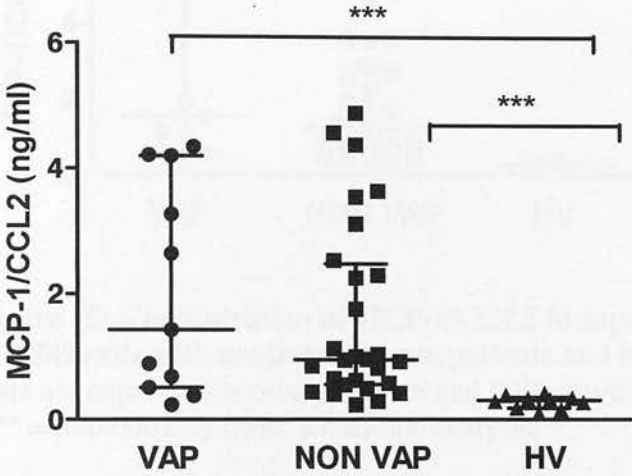


Figure 54: Concentration of MCP-1/CCL2 in supernatants from the “control-treated” A549 cells.

Data are expressed as median values and IQR, (p-value = 0.0001 by Kruskal-Wallis test), *** = p<0.0001 by Dunn`s post-hoc analysis.

Median concentration of MCP-1/CCL2 in the supernatants from “neutrophil-treated” A549 cells was 1.54 ng/ml (IQR: 0.6-5.2) in the VAP group, 1.24 ng/ml (IQR: 0.7-2.9) in the NON VAP group and 0.25 ng/ml (IQR: 0.1-0.3) in the healthy volunteers group (Figure 55).

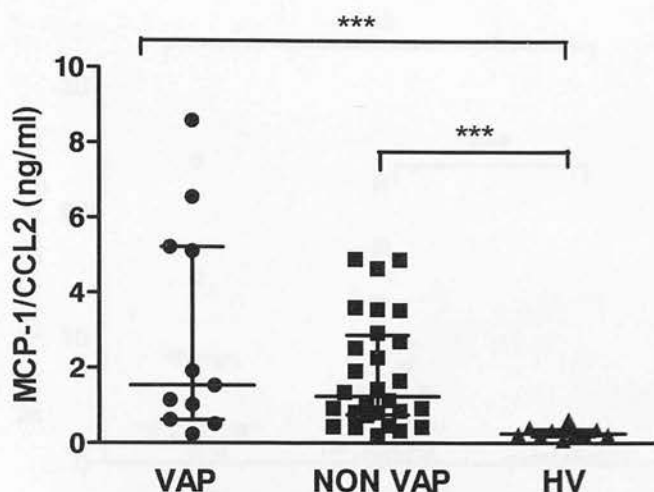


Figure 55: Concentration of MCP-1/CCL2 in supernatants after *in vitro* stimulation of A549 cells with neutrophils from patients and healthy volunteers.

Data are expressed as median values and IQR, (p-value <0.0001 by Kruskal-Wallis test), *** = p<0.0001 by Dunn`s post-hoc analysis.

MCP-1/CCL2 secretion was significantly higher when A549 cells were stimulated by neutrophils from VAP patients and NON VAP patients as compared with MCP-1/CCL2 secretion by A549 cells that were cultured with neutrophils from healthy volunteers.

The trend was the same when the non-VAP group was divided into ARDS (median 1.08 ng/ml, IQR: 0.5-2.8) and NEITHER group (median 1.35 ng/ml, IQR: 0.8-3.5): VAP v HV <0.0001, ARDS v HV p < 0.05, and NEITHER v HV p<0.001 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

For the “LPS-treated” A549 cells, median concentration of MCP-1/CCL2 was 2.95 ng/ml (IQR: 1.9-8.5) in the VAP group, 2.90 ng/ml (IQR: 1.5-7.1) in the NON VAP group and 0.5 ng/ml (IQR: 0.1-0.3) in the healthy volunteers group (Figure 56).

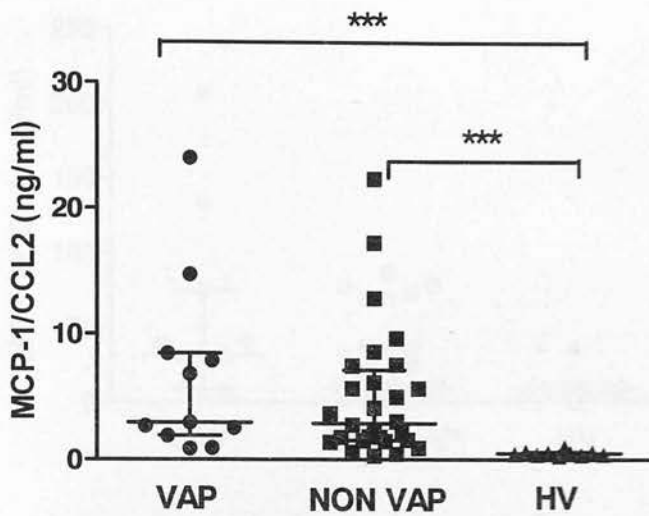


Figure 56: Concentration of MCP-1/CCL2 in supernatants from “LPS-treated” A549 cells.

Data are expressed as median values and IQR, (p-value <0.0001 by Kruskal-Wallis test), *** = p<0.0001 by Dunn`s post-hoc analysis.

MCP-1/CCL2 secretion was significantly higher when A549 cells were stimulated by LPS in the presence of serum from patients as compared with MCP-1/CCL2 secretion by A549 cells that were cultured with LPS in the presence of serum from healthy volunteers.

The trend was the same when the non-VAP group was divided into ARDS (median 2.75 ng/ml, IQR: 1.7-8.6) and NEITHER group (median 3.03 ng/ml, IQR: 1.5-6.1): VAP v HV <0.0001, ARDS v HV p < 0.001, and NEITHER v HV p<0.001 by Kruskal-Wallis test and Dunn`s post-hoc analysis.

Finally for “neutrophil and LPS-treated” A549 cells, median concentration of MCP-1/CCL2 was 31.80 ng/ml (IQR: 9.6-74.8) in the VAP group, 10.95 ng/ml (IQR: 5.9-36.6) in the NON VAP group and 8.7 ng/ml (IQR: 4.9-12.9) in the healthy volunteers group (Figure 57).

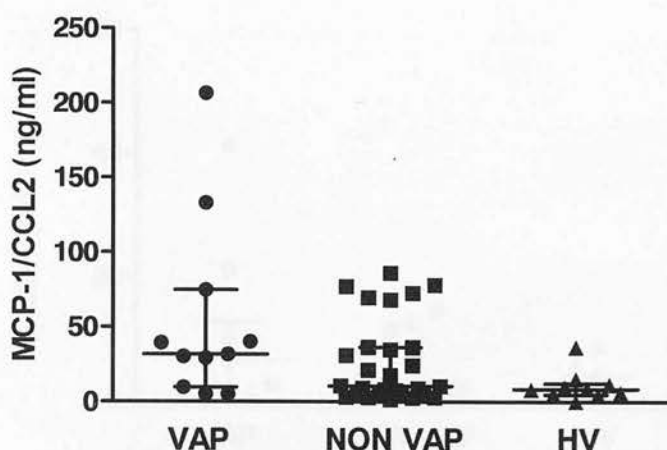


Figure 57: Concentration of MCP-1/CCL2 in supernatants after *in vitro* stimulation of A549 cells with LPS and neutrophils from patients and healthy volunteers. Data are expressed as median values and IQR, (p-value = 0.06 by Kruskal-Wallis test), $p > 0.05$ by Dunn's post-hoc analysis.

The trend was the same when the non-VAP group was divided into ARDS (median 6.27 ng/ml, IQR: 2.9-34.00) and NEITHER group (median 10.50 ng/ml, IQR: 7.7-68.4): $p > 0.05$ in comparison of all pairs of groups by Kruskal-Wallis test and Dunn's post-hoc analysis.

6.2 LDH activity

LDH activity was also measured in the supernatants of A549 cells co-cultured with neutrophils and/or LPS. It is expressed as LDH release above that from A549 cells alone. Results were available for 10 VAP patients, 28 NON VAP patients and 12 HV.

Median LDH activity in supernatants from *in vitro* stimulation of A549 cells with neutrophils was 6.73 mU/ml (IQR: 0.0-12.8) in the VAP group, 0.00 mU/ml in the NON VAP group (IQR: 0.0-3.5) and 0.00 mU/ml (IQR: 0.0-4.0) in the healthy volunteers group (Figure 58).

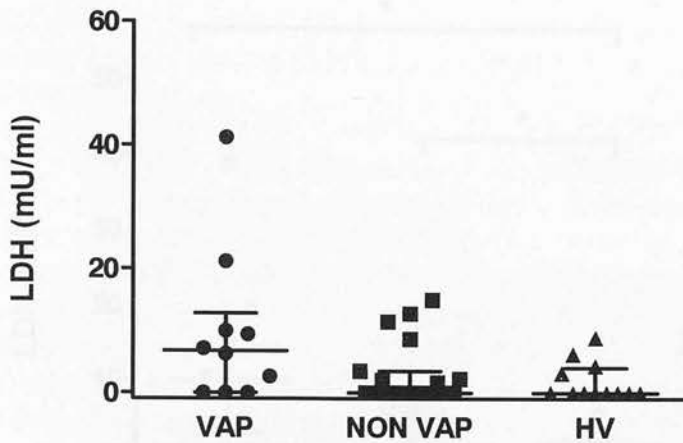


Figure 58: LDH activity in supernatants after *in vitro* stimulation of A549 cells with neutrophils from patients and healthy volunteers.

Data are expressed as median values and IQR, (p -value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

Median LDH activity in supernatants from *in vitro* stimulation of A549 cells with LPS in the presence of serum was 4.15 mU/ml (IQR: 0.0-23) in the VAP group, 0.00 mU/ml in the NON VAP group (IQR: 0.0-7.3) and 0.00 mU/ml (IQR: 0.0-0.1) in the healthy volunteers group (Figure 59).

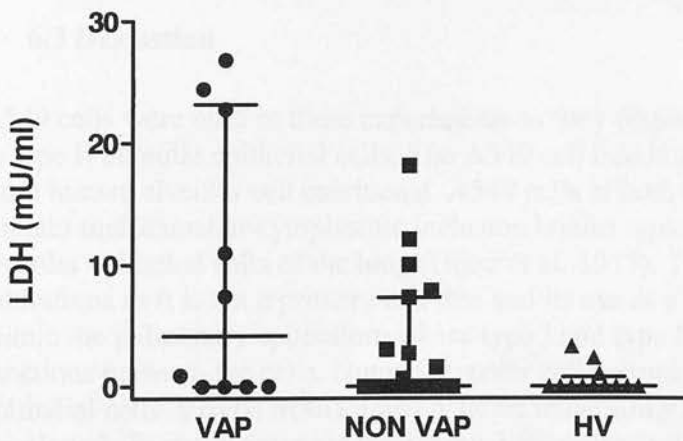


Figure 59: LDH activity in supernatants from "LPS-treated" A549 cells.

Data are expressed as median values and IQR, (p -value >0.05 by Kruskal-Wallis test and Dunn`s post-hoc analysis).

In the same set of experiments A549 cells were co-cultured with peripheral blood neutrophils and stimulated with LPS. Median LDH activity in the VAP group was 9.86 mU/ml (IQR: 2.9-18.8), 0.00 mU/ml in the NON VAP group (IQR: 0.0-6.3) and 1.31 mU/ml (IQR: 0.0-1.7) in the healthy volunteers group (Figure 60).

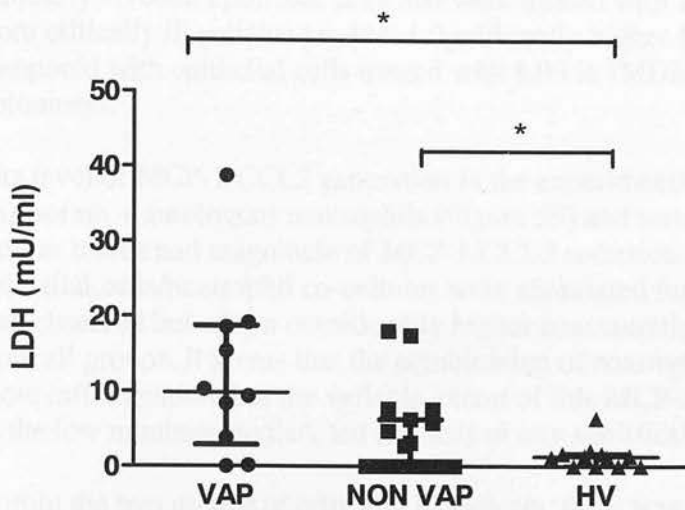


Figure 60: LDH activity in supernatants after *in vitro* stimulation of A549 cells with LPS and neutrophils from patients and healthy volunteers.
 Data are expressed as median values and IQR, (p-value = 0.008 by Kruskal-Wallis test), * = p<0.05 by Dunn's post-hoc analysis.

The trend was the same when the NON VAP group was divided in ARDS and NEITHER group in all sets of experiments.

6.3 Discussion

A549 cells were used in these experiments as they display morphological features similar to type II alveolar epithelial cells. The A549 cell line is a tumour-cell line that derived from human alveolar cell carcinoma. A549 cells at both early and late passage levels contain multilamellar cytoplasmic inclusion bodies typical of those found in type II alveolar epithelial cells of the lung (Giard et al. 1973). The A549 model has its own limitations as it is not a primary cell line and its use as a monolayer does not completely mimic the pulmonary epithelium where type I and type II cells coexist with tight junctions between the cells. Human alveolar cell culture models based on alveolar epithelial cells derived from human patients undergoing lung resection surgery have been developed. These cultures are limited by difficulties in distinguishing between type II and type I cells and the transient appearance of intermediate phenotype during differentiation, and various approaches are in progress to address the above problems (Forbes and Ehrhardt 2005).

Alveolar epithelial cells that were co-cultured with serum or neutrophils from critically ill patients exhibited a significantly higher response in secreting MCP-1/CCL2 when compared with epithelial cells co-cultured with neutrophils from healthy volunteers.

Similarly alveolar epithelial cells that were treated with LPS in IMDM containing serum from critically ill patients produced significantly higher MCP-1/CCL2 secretion when compared with epithelial cells treated with LPS in IMDM containing serum from healthy volunteers.

The level of MCP-1/CCL2 generation in the experiments studying serum alone (Figure 58), serum + autologous neutrophils (Figure 59) and serum + LPS (Figure 60) revealed similar trends and magnitude of MCP-1/CCL2 secretion. In contrast, when alveolar epithelial cells/neutrophil co-cultures were stimulated further with LPS, a similar pattern was observed but with a considerably higher concentration of MCP-1/CCL2 in samples from all groups. It seems that the combination of neutrophils/LPS generate substantially more inflammation, but the variable extent of this MCP-1/CCL2 release, possibly allied to the low numbers studied, led to a loss of any statistically significant difference.

Within the two groups of critically ill patients, there was a trend towards higher MCP-1/CCL2 levels after stimulation with neutrophils and neutrophils and LPS in the VAP group compared with the NON VAP, although the difference did not reach statistical significance.

Clearly, an *in vitro* system in which a cell line is co-cultured with serum and (autologous) neutrophils may not reflect the complex situation in patients. The alveolar compartment in patients contains a variety of different cell types and physico-chemical characteristics. Nevertheless, the apposition of serum and neutrophils (both from patients) and an epithelial cell line does go some way to mimicking crucial elements involved in lung inflammation. While recognising that this *in vitro* system cannot be immediately generalised to the situation in patients, it is interesting to speculate as to the biological processes reflected in these experiments.

It is known that MCP-1/CCL2 is produced by alveolar epithelial cells in response to infection and inflammation (Jansen et al. 1995; Olszyna et al 2001). Peripheral blood neutrophils from patients with critical illness appear to provoke a far greater response when co-cultured with alveolar epithelial cells. As MCP-1/CCL2 is a potent chemoattractant for monocytes these data suggest patients' neutrophils could potentially drive monocyte recruitment which is known to follow neutrophil accumulation at the site of infection or inflammation. However as the role of MCP-1/CCL2 in inflammation remains unclear, it is difficult to speculate whether the effect of circulating blood neutrophils and their ability to upregulate MCP-1/CCL2 secretion by alveolar epithelial cells could have a protective effect or not. The enhanced secretion of MCP-1/CCL2 may reflect a protective mechanism in the context of infection where circulating neutrophils recruited to the lung will set in motion monocyte recruitment, facilitating bacterial clearance, clearance of apoptotic neutrophils, and resolution of inflammation.

There is evidence to suggest that MCP-1/CCL2 is also implicated in exaggerating lung injury by enhancing alveolar macrophage activation and by contributing further to neutrophil recruitment and activation.

In that context one can speculate that in critically ill patients, recruited neutrophils not only display impaired phagocytic capacity as described earlier, but may also have the potential to promote macrophage and neutrophil activation resulting or contributing in inflammation and tissue damage of the ventilated lung. This suggestion is supported further by the data on LDH release from similar same set of experiments.

LDH activity has been used extensively in the literature as an index of increased membrane permeability and/or cell lysis. LDH is a cytoplasmic enzyme that is released in the extracellular space when the alveolar/capillary barrier is damaged and when true cell lysis occurs it can therefore provide information about the degree of cytotoxicity present (Drent et al. 1996; Cobben et al. 1999).

There was a trend towards higher LDH activity in the VAP group compared with the NON VAP and the HV group in samples taken from co-cultures of alveolar epithelial cells and neutrophils although the difference did not reach significance. Furthermore, circulating neutrophils from both groups of critically ill patients in the presence of LPS induced significantly higher LDH release as compared with neutrophils from healthy volunteers, suggestive of cellular damage.

There is a possibility that neutrophils per se could contribute to the LDH release measured. This was not tested as a separate group with neutrophils only and no A459 cells and the limitations of the experiment are acknowledged. However the importance of the findings reflects the interaction of neutrophils and alveolar epithelium and the resulting alveolar epithelial membrane damage, which is highly relevant in lung injury.

Membrane disruption followed by increased permeability and alveolar oedema has been traditionally described as the hallmark of lung injury. Circulating blood neutrophils from patients with critical illness appear to have the potential, especially upon contact with bacterial products in the alveolar compartment, to cause damage to the membrane barrier thereby playing an important role in the pathogenesis of lung injury.

In summary, this section provides evidence to suggest that patients with critical illness have enhanced potential to promote both damage and the secretion of inflammatory mediators from epithelial cells. The data do not support the original hypothesis that the pro-inflammatory/cytotoxic potential would be exaggerated in VAP specifically.

7 CONCLUSION

The aim of this study was to test the hypothesis that VAP was associated with impaired production of lung-protective antiproteases, and with functionally defective and 'over-activated' neutrophils, this in turn being associated with increased lung inflammation. In rigorously characterising patients with 'clinically suspected VAP' a very comprehensive analysis of the microbiology and innate immunity in patients with VAP was also performed.

In order to achieve the aims, healthy volunteers and a mixed surgical and medical cohort of critically ill patients were recruited from a general ICU, who were mechanically ventilated and developed clinical suspicion of VAP. Collection of clinical and microbiological data allowed a comprehensive analysis of the microbiological and demographic spectrum of critically ill patients with VAP. Both groups of patients had similar demographic parameters and displayed similar severity scores within the first 24 hours of admission. The incidence of VAP in this study was 27% with the majority of cases attributed to Gram-negative pathogens in keeping with the literature.

Central to the initial hypothesis was that an exaggerated pro-inflammatory response would be present in patients with VAP. This study has shown that BALF from patients with VAP displayed a trend towards a higher number of neutrophils/ml of BALF compared with the NON VAP group, while both groups displayed significantly higher counts of neutrophils compared with the healthy volunteers.

Neutrophil influx in the setting of VAP, driven by the presence of significant numbers of pathogens in the lung, was associated with a florid inflammatory response. While CXCL8 and IL-1 β levels were significantly elevated in both groups of critically ill patients compared with the healthy volunteers, IL-1 β and CXCL8 were significantly higher in the BALF from patients with VAP as compared with NON VAP, suggestive of an exaggerated and compartmentalised response that appears to be VAP-specific.

This pro-inflammatory reprogramming of the inflammatory cascade in patients with VAP may re-stimulate recruitment of phagocytes in the battle of the host defense against bacterial invasion. However unregulated recruitment can lead to neutrophil-mediated tissue injury through release of proteases and is dependent in part on secretion and inactivation by protease inhibitors.

It was anticipated that along with the pro-inflammatory profile in patients with VAP, the local antiprotease/antimicrobial expression would be impaired perhaps facilitating neutrophil-mediated tissue injury. This study has shown that BALF SLPI levels were not significantly different while elafin levels were significantly elevated in patients with VAP as compared with the healthy volunteers. More strikingly, HNE concentrations were significantly higher in the BALF of VAP patients as compared with NON VAP patients or healthy volunteers. In general, extracellular HNE is considered to be abnormal, and to reflect excessive activation, with inappropriate or excessive degranulation by neutrophils.

The above findings imply excessive activation of neutrophils in VAP possibly along with breakdown of the anti-protease shield. The consequence of this may be to facilitate neutrophil-mediated pulmonary inflammation and tissue injury. In addition, a defect in the recognised antimicrobial properties lung antiproteases cannot be excluded.

This may have implications in patients with critical illness as the battle between host defence and the virulence of the invading pathogens can determine the development of severe infection. A highly relevant contribution to this balance comes from the phagocytic capacity of neutrophils. It was hypothesised that neutrophils in patients with VAP may have impaired capacity to eliminate pathogens whilst they may have the potential to cause tissue damage. This study has demonstrated a clear defect of neutrophil phagocytosis in keeping with the presence of a less efficient mechanism of bacterial clearance in critical illness. Phagocytic capacity of circulating neutrophils was significantly impaired in all critically ill patients compared with the healthy volunteers (approximately 50% lower than the HV). It is not clear if neutrophil dysregulation can be explained by a unified hypothesis applicable both in the systemic circulation and the pulmonary compartment, or whether it is governed by more diverse and compartmentalised mechanisms.

Furthermore, circulating neutrophils from patients with critical illness not only exhibited impaired phagocytic capacity but appeared to have the potential, especially upon contact with bacterial products in the alveolar compartment, to cause damage to the epithelial membrane barrier thereby potentially playing an important role in the pathogenesis of lung injury.

In summary this study has, in keeping with the initial hypothesis, provided evidence that VAP is associated both with an up-regulation of IL-1 β and CXCL8 and with excessive HNE generation (implying excessive neutrophil degranulation) in the alveolar space. The hypothesis that lung-protective antiproteases would be down-regulated in VAP was not supported, though the excess of HNE does support an inherent imbalance in protease-antiprotease in the alveolar space in VAP. Similarly the hypothesis that neutrophil phagocytosis would be impaired in a VAP-specific manner was not supported, though I did identify a striking impairment in critically ill patients which has been the focus of further study.

This study has limitations as the number of patients recruited in the time available was small and perhaps not adequate to determine whether observed trends were biologically important. Within the group of suspected VAP, a number of patients with ARDS were included. Although inclusion of these patients may be interpreted as a confounding factor, results were analysed in subgroup analysis and no differences were detected. Healthy volunteers were used as a normal reference group instead of mechanically ventilated patients. The limitations have been acknowledged and cannot exclude the possibility that mechanical ventilation may explain some of the differences noted. The definition and recruitment of the ideal control group is not without considerable theoretical and practical difficulties.

It is therefore likely that this study would have been strengthened further by inclusion of a higher number of patients with redefinition of a different and perhaps more ideal control group.

However the above should not distract from the information that this study has provided in our further understanding of the biology of VAP, with emphasis on the dysfunction of neutrophils. Many questions remain unanswered and future work is needed to elucidate and dissect the mechanisms responsible for the defective phagocytic capacity of neutrophils and macrophages. Along similar lines, the role of endogenous SLPI and elafin in critical illness remains intriguing. The mechanisms responsible for the antimicrobial properties of these molecules and the effect of elafin and SLPI on phagocytosis of pathogens by neutrophils and alveolar macrophages require further clarification.

In an era where increasing numbers of infections are attributed to multiresistant pathogens, as shown in epidemiological studies, targeting of the functional impairment of the phagocytes and/or stimulation of endogenous production or exogenous administration of defense molecules can offer a novel therapeutic modality.

Finally the anti-protease and anti-inflammatory properties of elafin and SLPI can also provide alternative methods of attenuating tissue damage in other neutrophil-mediated diseases such as ARDS, a disease associated with a substantial morbidity, cost and mortality and disappointing therapeutic options (Wheeler and Bernard 2007).

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APPENDIX

Published papers arising from this work:

Conway Morris A, Kefala K, Wilkinson TS, Dhaliwal K, Farrell L, Walsh TS, Mackenzie SJ, Reid H, Davidson DJ, Haslett C, Rossi A, Sallenave JM, Simpson AJ. C5a mediates peripheral blood neutrophil dysfunction in critically ill patients. *Am J Respir Crit Care Med*. doi:10.1164/rccm.200812-1928OC. Published online 2009 Mar 26.

A Conway Morris, K Kefala, A J Simpson, TS Wilkinson, K Everingham, D Kerslake, S Raby, IF Laurenson, DG Swann, TS Walsh. Evaluation of the effect of diagnostic methodology on the reported incidence of ventilator-associated pneumonia. *Thorax* 2009;64;516-522; originally published online 12 Feb 2009.