Incidence and Pathogenesis of Acute Lung Injury and the Acute Respiratory Distress Syndrome in Humans

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Submitted towards the degree of Doctor of Medicine, University of East Anglia

Research conducted in the Department of Medicine, University of Cambridge

August 2013

Abstract

Acute lung injury (ALI) and it's more severe form, acute respiratory distress syndrome (ARDS), are conditions characterised by neutrophilic pulmonary inflammation, refractory hypoxaemia and diffuse alveolar damage. This work reports on the first UK based prospective study of the incidence and longer term mortality of ALI/ARDS in a general intensive care unit. Results reveal significant under recognition of this condition, which occurred in 12.5% of the ICU population (n=344). Hospital and 2 year mortality rates were 50-55% and 58-61% respectively.

Neutrophils are central in the pathogenesis of ALI/ARDS. Neutrophil priming, a reversible process whereby the response of neutrophils to an activating stimulus is up-regulated by prior exposure to a priming agent, is a pre-requisite for neutrophil-mediated tissue damage. Comparisons of the trans-pulmonary gradient of several markers of neutrophil priming were made in different patient groups. Patients with sepsis but healthy lungs (n=6) were found to have a positive trans-pulmonary gradient with respect to neutrophil expression of CD62L, suggesting that CD62L 'low' (primed) neutrophils are being de-primed in the pulmonary circulation. In patients with ARDS this gradient was reversed, suggesting that neutrophils are being primed within the lung. This leads to the novel hypothesis that, in conditions such as sepsis, neutrophils primed systemically may be held in the pulmonary capillary bed and there allowed to de-prime, before being released in a quiescent state. Failure of this process may allow net accumulation of primed neutrophils in the lung with consequent lung injury.

Currently there are no effective pharmacological therapies for ALI/ARDS, which is compounded by the relative lack of human models. An 18 month, prospective study of the incidence of ALI/ARDS post oesophagectomy revealed an incidence of 31% and suggested intra-operative one lung ventilation as a causative factor. Hence, patients undergoing oesophagectomy present an attractive model for future ALI/ARDS research.

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Acknowledgements

I would like to offer my sincere gratitude to my supervisors Professor Edwin Chilvers and Dr Andrew Wilson for making this all possible, in particular to Edwin for nudging me in the right directions, motivating me in times of despair and keeping faith in my abilities. Edwin's professionalism and respect for others is inspirational and has provided me with an education far beyond academia.

I would also like to thank Dr Charlotte Summers, who has been absolutely instrumental to this work. She introduced me to much of the background and trained me in many of the techniques involved, as well as working tirelessly in reviewing the outputs.

I would like to offer my further gratitude to; Ms Rosalind Simmonds and Ms Linda Worpole for helping compile the ICU database; Dr Andrew Johnston, Dr Vilas Navapurka, Dr Peter Bradley and Dr Razeen Mahroof for their support on the John V Farman Intensive Care Unit; Dr Christine Fiddler for her continued efforts in delivering the perfect intravenous bolus injection; Mr Peter Safranek, Mr Richard Hardwick, Dr Kevin Gunning, Dr Ian Munday, Dr Mark Abrahams and Dr David Tew for their patience in allowing me to conduct the neutrophil transit time experiments during their theatre lists; and Mr Chandra Solanki for his help with radio-labelling.

I would like to thank my wife, Kiran, and baby daughter, Gurbani, for their unconditional love, for their patience and forgiveness for missed times together, and for providing me with much needed encouragement and support throughout.

Lastly, I would like to thank my parents, whose ambition, hard work and sacrifices in prioritising our education have been immense. I am where I am today because of them.

1 Introduction

1.1 Acute Lung injury

1.1.1 Definition of ALI

Acute lung injury (ALI), and its more severe form, acute respiratory distress syndrome (ARDS), are conditions characterised by refractory hypoxaemia, diffuse alveolar damage, neutrophilic lung inflammation and protein-rich pulmonary oedema.

ARDS was first described in the literature in 1967 (Ashbaugh et al. 1967) after a case series of 12 patients with different underlying conditions, developed common features of tachypnoea, hypoxaemia, lung stiffness and diffuse infiltrates on chest radiograph. Although, initially termed the acute respiratory distress syndrome, this conditioned was erroneously called the adult respiratory distress syndrome (Petty and Ashbaugh 1971). However, as this condition can also occur in children the name was redefined as acute lung injury and the acute respiratory distress syndrome in 1994 (Bernard et al. 1994).

This initial definition was expanded in 1988 by Murray and colleagues, and incorporated (i) the relative acuteness of the disease process, (ii) whether there were any known risk factors, and (iii) attempted to measure disease severity (Murray et al. 1988). This was graded using the Lung Injury Score (LIS) which used physiological assessments of oxygenation, lung compliance (during mechanical ventilation), positive end-expiratory pressure (PEEP) during mechanical ventilation and the level of pulmonary infiltrates on the chest radiograph (Murray et al. 1988). However, this new score was not shown to correlate well with mortality (Monchi et al. 1998) and is heavily reliant on the use of ventilator parameters meaning that patients not mechanically ventilated are excluded.

In the 1990s the American Thoracic Society (ATS) and the European Society of Intensive Care Medicine (ESICM), with support from the National Heart, Lung and Blood Institute (NHLBI), convened a series of experts to establish a revised definition of ARDS, and in 1994 this joint American-European Consensus Conference (AECC) set out the following 4 criteria for a diagnosis of ALI or ARDS (Bernard et al. 1994):

1. Acute onset
2. Bilateral infiltrates seen on frontal chest radiograph consistent with pulmonary oedema (see figure 1.1A)

3. A pulmonary artery wedge pressure of less than 18 mmHg when measured or no clinical evidence of left atrial hypertension
4. Hypoxaemia, whereby the arterial oxygen tension to fraction of inspired oxygen ratio (PaO_2/FiO_2) is less than 300 mmHg (40 KPa) for ALI, and less than 200 mmHg (27 KPa) for ARDS

Hence, ALI was the term applied to the wide spectrum of this syndrome, and ARDS reserved for the most severe end of the spectrum based on severe hypoxaemia.

This new definition has now been adopted widely and used in all clinical trials for ALI/ARDS, and is still in current use today. It has resulted in a multitude of data from around the world, including numerous epidemiological and interventional studies. However, several issues have been raised with this AECC definition. Firstly, there is a lack of clarity for defining acute onset. In 2005 this was arbitrarily set at less than 72 hours in the Delphi consensus (Ferguson et al. 2005) although this has not been widely adopted in subsequent trials. Secondly, the oxygenation criteria do not account for the effects of PEEP on the PaO_2/FiO_2 ratio. Ferguson et al. studied 41 consecutive ICU patients that met AECC criteria and then placed them on a standardized ventilation setting of a PEEP 10 cmH_2O and FiO_2 of 1.0. The PaO_2/FiO_2 ratio was then rechecked 30 minutes later only to reveal that 58.5% of these patients had a PaO_2/FiO_2 ratio of greater than 200 mmHg and so therefore no longer fulfilled the AECC criteria for ARDS (Ferguson et al. 2004). Thirdly, measurements of pulmonary arterial wedge pressures are seldom made and clinical distinction of hydrostatic oedema is often difficult. Finally, chest radiograph interpretation is often highly variable (Angus 2012).

These difficulties led to a further consensus conference organised by the ESICM with support from the ATS, which produced in 2012 a revised definition: The Berlin Definition (Ranieri et al. 2012). This has now proposed to remove the term ALI and instead separate ARDS into mild, moderate and severe depending on PaO_2/FiO_2 ratio and levels of positive end-expiratory pressure (from invasive or non-invasive ventilation). Further changes, include defining the timing of onset of the condition as worsening respiratory symptoms within 1 week; defining radiological changes as bilateral opacities on chest radiograph or computed tomography (CT) (see figure 1.1B), which are not fully explained by effusions, lobar/lung collapse, or nodules; and finally the presence of respiratory failure that is not fully explained by cardiac failure or fluid overload, with objective assessment in regards to this (such as echocardiography) to exclude hydrostatic oedema (Ranieri et al. 2012).

This new definition is in its early days and has yet to be widely adopted, and hence all current epidemiology data are based on the 1994 AECC definition. For this reason and the fact that the work contained within this dissertation precedes the Berlin definition, all data presented in this current body of work are based upon the 1994 AECC definition.

Although ALI is an umbrella term that encompasses ARDS, to avoid any ambiguity, the term ALI will be used throughout this body of work to denote patients with ALI but not ARDS (i.e. those with a PaO_2/FiO_2 ratio of less than 40KPa but greater than 27KPa. The term ALI/ARDS will be used to encompass both conditions.

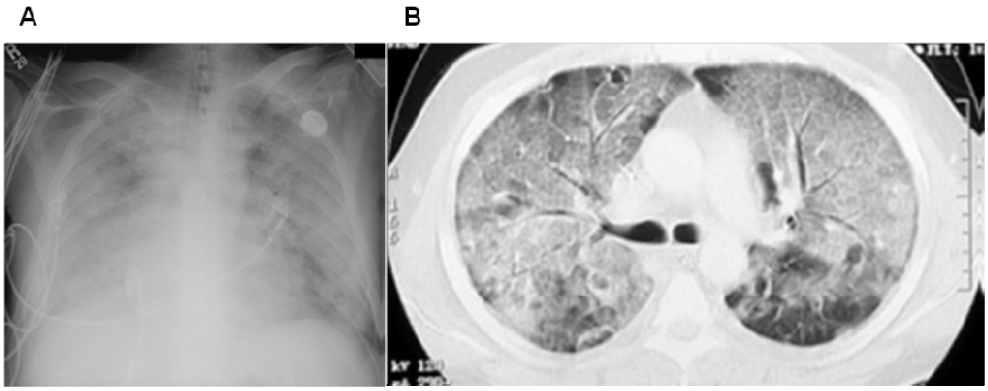

Figure 1.1 Acute lung injury causes diffuse pulmonary infiltration

Acute lung injury (ALI) is defined as a condition of acute onset, associated with bilateral pulmonary infiltrates consistent with pulmonary oedema and impaired gas exchange with a PaO_2/FiO_2 ratio of less than 40 KPa, in the absence of signs of left atrial hypertension. Panel A shows a chest radiograph from a patient with ARDS. Panel B shows a CT image from the same patient. Both demonstrate the diffuse bilateral pulmonary infiltrates that occur in ALI.

1.1.2 Incidence of ALI

An accurate estimation of the incidence of ALI and ARDS has been difficult, not least because of the previous lack of a uniform definition. Initial population based studies reported incidences in the region of 1.5 - 8.3 patients per 100,000 population per year (Thomsen and Morris 1995; Villar and Slutsky 1989; Webster et al. 1988). However, these used differing definitions of ARDS and arbitrary PaO_2/FiO_2 cut offs.

The first study to use the 1994 AECC definition showed considerably higher incidences of 17.9 patients per 100,000 population per year for all ALI/ARDS (13.5 for ARDS). This was a prospective study of 132 intensive care units (ICUs) in Sweden, Denmark and Iceland although covered only an eight week period (Luhr et al. 1999). A prospective Australian study, again only over a short time period of 2 months, showed an incidence of ALI/ARDS of 34 patients per 100,000 population per year (Bersten et al. 2002). Goss et al. estimated the incidence in the US as 64 patients per 100,000 population per year, after analysis of the ARDS Network database (Goss et al. 2003). The wide variation in these figures is thought to be due to extrapolating data from less than a complete year of observation and because of differences in the availability and utilisation of intensive care facilities around the world. The currently most widely cited reference for the incidence of ALI/ARDS is from a more robust study, performed prospectively over a whole year, within a county in the US, which reported an incidence rate of 78.9 patients per 100,000 population per year (Rubenfeld et al. 2005).

There is now some recent evidence to suggest that the incidence of ARDS may be reducing. In an 8 year prospective study, Li et al. has reported on the incidence of ARDS in a US county being reduced significantly from 82.4 patients per 100,000 population per year in 2001 to 38.9 in 2008 (Li et al. 2011). They found that the incidence of ARDS diagnosed on admission to hospital (i.e. 'community acquired ARDS') did not change over the same time period and that the reduced levels were entirely attributable to a reduction in hospital or ICU acquired ARDS. It was therefore felt that this was due to improved health care delivery such as the adoption of low tidal volume ventilation strategies, a significant reduction in the amount of blood transfusions, improved and prompt treatment of sepsis, and other general supportive ICU care (Li et al. 2011). Of note however, these findings have not been reproduced elsewhere.

Despite this, the incidence figures quoted in all the above studies are likely to represent a significant underestimate. Firstly, Rubenfeld et al. has shown that the incidence of ALI increases with age from 16 per 100,000 population per year for those 15-19 years of age to 306 per 100,000 population per year for those aged 75-84 years (Rubenfeld et al. 2005), thus a greater incidence would be expected as the population ages. Secondly, although ARDS is known to occur in children and is an important cause of respiratory failure in this group (Flori et al. 2005), none of the above mentioned studies take paediatric cases into account. Finally, all the above studies only focused on cases admitted to the ICU and most only on mechanically

ventilated patients. This undoubtedly represents a significant oversight as Cely et al. prospectively studied 11,465 acute medical and surgical admissions at a Veterans Affairs hospital over a 2 year period, finding that of the 156 cases of ALI/ARDS, 26% were admitted to ICU but not mechanically ventilated and as many as 17% were completely managed outside of the ICU (Cely et al. 2010).

1.1.3 Impact of ALI

As well as being common, ALI/ARDS is a devastating condition with mortality rates of 36-44% (Phua et al. 2009) and as high as 72% in some studies (Manktelow et al. 1997). Patients often develop multi-organ failure and the majority of deaths are related to this, or sepsis, rather than primary respiratory failure (Montgomery et al. 1985).

There has also been some suggestion that these mortality rates have been improving recently. In 2000, a multicenter trial involving 861 patients (The ARDSNet trial) showed that the use of lower tidal volumes (6 ml/kg) during mechanical ventilation in patients with ALI/ARDS resulted in a significant improvement in mortality when compared to the use of higher tidal volumes (12ml/kg), 31% vs 40% respectively (The Acute Respiratory Distress Syndrome Network, 2000). Caution must be used, however, when interpreting results from such randomised clinical trials, as they are often performed in specialized centres and have the potential for selection bias. Further evidence was obtained from a meta-analysis looking at 72 studies between 1994 and 2006, which reported a significant decrease in overall mortality rates of approximately 1.15% per year over the study period and suggested that improvements in ventilation strategies (such as those mentioned above) and general ICU care was the cause of this. However, this analysis has been criticised for including several studies that were primarily conducted in the late 1980s and 1990s and thus not using the 1994 AECC definition for ALI/ARDS. In fact, a more recent meta-analysis showed that although there seems to be a reduction in mortality when looking at observational studies prior to 1994, there has been no change in mortality over time when limiting the analysis to studies that were only conducted after 1994 (Phua et al. 2009). Further, a recent 1 year, multi-centred observational study from Spain reported a hospital mortality rate for ARDS as 47.8% even though all of these patients were receiving lung protective ventilation (Villar et al. 2011). This further highlights the severity of this condition with persistently high mortality rates.

Owing to the severity of this condition, the majority of patients with ALI/ARDS are managed on ICUs. Studies have shown that ALI/ARDS occurs in 3.8-19% of the case mixes of generalised ICUs (Bersten et al. 2002; Brun-Buisson et al. 2004; Fialkow et al. 2002; Hughes et al. 2003; Irish Critical Care Trials Group 2008; Vincent et al. 2010). On the ICU, these patients not only require ventilatory support, making up a significant proportion of ventilator bed days, but often also require multi-organ support.

ALI/ARDS is also associated with significant longer term morbidity. A 5 year follow up study of survivors of ARDS has shown persistent perceived weakness, a reduced 6 minute walk test and reduced physical quality of life (Herridge et al. 2011). Follow-up CT studies have also shown minor non-dependent pulmonary fibrosis, and although the patients in this study had normal or near normal volumetric and spirometric lung function results, similar studies have shown reduced gas transfer and mixed obstructive/restrictive lung defects on lung function testing (McHugh et al. 1994; Orme et al. 2003). Neuro-cognitive morbidity has also been reported, with symptoms of depression occurring in 17-43%, non-specific anxiety in 23-48% and post traumatic stress syndrome in 21-35% of survivors, even after an 8 year follow up period (Davydow et al. 2008). Many of these patients never return to their previous work, adding a significant long term economic burden.

1.1.4 Pathophysiology of ALI

ALI/ARDS has been shown to have many causes. These have traditionally been categorised as direct pulmonary insults (such as pneumonia, aspiration of gastric contents and pulmonary contusion) or indirect pulmonary insults (such as sepsis, shock from any cause, severe trauma, multiple blood transfusions, acute pancreatitis and burns). However the clinical utility of this differentiation is far from clear (Callister and Evans 2002) and these many seemingly disparate causes of ALI/ARDS all result in a similar overwhelming inflammatory process.

During the initial acute phase of ALI/ARDS, histologically, there is evidence of pulmonary vascular endothelial and alveolar epithelial injury, interstitial and alveolar protein-rich oedema and cellular exudate (mainly composed of neutrophils, red cells and macrophages) and prominent hyaline membrane formation in the alveoli (Bachofen and Weibel 1977). Here, as a result of acute inflammation at the alveolar capillary interface there is an initial injury to both the endothelial and epithelial surfaces, resulting in increased permeability with subsequent influx of protein-rich,

highly cellular fluid containing inflammatory cells and pro-inflammatory molecules. This leads to alveolar flooding and pulmonary oedema, increased risk of sepsis, and impaired surfactant synthesis with subsequent alveolar collapse (Ware and Matthay 2000).

After this acute phase, patients often recover gradually, however some with severe epithelial injury progress to a fibroproliferative phase. Here, the alveolar space becomes filled with mesenchymal cells and fibroblasts with resultant collagen deposition and neovascularisation and eventual clinical fibrosis (Proudfoot et al. 2011).

1.2 Neutrophils in ALI

1.2.1 Neutrophils in health

Neutrophils are the most abundant circulating leukocytes in man and play a fundamental role in shaping the innate immune response. The role of these cells in adaptive immunity including antigen processing and presentation, tumour immunology and thrombus formation is also now being recognised (Ostanin et al. 2012). The importance of neutrophils in preserving health is exemplified by patients with congenital or acquired neutropenia who are at high risk of life-threatening bacterial and fungal infections (Klein 2011). To undertake their vital pathogen-killing functions, neutrophils need to be highly motile, respond to the finest of chemotactic trails, and have the ability to kill bacteria even under the most adverse (e.g. hypoxic and acidotic) conditions (Walmsley et al. 2005). Neutrophils are recruited rapidly to sites of inflammation, where their primary role is to kill invading bacteria and certain fungal species. This involves phagocytosis and the release of preformed granular enzymes and toxic oxygen species. However, increased neutrophil accumulation and activation, especially if combined with any delay in their clearance, is also associated with a variety of chronic sterile or pauci-bacterial diseases, including ALI/ARDS, COPD, asthma and rheumatoid arthritis (Cowburn et al. 2008). This suggests that in certain conditions neutrophilic inflammation can become autonomous, chronic and clearly injurious to healthy tissue.

1.2.2 Evidence to support the central role of neutrophils in ALI

The neutrophil has become recognised as a key cell in the pathogenesis of ALI/ARDS. Clinical studies have shown that the accumulation of neutrophils within the pulmonary vasculature occurs early in the evolution of ALI/ARDS (Bachofen and Weibel 1982; Bachofen and Weibel 1977), and neutrophilic pulmonary inflammation is regarded as one of the histological hallmarks of human and experimental ALI/ARDS (Lee and Downey 2001; Matute-Bello et al. 2011). Neutrophilia is extremely common in the bronchoalveolar lavage fluid (BALF) of patients with ARDS. In one study of 16 patients with ARDS, it was found that the BALF taken from these patients within the first 12 hours of endotracheal intubation contained 85% neutrophils on differential counts compared to less than 3% neutrophils for BALF taken from 5 healthy, non-smoking, control subjects (Parsons et al. 1985). In patients with ARDS secondary to sepsis, a higher percentage of neutrophils were found in the BALF of non-survivors compared to survivors (Steinberg et al. 1994) and hence the extent of this BALF neutrophilia appears to correlate with clinical outcome. Furthermore, observational studies in neutropenic patients have demonstrated significant worsening of ALI following recovery of circulating neutrophil counts (Rinaldo and Borovetz 1985).

Studies in animal models offer further support for the importance of neutrophils in the pathogenesis of ALI/ARDS, in that neutrophil depletion or inhibition of neutrophil function invariably ameliorates the extent of ALI/ARDS. In mice, inducing neutropenia prior to hyperoxia (Folz et al. 1999), haemorrhage or experimental endotoxaemia (Abraham et al. 2000) results in attenuation of the increase in vascular permeability and lung injury, as well as reducing levels of pro-inflammatory cytokines such as interleukin 1 beta (IL-1β), macrophage inflammatory protein (MIP2) and tissue necrosis factor – alpha (TNFα). In an acid aspiration model of ALI in rabbits, blocking antibodies to IL-8 given as either before or 1 hour after acid inhalation, resulted in improved oxygenation and a 70% reduction in the extravascular protein accumulation (Folkesson et al. 1995). Similar observations have been made with TNFα-converting enzyme (TACE) blockade in a porcine model of ALI induced with intra-tracheal administration of LPS, which resulted in lower levels of soluble TNFα in the BALF from these animals and less protein leak (Shimizu et al. 2009). Likewise, in a hamster model of ALI, inhibition of neutrophil elastase caused a dose-dependent attenuation of protein accumulation in the airspace (Kawabata et al. 2000).

1.2.3 Accumulation of neutrophils within the lungs

Neutrophils within the blood compartment are distributed (under physiological conditions) between two distinct pools (Athens et al. 1961); firstly, a freely circulating population of cells, and secondly, a group of non-circulating (but still intravascular) cells located within poorly defined pools present in specific organs, notably the liver (Peters et al. 1985b), spleen (Peters et al. 1985b) and bone marrow (Ussov et al. 1995). The latter cells are in dynamic equilibrium with the freely circulating neutrophil population. Cells within the non-freely circulating intravascular pools are often referred to collectively as “marginated” neutrophils, however, this term is best reserved for the pathological pooling that takes place immediately before extravascular migration. The size of an organ pool of neutrophils is the product of the mean transit time of neutrophils across the organ in question and the organ’s blood flow: Neutrophil transit times, and hence the size of the physiological pools, within the liver, spleen and bone marrow are well established (22,23).

It is widely considered that the lung is also an important (and in certain studies the predominant) site of intravascular neutrophil pooling (Hogg and Doerschuk 1995; MacNee and Selby 1990). This view is based on morphometry data in dogs (Hogg et al. 1988) and humans (Hogg et al. 1994) showing a concentration of granulocytes, relative to erythrocytes, within pulmonary capillaries of between 80 and 100 times greater than the concentration of neutrophils seen within vessels in the systemic circulation. Video-microscopic studies in dogs, using fluorescein-labelled granulocytes observed through chest wall windows, showed pulmonary capillaries to be the site of neutrophil hold-up within the lungs (Lien et al. 1987). Complementary techniques utilising the injection of radio-labelled cells have also shown that up to 98% of neutrophils are retained within the lungs on first pass (Doerschuk et al. 1990; Doerschuk et al. 1987; Muir et al. 1984) and that neutrophils take 60-100 times longer to pass through the lungs than erythrocytes (Hogg and Doerschuk 1995). Estimates of the time neutrophils take to pass through the pulmonary circulation (mean transit time) have varied between 40 seconds and 8 minutes (Doerschuk et al. 1987; Hogg et al. 1994; Lien et al. 1987; Schütte et al. 1991; Ussov et al. 1995), which is markedly prolonged compared to erythrocytes (4-12 seconds) (Zavorsky et al. 2003). These prolonged neutrophil transit times have been explained by the need for neutrophils (mean diameter of 7.2 μm (Schmid-Schönbein et al. 1980)) to deform in order to pass through the smallest pulmonary capillaries (mean diameter of 5.5 μm (Guntheroth et al. 1982)) (Hogg 1987).

The pulmonary capillary bed is formed of a complex and multiple bifurcating lattice, with each alveolus engulfed by approximately 1000 capillary segments (Hogg and Doerschuk 1995). This generates an overall alveolar capillary surface area in the order of 60 m^2 (Hogg 1987). The proposed existence of a physiological pool of neutrophils within the lungs would result in the pulmonary capillaries being in significantly greater contact with these potentially destructive cells than other capillary beds, and the lungs perhaps as a consequence, being at particular risk of neutrophil-mediated injury, and hence ALI (Donnelly and Haslett 1992) (see figure 1.2A).

There are several difficulties with this model: Firstly, neutrophil pooling within the lungs of healthy individuals is not generally observed during routine clinical leukocyte scintigraphy (Peters 1998); these scans are most commonly undertaken using re-injected autologous Indium 111 (^{111}In) labelled 'mixed' white cells, which are predominantly but not exclusively comprised of neutrophils. Secondly, the reports of prolonged transit times of neutrophils across the lungs of healthy individuals may have been compromised by the use of techniques for ex-vivo cell purification and labelling that inadvertently damaged, or activated, the neutrophils. Hence deliberate activation of neutrophils has been shown to induce cytoskeletal changes, resulting in decreased neutrophil deformability and increased pulmonary retention. This has been most clearly observed in a rabbit model, where the increased cell stiffness and pulmonary retention induced by N-formyl-methionine-leucine-phenylalanine (fMLP) stimulation was completely prevented by prior treatment of the neutrophils with cytochalasin D (an agent that disrupts cellular actin organisation) (Worthen et al. 1989). Consequently, it is now well established that neutrophil pulmonary transit times may be altered by cell isolation and labelling techniques (Saverymuttu et al. 1983).

Leukocyte scintigraphy in subjects injected with ^{111}In-labelled autologous granulocytes purified using plasma-free labelling media show marked retention of neutrophils within the lungs 5 minutes post-injection; in contrast, scintigraphs obtained in subjects injected with granulocytes purified in the continuous presence of autologous plasma show rapid transit of neutrophils through the lungs with only minimal retention evident at 5 minutes (Peters 1998). Further, evidence to support the hypothesis that inadvertent cell stimulation/damage results in a prolongation of pulmonary transit time can be gleaned from the measurement of labelled neutrophil recovery in the freely circulating pool. Hence, it has been shown consistently that 35-40% of ^{111}In or Technetium-99m (^{99m}Tc) labelled neutrophils (prepared in the

presence of autologous plasma) are recoverable from the circulation 40 minutes post injection (Peters 1988; Peters et al. 1985a; Peters et al. 1983; Roddie et al. 1988; Saverymuttu et al. 1985; Saverymuttu et al. 1983) whereas in many of the studies reporting prolonged neutrophil pulmonary transit times 40 minute recovery values have been considerably lower (Doerschuk et al. 1987; Lien et al. 1987).

Neutrophil priming is a process whereby the response of neutrophils to an activating stimulus is enhanced by prior exposure to a priming agent (Condliffe et al. 1998). A large number of mediators are implicated in neutrophil priming including early-phase cytokines, such as TNFα and IL-1β, endotoxin, chemoattractants such as IL-8, growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), and even activated endothelial surfaces (Summers et al. 2010). Neutrophil activation is therefore very clearly a two-step process, as demonstrated by the observation that neutrophils exposed to the bacterial-derived ligand fMLP display a seven-fold increase in the release of superoxide anions when pre-treated with lipopolysaccharide (LPS), a ligand, which in itself does not activate the oxidase response (Guthrie et al. 1984). Besides enhancement of the neutrophil respiratory burst activity, priming causes inhibition of apoptosis, shedding of cell surface CD62L (L-selectin), up-regulation of cell surface CD11b (Mac-1) and neutrophil polarisation, evident as shape change (Condliffe et al. 1998), this is summarised in Table 1.1. Neutrophil shape change is the result of re-arrangements of the cytoskeletal actin, resulting in reduced deformability and consequently increased retention within the pulmonary circulation (Worthen et al. 1989; Worthen et al. 1987). This was demonstrated in healthy human subjects injected with radio-labelled neutrophils primed ex-vivo with GM-CSF which showed 50% retention within the lungs at 40 minutes compared to 0% retention when non-primed neutrophils were injected (Summers et al. 2009).

Markers of Neutrophil Priming
• Increased generation of reactive oxygen species to an activating agent, representing the enhancement of the neutrophil respiratory burst activity • Neutrophil cell shape change and cell stiffening as caused by modifications in cytoskeletal actin • Enhanced neutrophil cell surface integrin (CD11b/CD18) expression and function • Cell surface shedding of L selectin (CD62L) • Inhibition of neutrophil apoptosis

Table 1.1 Markers and functional consequences of neutrophil priming

Therefore, in contrast to the concept of a large physiological pool of neutrophils in the lung, it is now proposed that the main mechanism whereby neutrophils accumulate within the lungs involves neutrophils becoming primed in the systemic circulation (e.g. by a systemic inflammatory insult such as pancreatitis or sepsis), which then leads neutrophil cell stiffening, and subsequent pathological entrapment and margination within the pulmonary vasculature (see figure 1.2B). Evidence supporting this and the presence of primed neutrophils in ALI/ARDS was seen in a study of 15 patients with ARDS, within whom a subpopulation of neutrophils were identified that had an increased capacity to generate hydrogen peroxide after ex-vivo stimulation – hence, primed neutrophils (Chollet-Martin et al. 1992). A further study, showed an increase in respiratory burst activity of neutrophils (a marker of neutrophil priming) from patients with ARDS, which was far greater than in neutrophils from critically ill patients without ARDS (Zimmerman et al. 1983).

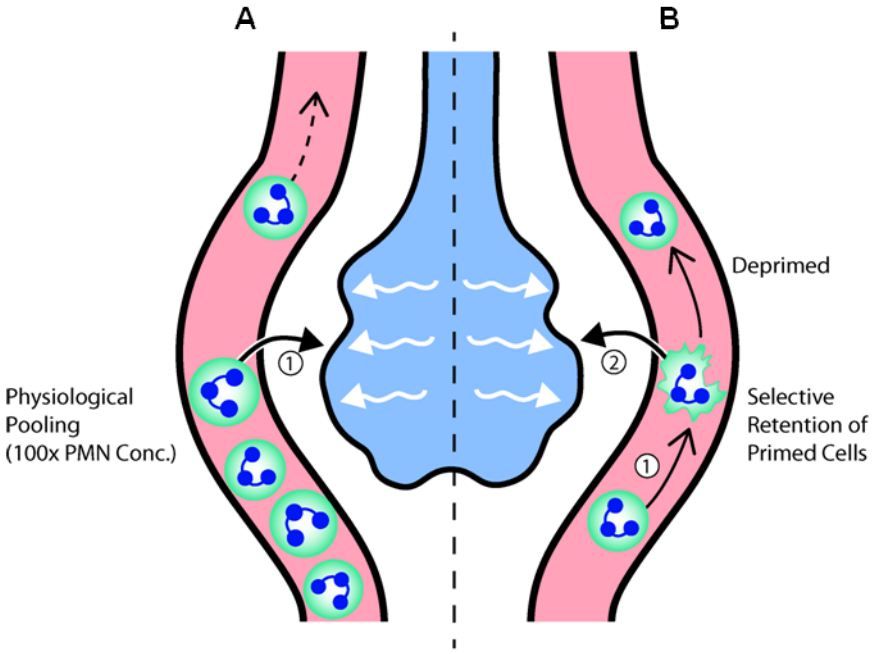

Figure 1.2 Hypothesis: Neutrophil recruitment into the lung is a two-step process.

It is widely reported that there is a substantial pool of neutrophils within the pulmonary vasculature even under physiological conditions, which are recruited readily into the lung in response to pulmonary injury (Panel A). However, clinical leukocyte scintigraphy of healthy individuals does not demonstrate physiological neutrophil pooling in the lungs. We propose that neutrophil recruitment in the lung is a two-step process, with systemic neutrophil priming precipitating intra-vascular neutrophil accumulation [1], and with a second stimulus causing activation and migration from the vasculature into the pulmonary interstitium [2] (Panel B). In the absence of the second insult, neutrophils are actively or passively de-primed and returned to the circulation in a quiescent state.

1.2.4 Neutrophil-mediated lung injury as a 'two hit' process

The priming of neutrophils, and their subsequent accumulation within the pulmonary vasculature, does not, per se, lead to ALI. This statement is best evidenced in sheep given high-dose alveolar endotoxin, which results in a ten-fold increase in the number of neutrophils within the air spaces, but no change in the permeability of the epithelial barrier and morphological studies confirming that the alveolar epithelial barrier was intact (Wiener-Kronish et al. 1991). Similar observations have been made in humans following remote head injury and in rabbits, where intravascular LPS or fNLP resulted in an increase in pulmonary neutrophil sequestration but no increase in vascular permeability, as measured by the pulmonary accumulation of radio-labelled albumin (Worthen et al. 1987).

It is proposed therefore, that ALI only occurs when there is a second, lung-directed insult, which causes actual migration of the primed 'trapped' neutrophils into the pulmonary interstitium and thereafter full activation (Perl et al. 2011). This hypothesis is supported by an in-vitro study, which showed minimal damage to endothelial monolayers when they were co-cultured with neutrophils and either LPS, fMLP or C5a alone, however there was extensive endothelial injury when the neutrophils were pre-treated with LPS and subsequently stimulated with fMLP or C5a (Smedly et al. 1986). This has also been demonstrated in-vivo in mice subjected to either haemorrhagic shock or caecal ligation alone. Here, despite showing signs of systemic neutrophil priming with an increase in neutrophil respiratory burst activity and delayed apoptosis ex-vivo, there was no evidence of lung injury. However, when these mice were given a subsequent septic challenge they developed lung oedema, increased levels of IL-6 in the BALF, and increased parenchymal cell infiltration and alveolar congestion on lung histology (Ayala et al. 2002).

1.2.5 ALI resulting from a failure in pulmonary neutrophil de-priming

The priming of neutrophils was previously considered an irreversible process, however in-vivo neutrophil de-priming is a logical conclusion: Assuming that all neutrophils that pass through an inflammatory focus become primed, and that the life span of a primed neutrophil is no shorter than that of an un-primed one; it can be expected that without de-priming of these neutrophils, all circulating neutrophils (>99%) will become primed within a short space of time. However, studies of inflammatory disorders such as inflammatory bowel disease, systemic vasculitis, and graft versus host disease; have found that only up to 60% of circulating

neutrophils to be in a primed state, as assessed by cell shape change (Ussov et al. 1999; Ussov et al. 1996).

Evidence of in-vitro reversible neutrophil priming has been shown with human neutrophils primed with platelet activating factor (PAF). These cells displayed a return to baseline values of superoxide anion generation, cell polarisation and CD11b activity after 120 minutes (Kitchen et al. 1996). Further, once these cells had de-primed, they were capable of re-priming with subsequent stimulation by PAF or TNFα, thus showing that neutrophils are capable of undergoing a complete cycle of priming, de-priming and re-priming. Reversible neutrophil priming has also been demonstrated in-vivo, where radio-labelled neutrophils primed with PAF were injected into healthy human subjects and shown to accumulate only transiently in the lungs (Summers et al. 2009). A further study where healthy human subjects inhaled PAF (representing pulmonary infection) showed similar results of only a transient sequestration of neutrophils within the lung (Tam et al. 1992). In both studies, after de-priming, the neutrophils were released back into the systemic circulation, without evidence of migration into the pulmonary interstitium.

The enormous size of the pulmonary capillary network, with a surface area in the region of 60 m^2, combined with the lungs being the only organ to receive the entire cardiac output, makes the pulmonary vasculature an ideal site for neutrophil de-priming, offering protection to the systemic circulation. It is proposed that the healthy pulmonary vasculature can retain primed neutrophils, facilitate their de-priming, and later release them into the systemic circulation in a quiescent state (see figures 1.3A and B). Further, it can be hypothesised, that when this protective mechanism fails, or is overwhelmed, pulmonary inflammation ensues (see figure 1.3C). This could explain the observation that whilst the priming status of circulating neutrophils correlates with the severity of lung injury, it does not correlate with the clinical severity in critical illnesses without lung injury (Chollet-Martin et al. 1992), as in these patients with a functional pulmonary vasculature, the primed neutrophils will be undergoing de-priming.

The concept of the pulmonary vasculature playing a protective role is not new and is thought to explain the phenomena of cerebral abscesses occurring in patients with macroscopic pulmonary arteriovenous malformations (Faughnan et al. 2000), as in these patients, a significant proportion of blood by-passes the capillary circulation in the lungs. However evidence to support this new hypothesis that the lungs might

act as a site for neutrophil entrapment and de-priming can be found in studies looking at gradients of neutrophil priming across the pulmonary circulation.

A study in rats examined the gradient of primed neutrophils across the lungs based on cytoskeletal F-actin rearrangement (62). Four hours after the instillation of Streptococcus in the lungs, it was observed that neutrophils entering the lungs were more primed than those leaving, and that this was associated with a fall in the circulating neutrophil count. As no trans-pulmonary priming gradient was seen prior to the inducement of pneumonia, this suggested that primed neutrophils were being selectively taken up by the pulmonary circulation (Yoshida et al. 2006). A further study in patients with sepsis without lung injury, patients with lung injury, and perioperative control patients examined trans-pulmonary neutrophil priming gradients by measurement of neutrophil hydrogen peroxide (H_2O_2) production after ex-vivo activation with zymosan (Nahum et al. 1991). In keeping with the previous study, the perioperative controls displayed no trans-pulmonary priming gradient. However, in the patients with sepsis alone, neutrophils in the mixed venous blood (i.e. blood entering the lungs) produced more H_2O_2 in response to zymosan than neutrophils in arterial blood (i.e. blood exiting the lungs), suggesting again that in patients with sepsis neutrophils are primed predominantly in the peripheral circulation and then extracted and/or de-primed within the lungs. This trans-pulmonary gradient was reversed in the patients with lung injury, where neutrophils leaving the lungs were more primed than neutrophils entering, suggesting that in these circumstances, the lungs themselves can act as a site of neutrophil priming.

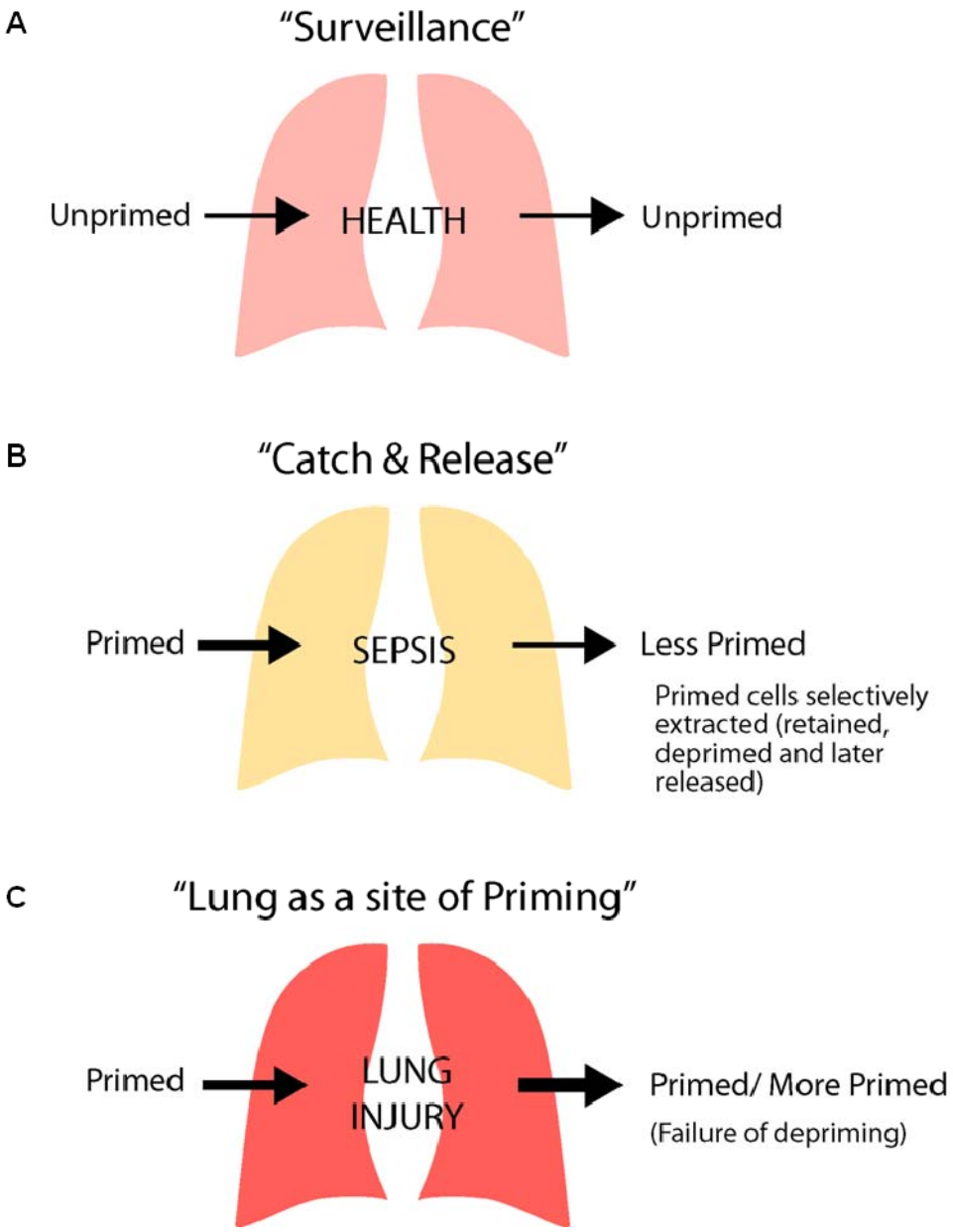

Figure 1.3 Hypothesis: ARDS results from a failure of neutrophil de-priming

The healthy pulmonary vasculature is able to selectively retain primed neutrophils, allow them to de-prime, and later release them into the circulation in a quiescent state. This "catch and release" mechanism is effective in disease states such as systemic sepsis, which result in high levels of primed neutrophils entering the pulmonary circulation. However when it fails, as a result of pulmonary insults such as infection or ventilator-induced barotrauma, or is overwhelmed, neutrophil-mediated pulmonary inflammation, such as that seen in ARDS occurs (Panel C) and the lung itself becomes a site of neutrophil priming.

1.3 Experimental models of ALI

1.3.1 Animal models

Much of our knowledge of the pathophysiology of ALI has come from animal models, where lung injury has been induced by insults targeted at either the alveolar epithelium (to represent pulmonary causes of ALI), or the capillary endothelium (to represent extra-pulmonary causes of ALI) (Matute-Bello et al. 2008) (see table 1.2).

Direct Lung Injury	Indirect Lung Injury
Intratracheal/intranasal bacteria or LPS Intratracheal acid High tidal volume ventilation Hyperoxia Intratracheal bleomycin	Intravenous bacteria or LPS Caecal ligation and puncture Peritonitis Intravenous oleic acid Haemorrhagic shock

Table 1.2 Experimental methods used to reproduce acute lung injury in animal models

However, extrapolation of animal data to humans is less than straightforward. Many of the clinical observations required to define ALI in a human setting are impractical in animal models, and hence most animal studies rely on histopathological criteria of diffuse alveolar damage (these include parenchymal inflammatory infiltration, thickened alveolar septae, the deposition of hyaline membranes and the presence of microthrombi) and assessment of increased permeability of the alveolar-capillary membrane (Matute-Bello et al. 2011). Although experimental models can reproduce components of the changes observed in ALI/ARDS, none have been able to recapitulate the human disease in its entirety. For example, although, administration of E.coli endotoxin in sheep causes a neutrophilic inflammatory response with an increase in intrapulmonary cytokines, there is only mild alveolar neutrophilia and only minimal changes in alveolar-capillary permeability (Wiener-Kronish et al. 1991).

Most models of ALI are based on one or very occasionally two methods for causing injury, however, in humans ALI/ARDS is often the result of multiple primary insults, clouded by co-morbidities, as well as secondary insults from therapeutic

interventions such as mechanical ventilation and secondary infection (Matute-Bello et al. 2011).

Further, marked differences exist in lung anatomy and physiology between the animals and humans. For example mice have no bronchial arteries and have a larger number of Clara cells in the distal airways compared to humans, they also rarely demonstrate hyaline membrane deposition during ALI (Matute-Bello et al. 2011). Fundamental differences also exist, in neutrophil function and the inflammatory response. For example, mice have far fewer circulating neutrophils and a different set of neutrophil CXC chemokines as compared with humans (Matute-Bello et al. 2008) and do not 'prime' to anything like the extent observed in human cells (Condliffe et al. 2005).

Thus animal models of ALI correlate poorly with the human condition; this is highlighted by the fact that although several agents have shown benefit in various experimental models of ALI, to date no pharmacological therapy has proved successful in a clinical setting (Matthay and Zemans 2011).

Finally, problems with animal size often cause difficulty with accurate physiological measurements such as arterial oxygen tension, and also obtaining repeated samples such as blood or BALF.

1.3.2 Human models

Elective oesophagectomy, an operation normally performed for resection of oesophageal carcinomas, is associated with high post-operative rates of respiratory complications and commonly ALI/ARDS (Baudouin 2003). Retrospective studies have shown an incidence of 15-17% for ARDS (Millikan et al. 1995; Tandon et al. 2001) and 24% for ALI. Small prospective studies have shown higher incidences still, of 33% for ARDS (Schilling et al. 1998) and 41% for ALI (Morita et al. 2008). Further, Rocker et al. looked at pulmonary vascular permeability to transferrin in 9 subjects undergoing oesophagectomy, and showed a significant increase in permeability in both lungs at 8 hours post surgery, which was accompanied by increased intra-alveolar neutrophil elastase release and arterial hypoxaemia. Hence, although, none of these subjects developed overt ARDS, all showed sub-clinical evidence of lung injury (Rocker et al. 1988).

Most oesophagectomy procedures require a period of one lung ventilation, whereby, for a significant proportion of time during the operation only one lung is being

ventilated and the other is allowed to collapse, thus allowing better surgical access. The main causes of ALI/ARDS in this setting appear to be:

1) Barotrauma and volutrauma to the ventilated lung. Mechanical ventilation at high volumes and pressures is well known to cause ventilator-induced lung injury. Webb et al. have shown that rats ventilated with peak airway pressures of 45 cmH_2O become markedly hypoxic and die within an hour having developed marked perivascular and alveolar oedema. In contrast, rats ventilated with a peak airway pressure of 30 cmH_2O showed only mild perivascular oedema while those ventilated at 14 cmH_2O showed no histological changes (Webb and Tierney 1974). When lung pressure was dissociated from volume in rats whose tidal excursion was limited by strapping the chest and abdomen, it was found that high airway pressure alone without a high tidal volume did not produce lung injury (Dreyfuss et al. 1988). This is in contrast to animals that developed severe lung injury after high tidal volume ventilation achieved by negative pressure in an iron lung (Whitehead and Slutsky 2002). Not only does barotrauma and volutrauma lead to mechanical damage by physical disruption but they also induce a pro-inflammatory state that causes further damage.

   Vlahakis et al. showed that, in cultured human alveolar epithelial cells, cyclic cell stretch (in a stimulus or rate-dependent manner) upregulated the production and release of IL-8 even in the absence of structural cell damage or paracrine stimulation (Vlahakis et al. 1999). Wilso et al. have shown in an in-vivo mouse model that high tidal volume ventilation results in increased protein and cytokine concentration in lung lavage fluid, hyaline membrane formation, and increase pulmonary neutrophil sequestration (Choudhury et al. 2004; Wilson et al. 2003). Further clinical evidence of barotrauma/volutrauma is seen in the results of the ARDSNet trial (mentioned above) showing a worsened mortality in patients receiving high tidal volume ventilation (12 ml/kg) compared to those receiving lower volumes (6 ml/kg) (The Acute Respiratory Distress Syndrome Network 2000). This has been correlated with reduced levels of pro-inflammatory mediators and neutrophils in the BALF of patients with ARDS after receiving 36 hours of low tidal volume ventilation compared to conventional (higher tidal volume) ventilation (Ranieri et al. 1999). Hence, during one lung

ventilation, relatively high tidal volumes are often used and could result in significant barotrauma/volutrauma, especially to the residual inflated lung.

2) Ischaemia-reperfusion injury to the collapsed lung. During one lung ventilation, the collapsed lung is subjected to hypoperfusion and hence ischaemia. Hypoperfusion by itself does not necessarily result in lung injury. Hence, in a study looking at isolated blood-perfused rodent lungs, there was no appreciable change in pulmonary vascular permeability after 30 minutes of ischaemia compared to continuously perfused lungs. However, reperfusion for a further 30 minutes resulted in significant increase in pulmonary permeability to albumin (Messent et al. 1993). This has been further demonstrated in an in-vivo rodent study, whereby rodent lungs underwent right sided one lung ventilation for 30 minutes followed by either right sided pneumonectomy or right sided re-inflation. Both of these groups developed hypoxaemia, a rise in pulmonary artery pressure, and significantly increased extra-vascular albumin accumulation of both lungs compared to lungs that underwent continuous ventilation. However, the extra-vascular albumin accumulation (and hence increase vascular permeability) was significantly greater in the left lung following right lung re-inflation compared to the left lung following right pneumonectomy (Williams et al. 1999), suggesting that injury occurs during reperfusion. It was also suggested that reactive oxygen species (ROS) may be responsible as there was an increase ROS production in both experimental groups and the lung injury was attenuated by pre-treatment with superoxide dismutase (a scavenger of ROS) (Williams et al. 1999). Clinically, ischaemia-reperfusion injury has been seen after rapid re-expansion of a collapsed lung after pulmonary resection (Williams et al. 1998), removal of thrombus in massive pulmonary embolus (Jamieson et al. 1993) and pulmonary transplantation (Alam and Chan 1996).

Other possible causes of ALI/ARDS following oesophagectomy include endotoxaemia caused perioperatively by GI tract resection or postoperatively by anastomotic leaks. This is supported by observational studies showing higher rates of ALI in patients with antastomotic leakage (Morita et al. 2008) and increased levels of serum endotoxin levels in patients who developed ARDS post oesophagectomy (Moriyama et al. 1995). However, it has also been proposed that

some cases of anastomotic leakage may be the result of, rather than the cause of, ALI/ARDS, due to an adverse influence of ALI/ARDS on wound healing (Morita et al. 2008).

Finally, as with most major surgery, there is release of pro-inflammatory cytokines, which may also add to the lung injury burden (Tandon et al. 2001).

Elective oesophagectomy therefore, offers a useful model of ALI/ARDS where it is possible to time the insult. Similarly, subjects undergoing one lung ventilation for pulmonary resections also may offer a suitable experimental model of human ALI/ARDS, although the incidence of ALI in these patients are considerably less (4-7%) (Jordan et al. 2000).

Inhalation of LPS has been used widely in animal models of ALI/ARDS, and cautious use in humans has also shown subclinical lung inflammatory changes including BALF neutrophilia and elevated cytokine and chemokine profiles, as well as increased pulmonary vascular permeability to albumin (O'Grady et al. 2001; Sandström et al. 1994). Repeated LPS administration has been associated with minor local side effects (e.g. cough) and systemic effects (e.g. fatigue) all of which resolve within 10 hours, and its use therefore, is considered a safe and tolerable tool to investigate lung inflammatory responses (Kitz et al. 2008). Further, LPS inhalation in healthy subjects was used in a recent study to demonstrate the anti-inflammatory effects of the HMGCoA-reductase inhibitor, simvastatin (Shyamsundar et al. 2009), the positive results of which have led to a randomised clinical trial of simvastatin in patients with established ALI (Craig et al. 2011).

Finally, the use of ex-vivo perfused human donor lungs is now also being developed as an experimental model of ALI/ARDS (Proudfoot et al. 2011). Here, lungs that are unsuitable for transplantation are maintained with ongoing ventilation and perfusion systems. ALI/ARDS can then be simulated by insults similar to those used in animal models. This negates the problems of species specific differences associated with animal models but still allows for timed insults and control of single and multiple variables.

1.4 Hypotheses and Aims

1.4.1 Hypotheses

1) ALI/ARDS remains under-recognised and under-reported, and is an ongoing cause of morbidity and mortality in critical care.
2) Systemic neutrophil priming/activation represents a unified pathogenic common factor in ALI. It is proposed that while the healthy pulmonary vascular bed can extract these cells and facilitate de-priming, failure of this protective mechanism can lead to fulminate lung injury.
3) Oesophagectomy is a common cause of ALI and can be utilised as a model to study trans-pulmonary neutrophil trafficking in ALI.

1.4.2 Aims

1) Prospective evaluation of the incidence and impact of acute lung injury in a UK adult ICU.
2) Measurement of trans-pulmonary gradients in neutrophil priming in (i) pre-operative subjects, (ii) subjects with peripheral sepsis but no evidence of lung involvement, and (iii) subjects with ARDS from any cause.
3) Prospective evaluation of incidence of ALI post oesophagectomy.
4) Establishing methodology allowing to the assessment of neutrophil transit times pre and post-oesophagectomy.

2 Incidence and Impact of Acute Lung Injury in a UK Teaching Hospital Intensive Care Unit

2.1 Introduction

ALI/ARDS is associated with significant morbidity and mortality and these conditions utilise a significant proportion of critical care facilities (Bernard et al. 1994). Although the current definition of ALI/ARDS has not changed since the American-European Consensus Conference (AECC) in 1994 (Bernard et al. 1994), there is a 7 fold difference in the reported incidence of this condition, with ALI/ARDS being recognised in 2.5-19% of ICU patients (Bersten et al. 2002; Brun-Buisson et al. 2004; Fialkow et al. 2002; Hughes et al. 2003; Irish Critical Care Trials Group 2008; Vincent et al. 2010; Webster et al. 1988). The reported death rates for ALI/ARDS also vary substantially, with values for in-hospital mortality ranging from 23 to 72% (Zambon and Vincent 2008). Further, there is some evidence that following the introduction of low tidal volumes in the management of patients at risk of ALI, and improvements in general supportive ICU care, that there has been a reduction in the incidence and mortality rates amongst these patients (Li et al. 2011).

Despite the above, there have been no recent or current UK prospective population based studies aimed at identifying the true incidence and impact of ALI and ARDS. Of note, the Intensive Care National Audit and Research Centre (ICNARC) Case Mix Programme, which collects data from a majority of general ICUs in England, Wales and Northern Ireland, is unable to accurately identify patients with lung injury (Dushianthan et al. 2011). To address this we undertook a 6 month, prospective study to determine the incidence and outcome of ALI and ARDS in a general adult ICU within a large UK teaching hospital with a particular focus on accurate case validation, ICU, in-hospital and long term (900 day) mortality, and the specific recognition of this condition at the time of ICU and hospital discharge, hospital coding and death certification.

2.2 Methods

This study was approved by the Cambridgeshire 3 Ethics Committee (08/H0306/17) and conducted in the John V Farman general adult ICU of Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust. This is a 20-bedded general ICU, which serves a 1250-bedded University Hospital with a local

catchment of 350,000 and a wider regional referral base of approximately 2 million people in the East of England. Addenbrooke's Hospital forms part of Cambridge University Hospital NHS Foundation Trust and has the second lowest standardised mortality rate of any hospital in the UK at 0.80 (www.drfosterhealth.co.uk).

A dedicated clinical research team, not involved in routine patient care, undertook a prospective study of all patients admitted onto the John Farman ICU between 1st January – 30th June 2009. The clinical staff on the unit were not aware of the aim of this data collection exercise. The initial data set for each patient included; age, gender, date of hospital admission, date of ICU admission, primary reason for admission, source of admission (emergency vs. elective), and the admission Acute Physiology and Chronic Health Evaluation (APACHE II) score. Ongoing data were collected on a daily basis for each patient and included arterial blood gas results, details regarding the mode of ventilation and fraction of inspired oxygen (FiO_2) levels, and the appearances of anterior-posterior (frontal) chest radiographs. This continued daily for each patient until they were discharged from the ICU. Patients were followed up to determine their date of hospital discharge and their survival status at 28 days, 6 months and 2 years. This was done using the hospital electronic medical records for each patient and by telephoning the patients known last GP. If the survival status was unable to be determined at any of these times than the data for the last known survival status was used. Patients who had recurrent ICU admissions for the same complaint during their hospital stay were counted as single admission with a combined total length of stay and ventilator days. This data collection was carried out by 2 dedicated research nurses; Ms Linda Worpole and Ms Rosalind Simmonds.

The above information was populated onto a database by Ms Rosalind Simmonds and screened initially for all patients with a PaO_2/FiO_2 ratio of less than 40 KPa (300 mmHg) and bilateral opacities on their chest radiographs. All of the patients identified, then had their past and current medical notes, including ICU charts, and the results of all hospital investigations and chest radiographs reviewed by myself (a trainee respiratory physician - independent of the initial data collection), to confirm or exclude the diagnosis of ALI or ARDS as per the 1994 AECC criteria. These were disease of acute onset, associated with bilateral infiltrates on a frontal chest radiograph, the absence of clinical left atrial hypertension, and a PaO_2/FiO_2 ratio of less than 40 KPa for ALI and less than 27 KPa for ARDS (Bernard et al. 1994). An additional criterion was included in that the PaO_2/FiO_2 ratio criteria had to be met on 2 arterial blood gas results taken at least 6 hours apart. This is in-line with other

clinical studies and drug trials in ARDS (Gao Smith et al. 2012) and was initiated to ensure that the result of hypoxaemia was not transient as may be the case, for example, in patients with acute airway obstruction secondary to sputum plugging. Assessment of left atrial hypertension was based on objective criteria when available, and patients were excluded if they had biochemical or electrical evidence of an acute myocardial infarction, previous or current echocardiogram reports showing moderately (or greater) dilated left atrium or moderately (or greater) left ventricular dysfunction, or an enlarged cardiac silhouette on a recent posterior-anterior chest radiograph.

Patients who were not identified by the database screening tool were considered to have no acute lung injury (NLI). A randomly generated sample of 20% of these patients also had their cases reviewed by myself, in the same manner as above to confirm the absence of ALI/ARDS.

For those patients who had a confirmed diagnosis of ALI/ARDS, I obtained copies of their hospital discharge summaries and official medical coding and determined if they had received hospital follow-up following discharge. The death certificates for those patients who died in hospital were also reviewed.

2.2.1 Statistical analysis

Data were analysed using GraphPad Prism 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Categorical variables were described using proportions and analysed using Fisher exact and chi-squared tests. Continuous variables were described using median (inter-quartile range) unless otherwise stated, and analysed using Mann-Whitney and Kruskal-Wallis tests with Dunn's post test. All tests were 2 tailed. Survival curves were compared using log-rank (Mantel-Cox) test. A *P* value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 Incidence of ALI and ARDS

Three hundred and forty four patients were admitted onto the ICU during the study period. Of these, 115 patients were identified as potentially having ALI/ARDS, however after these patients had their past and current medical notes, including ICU charts, and the results of all hospital investigations and chest radiographs reviewed,

72 of these cases were subsequently excluded. Reasons included; 28 cases showing evidence of cardiogenic pulmonary oedema, 12 cases had venous blood gases recorded as arterial and therefore were not as hypoxic as initially thought, 12 cases had significant pleural effusions as oppose to infiltrates on their chest radiographs, 9 cases had evidence of chronic radiological changes such as interstitial lung disease (ILD), 5 cases had unilateral changes on their chest radiographs only, 2 cases had evidence of pneumothoracies with evidence of surgical emphysema hence making interpretation of pulmonary infiltrates difficult and giving an additional reason for hypoxia, 2 cases had evidence of pulmonary haemorrhage, 1 case was the result of fluid overload in the context of anuria and 1 case was suffering from hypoxia secondary to an exacerbation of COPD. Thirty one patients (9%) were confirmed as having developed ARDS and 12 (3.5%) as having ALI, giving a combined ALI/ARDS incidence of 43 (12.5%) patients, (see figure 2.1). The baseline characteristics of each patient group is summarised in Table 2.1. There were no differences between patients with ARDS, ALI and NLI in terms of age-sex distribution with a mean age of 60.7 years (+/-18.3) for all ICU patients,

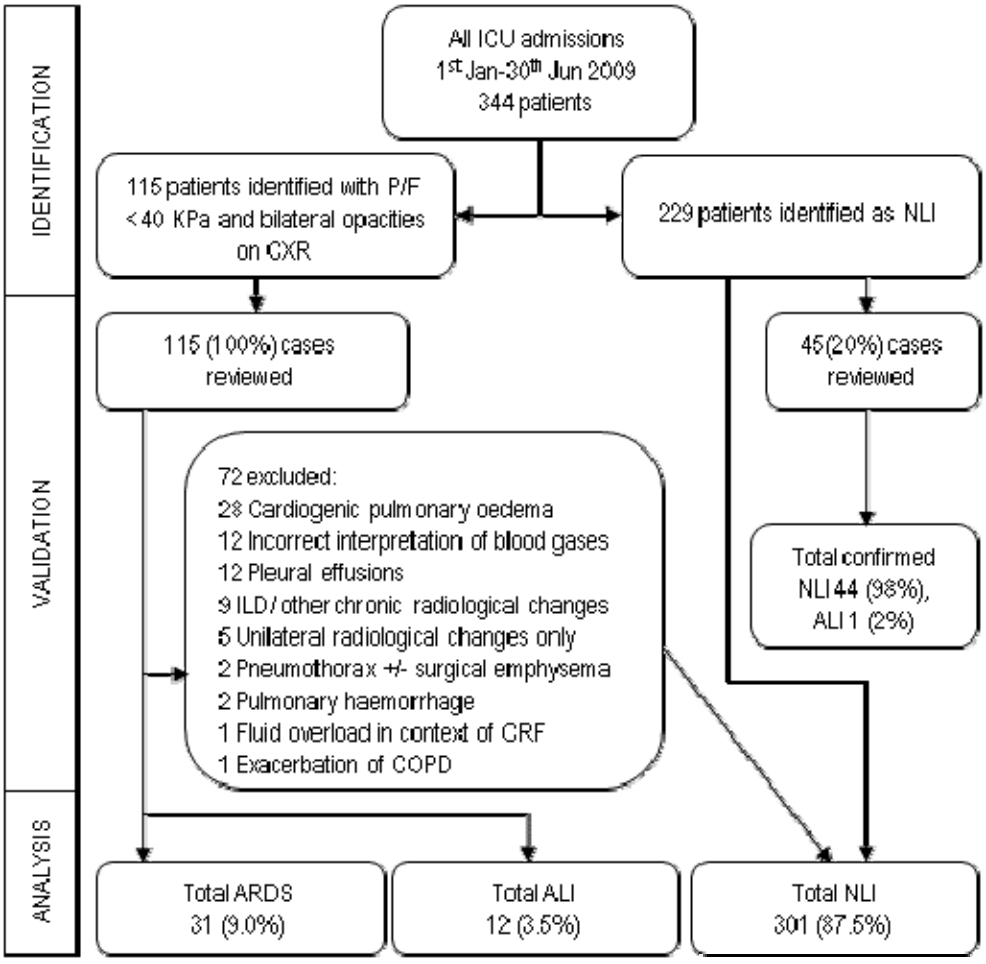

Figure 2.1 Schematic showing the identification and validation of ALI (n= 12), ARDS (n = 31) and no lung injury (NLI; n = 301) patients

	NLI	ALI	ARDS
n (%)	301 (87.5)	12 (3.5)	31 (9.0)
Mean age (years (median))	60.7 (63)	67.2 (70.5)	57.1 (57)
Male (%)	56.8	58.3	48.4
Patients admitted to ICU electively (%)	31.2	41.7	22.6
APACHE II score (mean (median))	17.4 (16)	21.4 (19.5)**	21.2 (20)**

Table 2.1 Baseline patient characteristics

NLI - no lung injury, ALI – acute lung injury, ARDS – acute respiratory distress syndrome, APACHE II - Acute Physiology and Chronic Health Evaluation II on ICU admission. Values represent absolute or percentage values as indicated. (**p<0.01 comparing ARDS and ALI with NLI).

and with males making up 56.3%. The majority of ICU admissions were emergency in nature, with elective ICU admissions making up only 31% of the ICU population. The admission APACHE II score however, was significantly greater for patients who either had, or developed, ARDS with a median score of 20 (17-25), compared to patients with NLI who had a median score of 16 (13-21), ($p<0.01$).

Despite the higher APACHE II scores, patients identified as having ARDS spent a median of 4 (2-18) days on the hospital wards prior to ICU admission, which was significantly greater than that for NLI patients who spent a median time of 2 (1-7) days ($p<0.05$), (Figure 2.2A). Sixty eight percent of these ARDS patients already had evidence of ALI on ICU admission (Figure 2.2B). No differences were found when comparing the time to ICU admission of patients who had developed ALI (median 3 (2-18.5) days), with the NLI group (Figure 2.2A), however 50% of these ALI patients also had evidence of ALI on ICU admission (Figure 2.2B).

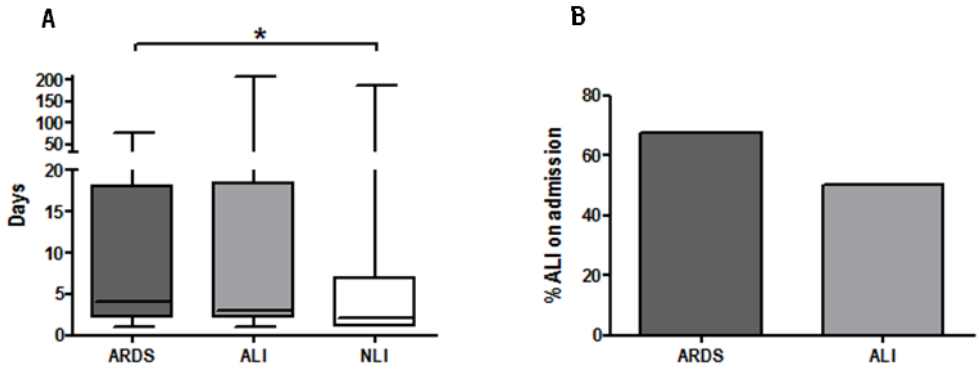

Figure 2.2 Days in hospital prior to ICU admission and percentage of patients with ALI/ARDS on admission to ICU

Panel A - Time from hospital admission to ICU Admission for patients with an eventual diagnosis of ALI (n=12), ARDS (n=31) or no lung injury (n=301). Data represent median, inter-quartile ranges and full ranges. (*$p<0.05$ comparing ARDS and NLI groups).

Panel B Percentage of patients with a final diagnosis of ALI/ARDS (n=43) who had evidence of ALI on ICU admission.

Chest sepsis secondary to community, hospital, ventilator or aspiration acquired pneumonia was the biggest cause of both ALI and ARDS, contributing to 67% and 68% of diagnoses respectively. Other causes included sepsis from a non-

pulmonary source, including septic shock, transfusion related acute lung injury (TRALI) and trauma (see Table 2.2).

	ARDS	ALI
Chest sepsis	21 (67.7%)	8 (66.7%)
Sepsis, non-pulmonary source	6 (19.4%)	3 (25%)
Transfusion related ALI	3 (9.7%)	1 (8.3%)
Trauma	1 (3.2%)	0

Table 2.2 Underlying cause of ARDS and ALI - Values represent; n (%)

2.3.2 Outcome of ARDS and ALI

Patients with either ARDS or ALI required longer periods of invasive ventilation with medians of 9 (3-20.5) and 11 (2-15) days respectively, compared to NLI patients, median 0 (0-2) days (p<0.001 and p<0.01 respectively) (Figure 2.3A). This corresponds with significantly less ventilator free days within the first 28 days following ICU admission for patients with ARDS and ALI with medians of 5 (0-10.5) and 15 (0-26) days respectively, compared to NLI patients, median of 27 (24-28) days (p<0.001 and p<0.05 respectively) (Figure 2.3B).

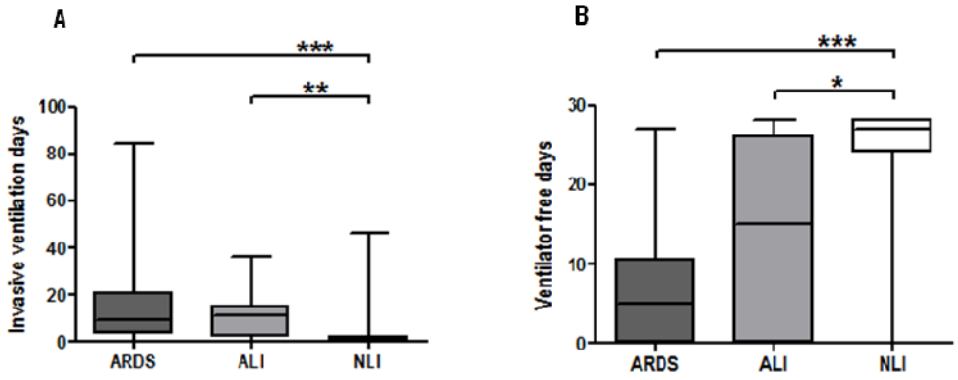

Figure 2.3 Number of days ventilated and ventilator free days on the ICU for patients with ARDS, ALI and NLI

Panel A - Number of days patients spent on invasive mechanical ventilation; Panel B - Number of ventilator free days during the patients first 28 days of ICU admission. Data represent median, inter-quartile ranges and full ranges. (*p<0.05, **p<0.01, ***p<0.001 comparing ALI or ARDS and NLI groups).

Patients with ARDS and ALI spent significantly longer on the ICU with medians of 13 (5-23) and 15 (3.25-22.25) bed days respectively, compared to NLI patients, median of 3 (2-6.5) bed days (p<0.001 and p<0.01 respectively) (Figure 2.4A). However, there were no significant differences between the 3 patient groups in total lengths of hospital stay (Figure 2.4Bi); however when the effect of the increased mortality rate in the ALI/ARDS groups was accounted for (by looking at the length of hospital stay in survivors only), patients with ARDS had significantly increased hospital lengths of stay with a median of 45 (29-99) bed days, compared to NLI patients who had a median of 24 (12-41) days (p<0.05) (Figure 2.4Bii).

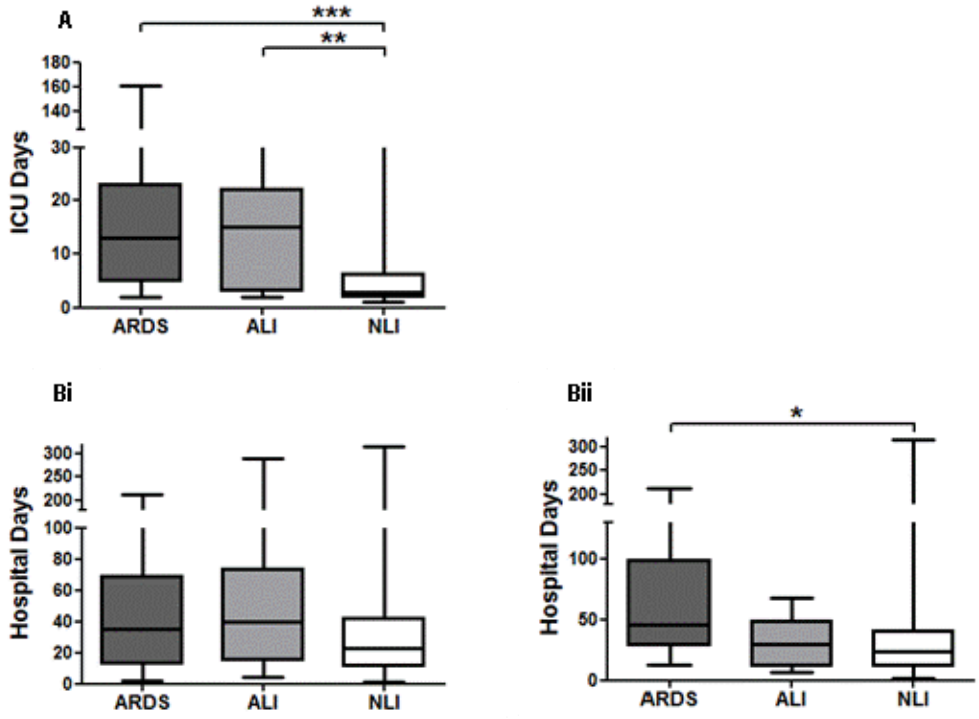

Figure 2.4 Lengths of ICU and hospital stay for patients with ALI, ARDS and NLI

Panel A - Total number of days patients in each group spent on the ICU during their entire hospital admission, Panel Bi - Total length of hospital stay in days including deaths, and Panel Bii – Total length of hospital stay in days for patients alive on hospital discharge. Data represent median, inter-quartile ranges and full ranges (*p<0.05, **p<0.01, ***p<0.001 comparing ALI or ARDS and NLI groups).

Patients with ARDS had ICU and hospital mortality rates of 42% and 55% (42.5% and 54% age adjusted) respectively; patients with ALI had similar rates of 42% and 50% (31% and 35.5% age adjusted). Both of these were significantly greater than

the ICU and hospital mortality rates for NLI patients of 11% and 21% (11% and 21% age adjusted) (p<0.001, p<0.05), (Figure 2.5Ai and Aii). The 28 day and 6 month ICU mortality rates were 45% and 58% for patients with ARDS respectively, 25% and 50% for ALI patients and 15% and 24% for NLI patients. Only three of the 301 patients in the NLI group were lost to follow up and allowing for this, by 2 years the mortality rates had increased to 61% for patients with ARDS, 58% for patients with ALI and 35% for NLI patients.

Figure 2.5B shows the survival profiles of each patient group. Patients with ARDS and ALI have a significantly reduced survival with a median survival of 45 and 282 days respectively, compared to NLI patients who have an overall survival rate of 64% at 900 days post ICU admission (p<0.001 and p<0.05 respectively).

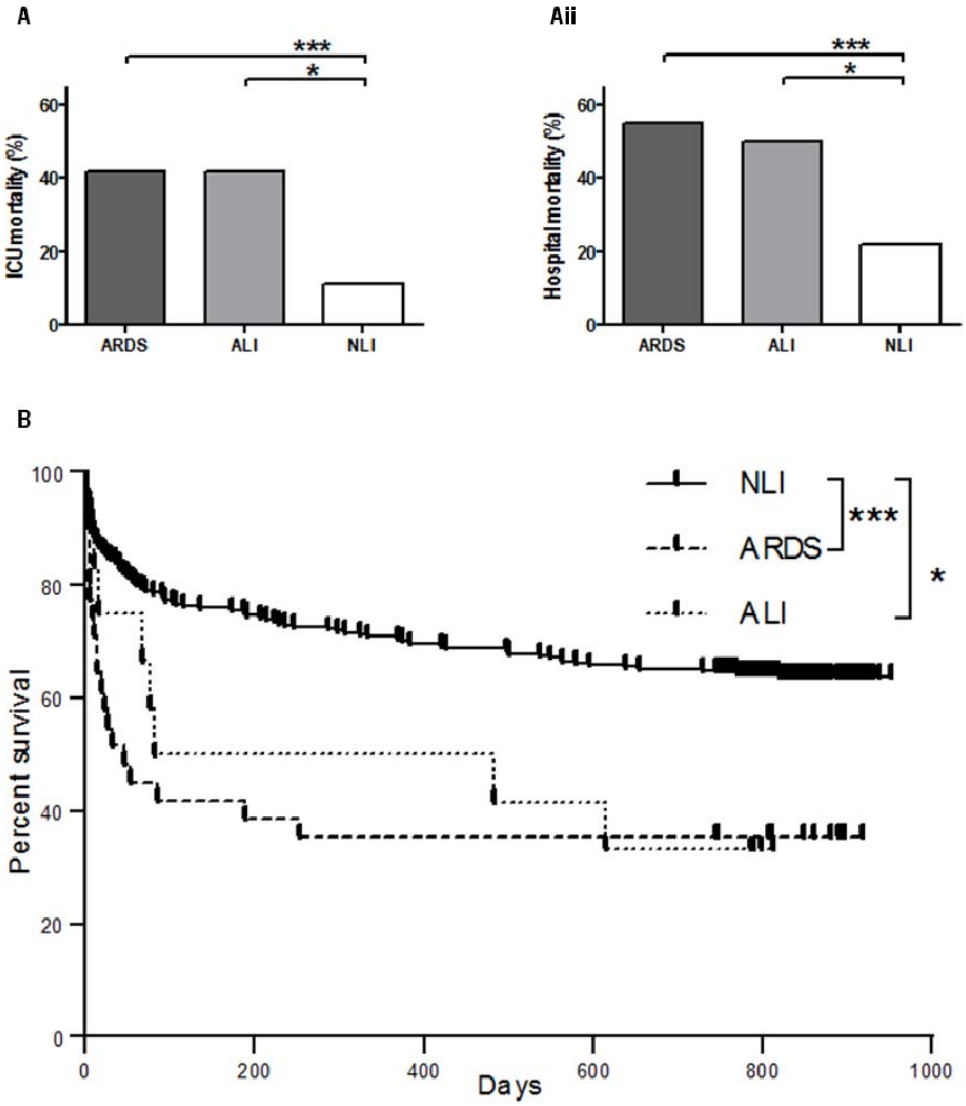

Figure 2.5 Survival and mortality data for patients with ALI, ARDS and NLI

Panel Ai –Mortality rates in ICU (%), Panel Aii –mortality rates in hospital (%), and Panel B – Kaplan-Meier plot showing the percent survival over time (days) for the three patient groups up to 900 days. Data represent the absolute percentage values for n = 12 ALI, n = 31 ARDS and n = 301 NLI patients (reduced to 298 patients at 900 days). (*p<0.05, ***p<0.001 comparing ALI or ARDS and NLI groups).

2.3.3 Recognition of ARDS and ALI

Despite a hospital protocol that all patients admitted to our hospital should receive a discharge summary irrespective of the mode of discharge including death, of the 43 patients with ALI/ARDS only 20 had a completed electronic hospital discharge summary. Nearly all of the patients with missing discharge data had died in hospital. Moreover, of these 20, only 1 (5%) made reference to the development of ARDS during the admission. Of the patients that died prior to hospital discharge only 1 (4%) had a medical death certificate where ALI/ARDS was specified as a contributing cause of death, although many of the other death certificates (48%) had 'multiple organ failure' as a cause of death. The national coding system only codes for ARDS and has no designation for ALI. Of the 31 patients with ARDS only 2 (7%) were coded as such by the hospital's coding department. Of the 19 patients with ALI/ARDS that were alive on hospital discharge, 2 (11%) were followed up in the respiratory clinic (Figure 2.6).

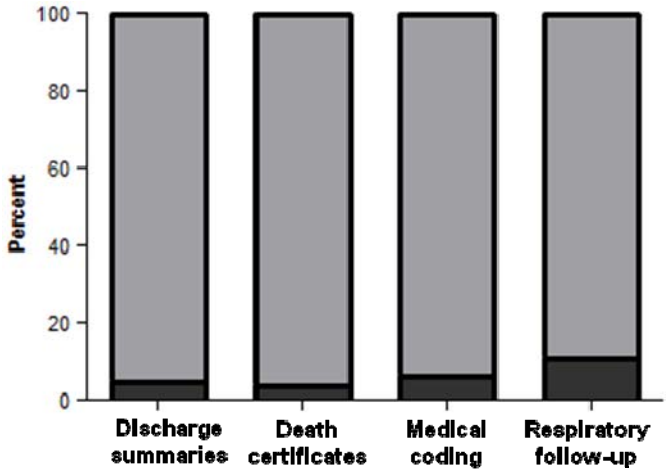

Figure 2.6 Recognition and documentation of the diagnosis ALI/ARDS

Dark shading represents percentage of patients with ALI or ARDS whose diagnosis was included in their hospital electronic discharge summary (n = 20), death certificate (for those patients who died in hospital, n = 19), official hospital medical coding (n = 31), and for those discharged alive who were followed up in a respiratory clinic post discharge (n = 19). Grey shading represents total percentage of patients with ALI or ARDS.

2.4 Discussion

The incidence of ALI/ARDS varies greatly in the literature, ranging between 2.5-19% of patients admitted onto ICUs (Bersten et al. 2002; Brun-Buisson et al. 2004; Fialkow et al. 2002; Hughes et al. 2003; Irish Critical Care Trials Group 2008; Vincent et al. 2010; Webster et al. 1988). This may be due in part to the difficulties in diagnosing ALI/ARDS using the AECC criteria, as exclusion of left atrial hypertension by clinical assessment alone is often quite subjective and interpretation of chest radiographs by different observers is inconsistent. This is evident in a study where 21 assembled experts agreed on the findings of only 43% of 28 randomly selected chest radiographs from critically ill, hypoxaemic patients (Rubenfeld et al. 1999). There may also have been a change in the incidence of ALI/ARDS over time. Studies from 2000 have shown that patients with ARDS and those at risk of ARDS benefit from low tidal volumes during mechanical ventilation (Determann et al. 2010; The Acute Respiratory Distress Syndrome Network 2000), therefore implementation of these new ventilation strategies over the past decade as well as improvements in other general supportive ICU care may have affected the incidence of ALI and ARDS. In fact, a recent US study found that the incidence of ARDS had halved over an 8 year period (Li et al. 2011). Finally, the large variations in the reported rates of ALI/ARDS may also reflect the differences in health care provision across the world and the availability of critical care facilities, for example the US have seven times the number of ICU beds per capita than the UK (Wunsch et al. 2011). This too, may explain the many (largely unreported), cases of ALI/ARDS occurring in self ventilating patients managed outside an ICU (Cely et al. 2010). Recent reports covering UK regions are extremely sparse. An audit study in 2003 looking at 23 Scottish ICUs reported a frequency of ARDS of 8.1% (Hughes et al. 2003) and the only study conducted in England is from 1988 (Webster et al. 1988) and undertaken at a time before the current diagnostic criteria were published.

This study therefore represents the first prospective study detailing the incidence, impact and longer term mortality of ALI/ARDS in a UK teaching hospital general adult ICU. Patients with ARDS are shown to make up 9% of all ICU admissions and patients with less severe ALI constitute a further 3.5% of patients. This is consistent with the previous multicentre Scottish study described above and the multicentre European study in 2002 where 12.5% of ICU patients had ALI/ARDS (Hughes et al.

2003; Vincent et al. 2010), and suggests that in the UK there may not have been a major decline in the incidence of ALI/ARDS as reported in the US (Li et al. 2011).

The ICU and hospital mortality rates for patients with ARDS were 42% and 55% respectively. This is slightly higher than the values reported in a recent, multicentre Irish study, which reported an ICU mortality of 32%, and in a large multicentre US study which reported a hospital mortality of 41% (Irish Critical Care Trials Group 2008; Rubenfeld et al. 2005). However, this may be explained by differences in the case mix as patients with ARDS secondary to sepsis are known to have a significantly greater mortality than patients with ARDS secondary to trauma (Rubenfeld et al. 2005). In this study, there was only one case of trauma-related ARDS, which may reflect the presence of a separate neuro-critical care unit within the hospital, which admits the majority of trauma patients. Further owing to the transplant services at the hospital there are higher levels of sepsis owing to immunosuppression. Despite this, a recent large multicentre Spanish study demonstrated almost identical mortality rates with an overall reported ICU mortality rate of 42.7% (Villar et al. 2011).

Of interest, patients with ALI had similarly high crude ICU and hospital mortality to ARDS patients with rates of 42% and 50% respectively. This has been seen in a number of other studies (Fialkow et al. 2002; Luhr et al. 1999) and highlights the severity of the entire spectrum of ALI/ARDS even in its more mild form. This is further demonstrated by comparing the survival curves of patients with ARDS and ALI (figure 2.5B), which over time converge so that by 600 days both cohorts have identical death rates. This is in complete contrast to patients with NLI who had ICU and hospital mortality rates of 11% and 21% respectively. Age-adjusted ICU and hospital mortality rates are similar to the crude mortality rates, accept for the ALI group, which appear to have a lower age adjusted ICU and hospital mortality rates (30.5% and 35.5% respectively). This may represent the very small sample size of this group (n=12) or in contrast to above, represent a true reduced mortality compared to ARDS. However, no other previous study has reported on age-adjusted mortality.

Although patients with ALI/ARDS make up only 12.5% of all ICU admissions, they utilise considerably more ICU and hospital resources than NLI patients. We have shown that patients with ALI/ARDS spend 3 times as many days on the ICU compared to NLI patients and require invasive ventilation for more than 3 times as long as NLI patients. Likewise, patients who survive to hospital discharge spend

approximately double the length of time in hospital compared to patients without lung injury.

This study also revealed the poor recognition and documentation of ALI/ARDS by health care professionals. In this study, the diagnosis of ALI or ARDS was noted in the electronic discharge summary of only 5% of cases, and ARDS was officially coded for in only 7% of cases. To see if this just represented poor coding as compared to poor recognition, the ICU notes of half of the patients with ALI/ARDS were subsequently re-reviewed (21 of 43) and reference to ALI/ARDS was only included in 14% of these. Further, in the patients that died before hospital discharge, only 4% of death certificates recorded ARDS or ALI as a cause of, or factor contributing towards, death. This under-recognition has wide ranging implications, not only in terms of the immediate management for individual patients but also in their follow-up care. Two and 5 year follow up studies have shown that surviving ARDS patients suffer ongoing reduction in their exercise capacity in part due to reduced defusing capacity and a mixed pattern of airflow restriction and obstruction (Herridge et al. 2011; McHugh et al. 1994; Orme et al. 2003). This suggests that surviving patients may benefit from specialist follow up care; however, only 2/19 (11%) of surviving patients with ALI/ARDS were followed up in the respiratory clinic. Under-recognition also has implications for future resource planning in regards to ensuring critical care facilities meet demands, and this in part may explain our finding that patients with ARDS spent significantly more time on the hospital wards prior to ICU admission (Figure 2.2A). Accurate incidence rates of ARDS will also inform regional resource planning such as the provision of extracorporeal membrane oxygenation (ECMO) services.

A clear limitation of this study as with other studies mentioned previously is in the potential for misclassification of ALI/ARDS; this is due in part to the subjective nature of the AECC diagnostic criteria. To overcome this, a physician independent of the initial data collection team reviewed all the radiology and assessed left atrial hypertension and the likely presence of left ventricular failure using pre-defined and objective criteria. A representative 20% sample of all patients in the NLI group was also re-reviewed to ensure that these patients had been appropriately ascribed. The other relative weakness of this study was the potential for the study nurse involved in the prospective data collection to influence the outcome and the lack of inclusion of other high-dependency patients within the hospital. Finally, as commented, we had a lower than anticipated rate of ALI/ARDS secondary to trauma and pancreatitis.

In conclusion, this is the first prospective study of the incidence and long term mortality of ALI/ARDS in a general ICU in the UK. There is significant under recognition and documentation of this condition with mortality rates that remain high and similar in both ALI and ARDS patients.

3 Trans-Pulmonary Neutrophil Priming Gradients in Sepsis and ARDS

3.1 Introduction

The primary role of neutrophils is to kill invading bacteria and certain fungal species. In facilitating this role, neutrophils are recruited rapidly to sites of inflammation, where they initially become adherent to activated vascular endothelium, transmigrate out towards the inflammatory foci and then finally kill their targets by phagocytosis, generation of reactive oxygen species and release of an arsenal of microbicidal substances. This arsenal is released from preformed granules and includes proteinases such as neutrophil elastase, collagenase and gelatinase; enzymes such as myeloperoxidase and lysozyme; and hydrolases such as lactoferrin (this is by no means an exhaustive list) (Faurschou and Borregaard 2003).

Clearly, this destructive force can also give rise to host tissue damage following unregulated and inappropriate neutrophil activation. As a result there are tight controls and safety mechanisms in place. One of these key mechanisms is neutrophil priming. This is a process whereby the response of neutrophils to an activating stimulus is potentiated by prior exposure to a priming agent (Condliffe et al. 1998), yet these agents in themselves do not initiate degranulation or superoxide formation (or do so only a minimal extent). Therefore, for a neutrophil to go from its normal quiescent state to a fully activated state capable of maximal respiratory burst and degranulation, it must first be primed. A wide variety of pharmacological and physiological substances have been shown to act as priming agents (see table 3.1).

Priming agent	Time required to induce maximal priming	Reference
L-selectin cross-linking	3 min	(Waddell et al. 1994)
CD18 cross linking	5 min	(Liles et al. 1995)
PAF	5 min	(Vercellotti et al. 1988)
TNF-α	10 min	(Berkow et al. 1987)
IL-8	10 min	(Daniels et al. 1992)
LPS	120 min	(Guthrie et al. 1984)
GM-CSF	120 min	(Weisbart et al. 1987)
IFN-γ	120 min	(Tennenberg et al. 1993)

Table 3.1 Common priming agents - Adapted from (Condliffe et al. 1998)

Neutrophil priming has a number of effects on neutrophil functions:

1) Enhancement of neutrophil respiratory burst activity. This is considered the 'gold standard' priming response (Condliffe et al. 1998). The generation of reactive oxygen species is performed by the enzyme, phagocyte NADPH oxidase, this is a multi-component enzyme with a redox center that transfers electrons from cytoplasmic NADPH onto extracellular oxygen, creating superoxide anions (Roos et al. 2003). These superoxide anions are converted to reactive oxygen species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid, and react with nitric oxide to form reactive nitrogen species. The result of this respiratory burst activity is efficient bacterial killing, which is highlighted by the rare genetic condition; chronic granulomatous disease. In this condition there are mutations in genes coding for the NADPH oxidase components leading to failure of generation of reactive oxygen species and subsequent profound immunodeficiency characterised by recurrent life threatening bacterial and fungal infections and formation of granulomas (Quinn and Gauss 2004).

2) Shape change and deformability. Normal quiescent neutrophils are spherical in shape (Ehrengruber et al. 1996) and although their mean diameter is in the order of 7.2 μm (Schmid-Schönbein et al. 1980) they have been shown capable of passing through Nuclepore filters with a pore size of just 5 μm (Downey and Worthen 1988), and hence are highly agile and deformable cells. However, once they become primed they change their shape to a more polarized, elliptical form with cellular protrusions (Ehrengruber et al. 1996), this is the result of changes in the cytoskeleton of the neutrophil. Wallace et al. demonstrated a 220% increase of F-actin within neutrophils primed with fMLP compared to control cells (Wallace et al. 1984); it was shown that this polymersization of F-actin occurred rapidly within 45 seconds of stimulation and although initially occurred diffusely, eventually was focal to the leading edge of the cells (Howard and Oresajo 1985). This rapid formation of F-actin causes a significant reduction in deformability. Using a "cell poker" which measures the force required to indent the surface of a cell, it was shown that unstimulated neutrophils required a force of 0.054 mdyne/μm for indentation, but after priming with fMLP the neutrophils rapidly stiffened and required a significantly larger force of 0.23 mdyne/μm; this was also accompanied by an inability to pass

through 5μm pore filters. Both of these features were abolished with the use of cytochalasin D, which causes disruption of actin filament organisation (Worthen et al. 1989).

3) Shedding of the cell surface adhesion molecule, L selectin (CD62L).
Neutrophil-endothelial cell interactions are regulated by cell adhesion molecules. The selectin family of adhesion molecules mediates the initial attachment of neutrophils to the vascular endothelial cells. L selectin (CD62L), is expressed on most leukocytes and consists of a large, highly glycosylated extracellular domain, a single transmembrane spanning domain and a small cytoplasmic tail. Its expression on the surface of neutrophils supports a highly effective interaction with glycosylated ligands on the endothelium and mediates initial tethering and rolling along the vascular wall at sites of inflammation (Smalley and Ley 2005). Besides, cell adhesion, CD62L has also been implicated in cell signalling directly. Antibody cross-linking of CD62L has been shown to result in increases in cytosolic free calcium (Laudanna et al. 1994), activation of the respiratory burst (Crockett-Torabi et al. 1995), induction of mRNA for cytokine production (IL-8 and TNFα) (Laudanna et al. 1994) and up-regulation of the cell surface β_2-intergrin adhesion complex CD11b/CD18 (Mac-1) (Simon et al. 1995). On neutrophil priming with fMLP, C5a or PMA, rapid downregulation of cell surface CD62L expression is observed (Jutila et al. 1990) with an corresponding increase in soluble L-selectin (Kishimoto et al. 1989). This is the result of proteolytic cleavage of CD62L near the cell surface by the shedase; TNFa converting enzyme (TACE or ADAM17). Other priming agents have induced similar findings (Condliffe et al. 1996) as well as CD18 cross linking (Walzog et al. 1994). The functional implications of CD62L shedding have been investigated using inhibitors that prevent shedding (e.g. hydroxamic acid-base peptide inhibitors) and these studies have shown to a varying degree of success a reduction in neutrophil rolling velocities (Walcheck et al. 1996). Transgenic mice expressing shedding resistant CD62L showed a reduced ability of neutrophils transmigrating into tissue following activation with keratinocyte-derived cytokine (Venturi et al. 2003); this was not observed however, with TNFα activation, and so may be agonist specific.

4) Up regulation of cell surface CD11b adhesion molecule. If CD62L is responsible for the initial neutrophil-endothelial cell adhesion, the β_2 integrin adhesion molecule CD11b/CD18 is well recognised for the subsequent tight neutrophil-endothelial adhesion and consequent transmigration into inflamed tissues. The β_2 integrins are composed of a common heterodimer β chain (CD18) that is non-covalently associated with any of four types of α subunits: CD11a (lymphocyte function-associated antigen-1 (LFA-1)), CD11b (Mac-1), CD11c or CD11d80. The CD11b/CD18 integrins are found in large intracellular myeloperoxidase negative secretory granules, and upon neutrophil priming these are mobilised to the cell surface, such that neutrophil priming with agents such as C5a, IL-8, PAF and fMLP can cause a several fold upregulation of cell surface CD11b/Cd18 expression (Mazzone and Ricevuti 1995). This together with a conformational change/activation of the adhesion molecule itself leads to a significant increase in adhesion (Springer 1994). Blocking antibodies against either subunit result in marked inhibition of neutrophil adhesion and transmigration (Parkos et al. 1991). CD11b/CD18 binds with the endothelial surface ligand; intercellular adhesion molecule-1 (ICAM-1; CD54), whose endothelial surface expression is also increased by pro-inflammatory cytokines. As well as neutrophil adhesion and migration, CD11b/CD18 interactions, through outside-in signalling with a concomitant activation of several intracellular signalling pathways, have been implicated in respiratory burst activity (Nathan et al. 1989), phagocytosis (Mayadas and Cullere 2005), inhibition of apoptosis (Whitlock et al. 2000) and after neutrophil phagocytosis, enhancement of apoptosis (Zhang et al. 2003). The functional importance of β_2 integrins is illustrated by the rare human disorders known as leukocyte adhesion deficiency syndrome 1 (LAD-I): LAD-I is caused by a mutation in the gene that encodes the integrin β_2 subunit, which results in failure to express any of the 4 members of the β_2 integrin family; this leads to defects in the ability of neutrophils to leave the circulation and phagocytose pathogens, as well as neutrophil apoptosis. Patients with this autosomal recessive disorder suffer from frequent life threatening infections (Evans et al. 2009).

5) Inhibition of apoptosis. Neutrophils are known to undergo rapid programmed cell death in-vitro, with only c. 30% survival at 24 hours whilst in culture. However the rate of constitutive neutrophil apoptosis has been observed to

be markedly delayed when primed with a range of agents such as IL-1β, TNFα-, GMCSF and LPS, with reports of up to 90% survival at 72 hours (Colotta et al. 1992). This finding is not universal however, as PAF, fMLP and IL-8 appear to have no or only minimal effects on apoptosis.

Although originally considered to be an irreversible event, there is a growing body of in-vitro and now in-vivo data to support the idea of reversible neutrophil priming. The evidence for neutrophil 'de-priming' as termed by Chilvers and co-workers (Kitchen et al. 1996), as well as the pulmonary vasculature being a potential site for this process physiologically, has been discussed in chapter 1 (1.2.5); this suggests the possibility of neutrophils that become primed in the systemic circulation (e.g. in the context of peripheral sepsis or pancreatitis etc.) and then become trapped in the pulmonary circulation, may be able to de-prime and be released back into the circulating pool in a quiescent state. Further, in conditions in which gross pulmonary inflammation exists, such as in ARDS, this de-priming mechanism may fail.

The aim of this chapter was to perform a feasibility study to initially investigate this hypothesis, by attempting to assess the priming status of neutrophils entering the lungs and comparing this simultaneously to the priming status of neutrophils leaving the lungs (hence determine the trans-pulmonary gradient of primed neutrophils) in patients with (i) peripheral sepsis but healthy lungs, (ii) ARDS, and (iii) a control population of perioperative subjects. By using flow cytometry, the priming status of neutrophils could be established in small volumes of blood, specifically looking at the shape change of neutrophils, the upregulation of cell surface C11b and shedding of cell surface CD62L.

The hypothesis for this work was:

(i) Patients with peripheral sepsis but healthy lungs would have a measurable gradient across the pulmonary circulation, such that neutrophils entering the lung would be more shape changed, and express more CD11b and less and less CD62L on their cell surface than neutrophils leaving the pulmonary circulation.

(ii) Patients with ARDS, would have the reverse, such that neutrophils entering the lung would be less shape changed, and express less CD11b and more CD62L on their cell surface than neutrophils leaving the lungs.

(iii) In the control population, no gradient would be expected for any of the measured variables

3.2 Methods

3.2.1 Experimental design

This study was approved by the Cambridgeshire 3 Ethics Committee (08/H0306/17) and by the Research and Development department of Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge (A091225).

Subjects were recruited from 3 different groups:

1) **Patients with systemic sepsis but without evidence of lung involvement**

   Sepsis was defined as a known infective source with 2 or more of the following: A body temperature of less than 36ºC or greater than 38ºC; a heart rate of greater than 90 beats per minute; a respiratory rate of greater than 20 breaths per minute or a $PaCO_2$ of less than 4.3 KPa; and a white cell count of less than 4 x 10^9/L or greater than 12 x 10^9/L. Patients were required to have had a recent frontal chest radiograph confirming no pulmonary infiltrates and a PaO_2/FiO_2 ratio of greater than 40KPa.

2) **Patients with ARDS from any cause**

   ARDS was defined using the 1994 AECC definition. Patients were required to have an acute onset of their symptoms, a frontal chest radiograph showing bilateral pulmonary infiltrates, no clinical evidence of left atrial hypertension, and a PaO_2/FiO_2 ratio of less than 27 KPa. An additional criterion was included in that the PaO_2/FiO_2 ratio criteria had to be met on 2 arterial blood gas results taken at least 6 hours apart, this is in-line with other clinical studies and drug trials in ARDS (Gao Smith et al. 2012) and was initiated to ensure that the result of hypoxaemia was not transient, as may be the case, for example, in patients with acute airway obstruction secondary to sputum plugging. Assessment of left atrial hypertension was based on objective criteria when available, and patients were excluded if they had biochemical or electrical evidence of an acute myocardial infarction, previous or current echocardiogram reports showing moderately (or greater) dilated left atrium or moderately (or greater) left ventricular dysfunction, or an enlarged cardiac silhouette on a recent posterior-anterior chest radiograph.

3) **Perioperative, elective oesophagectomy patients**
   These patients were required to have normal lung spirometry and gas transfer measurements, a normal chest radiograph, and to have been abstinent from tobacco smoking for the preceding 6 months.

All patients were required to have an internal jugular, or subclavian catheter and a radial artery catheter in-situ as part of their normal clinical care. They were required to give written consent to the study, or in the case of critical care patients; their relatives were required to give written assent.

Subjects were excluded if they were neutropenic (absolute neutrophil count of less than 1 $x10^9$/L), were on immunosuppressive therapy prior to their admission to the ICU or if they had a haematological malignancy.

Ten millilitres of blood was taken from the central venous catheter of recruited subjects and discarded, a further 5 ml blood sample was then taken and collected into x2 EDTA blood tubes in an aseptic fashion. Immediately after this, 10 ml of blood was taken from the radial arterial catheter and discarded, followed by a 5 ml sample which was again collected in x2 2.5 ml EDTA blood tubes in an aseptic fashion. One pair of central venous and arterial blood samples was sent to the main hospital haematology laboratory for assessment of absolute neutrophil counts. The other pair of blood samples was then assessed for neutrophil priming status by measurement of neutrophil shape change, cell surface CD62L expression and cell surface CD11b expression. To ensure there was no order effects of how the blood samples were taken i.e. venous samples taken before arterial samples, the order was varied throughout the study.

The perioperative patients then went on to have their surgery as planned. For the septic patients and patients with ARDS, further samples were taken from these patients as indicated during the first 7 days when their clinical condition had changed or until their central venous or arterial catheters were removed.

3.2.2 Purification of human neutrophils

Ethical permission was granted by the local ethics committee (REC reference 06/Q0108/281) to obtain peripheral venous blood from healthy volunteers. Neutrophil isolation was undertaken at room temperature, using endotoxin-free reagents and plasticware, under sterile conditions in a laminar flow cell culture hood (Microflow Class II cabinet) as described (Haslett et al. 1985).

Venepuncture was undertaken using a sterile 19 gauge Butterfly needle (Hospira Venisystems) and 50 ml Plastipak disposable syringes. The blood was immediately and gently transferred into 50 ml Falcon tubes (BD) containing 4 ml (3.8%) sodium citrate (Phoenix Pharma, UK) to a total volume of 40 ml. The tubes were inverted a couple of times and then centrifuged at 320 *g* for 20 min at room temperature. The platelet-rich plasma (PRP) supernatant layer was removed and used to make platelet poor plasma (PPP) and autologous serum. To prepare PPP, the PRP was centrifuged at 2652 *g* for 20 min and the pellet discarded. Simultaneously, the pelleted cells from the initial blood centrifugation were subjected to dextran sedimentation using 6% dextran (2.5 ml/10 ml of cell pellet). The volume was made up to 50 ml with 0.9% pre-warmed (37ºC) sterile 0.9% saline and the tubes mixed gently and allowed to stand for 30 min to allow the erythrocytes to sediment. The leukocyte-rich plasma was aspirated and centrifuged at 320 *g* for 6 min.

The leukocyte pellet was gently re-suspended in 2 ml PPP and transferred to a 15 ml Falcon tube, where it was under-layered with 2 ml freshly prepared 42% Percoll in PPP, which was in turn under-layered with 2 ml of freshly prepared 51% Percoll in PPP. Under-layering was performed using a glass pipette, which had been sterilised by autoclaving (240ºC for 4 h). The gradients were centrifuged for 10 min at 205 *g*, with the brake and acceleration speed set to zero. Mononuclear cells and some platelets remained at the upper interface between the plasma and 42% Percoll layer, with neutrophils in a wider band at the interface of the 42% and 51% layer Percoll layers and extending into the 51% Percoll layer to a few millimetres above the erythrocyte pellet. Each band was aspirated with a Pasteur pipette (Appletonwoods). The neutrophils were sequentially washed in PPP, phosphate buffered saline without Ca^{2+}/Mg^{2+} (PBS-/-) and phosphate buffered saline with Ca^{2+}/Mg^{2+} (PBS+/+) (centrifuged at 205 *g*, 6 min) prior to re-suspension at 5×10^6 cells/ml in PBS+/+.

This method has been reported to result in an efficiency for neutrophil recovery of >80%, with the resulting neutrophils being >99% viable (trypan blue negative) and >95% pure (eosinophils being the dominant non-neutrophil contaminant), with less than 0.1% monocytes (Haslett et al. 1985). I can report similar results after an n=3 of neutrophils being 98.6% (+/- 1.65) viable (trypan blue negative) and 96% (+/- 1.65) pure as determined by May-Grunwald-Giemsa-stained cell cytospins.

3.2.3 Isolation of neutrophils in whole blood

The above method of neutrophil isolation requires relatively large volumes of blood, this is inappropriate when assessing neutrophils from critically ill patients on multiple occasions. Further, various neutrophil purification techniques have been suggested to cause inadvertent alterations in neutrophil function/priming status, for example, the use of dextran sedimentation (Macey et al. 1992), and centrifugation on density gradients (Kuijpers et al. 1991) have both been reported to cause upregulation of cell surface CD11b; and the use of ammonium chloride erythrocyte lysis may cause shape change (Haslett et al. 1985). The Chilvers laboratory has therefore developed techniques where the neutrophil phenotype may be studied in small volume, minimally manipulated, whole blood samples by the use of a nuclear stain and flow cytometry techniques (Summers 2010), based on the work of Alvarez-Larran (Alvarez-Larrán et al. 2005). The detailed method can be found below but is based on whole blood being stained with the far red fluorescent DNA dye "DRAQ 5". Since red blood cells and platelets do not contain nuclear DNA only nucleated leukocytes are labelled.

3.2.4 Assessment of shape change, CD62L and CD11b expression

Ninety µl of freshly collected whole blood anti-coagulated with EDTA (or purified granulocytes at $5x10^6$/ml), was added to Eppendorf tubes containing 1 µl DRAQ 5 and either 10 µl GM-CSF (100 ng/ml) or PBS-/- and incubated at 37ºC for 30 minutes. 250 µl of ice cold optimised CellFIX solution (1 ml CellFIX, 9 ml water, 30 ml FACSflow sheath fluid) was added to each Eppendorf and the tubes placed on ice for 1 minute. Forty µl of each sample was then transferred to a FACS tube containing either (i) 10 µl anti-human CD62L RPE conjugate, (ii) 10 µl anti-human CD11b FITC conjugate and 5 µl anti-human CD16 RPE conjugate that had been premixed, or (iii) their respective isotype-matched antibody controls. Samples were incubated on ice for 30 minutes in the dark before the addition of 2 ml of ice-cold PBS-/- to each sample followed by immediate analysis.

Samples were analysed using a flow cytometer (initially this was with a BD Facsort, however after a significant problem with the laser, a BD FACSCaliber was used). A threshold for FL-3 that allowed use of DRAQ 5 to distinguish the nucleated cell populations was set. Three thousand cell events were counted for each sample. Granulocytes were identified by their characteristic forward and side scatter profiles.

Shape change was analysed by measurement of mean forward angle light scatter, this has been shown to correlate closely with microscopic evaluation of neutrophil shape change in concentration-response stimulation experiments with fMLP and IL-8 (Cole et al. 1995).

Neutrophil cell surface CD62L expression was measured in the CD62L labelled/ isotype-matched antibody control samples, and was determined by mean fluorescence in the FL-2 channel.

Neutrophil cell surface CD11b expression was measured in the CD11b and CD16 labelled/ isotype-matched antibody control samples. CD11b are also found on eosinophils, however eosinophils do not express CD16. Neutrophils were therefore identified by gating on CD16+ve cells in the FL-2 channel. CD11b expression was then determined by mean florescence of these cells in the FL-1 channel.

3.2.5 Measurement of absolute neutrophil counts

Two point five ml (2.5 ml) of whole blood samples collected in clinical grade EDTA blood tubes were measured for absolute neutrophil counts by use of a Beckman Coulter UniCel DXH800 Coulter Cellular Analyser, this uses the Coulter principle of impedance counting with a published accuracy rate of +/- 2% (Beckman Coulter website - https://www.beckmancoulter.com).

3.2.6 Statistics

Mean values for neutrophil shape change, CD62L, CD11b and absolute neutrophil counts from arterial blood samples were compared to that of the paired central venous blood samples to determine the presence of a trans-pulmonary gradient.

Half way through the study the laser from the BD FACSort flow cytometer failed making its further use impossible. Subsequent blood samples were therefore analysed using the BD FACSCaliber flow cytometer. This makes a comparison of absolute mean fluorescence values from the different machines difficult. As a result, to allow for meaningful comparison between all data, results have been expressed as a ratio compared to the paired central venous sample.

Data were analysed using GraphPad Prism 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All data were analysed using non-parametric methods including Mann-Whitney and Kruskal-Wallis tests with Dunn's post test. All tests were 2 tailed. All results are expressed as mean (+/- standard

deviation), unless otherwise stated. A P value of <0.05 was considered statistically significant.

3.2.7 Materials

- Dulbecc's PBS with calcium and magnesium ($PBS^{+/+}$), Dulbecc's PBS without calcium and magnesium ($PBS^{-/-}$), low endotoxin bovine serum albumin, sterile water and fMLP were obtained from Sigma (Poole, Dorset, UK).
- Anti-human CD11b-FITC (Clone Bear1) and IgG2a-FITC isotype control (Clone 7T4-IFS) were obtained from Beckman Coulter (High Wycombe, UK).
- Anti-human CD62L-RPE (clone FMC46) and IgG2b-RPE isotype control (clone TEN/0) were obtained from AbD Serotec (Oxon, UK).
- Anti-human CD16-RPE (clone DJ130c) and IgG1-RPE isotype control (clone DAK-GO1) were obtained from DAKO (Ely, Cambs UK).
- DRAQ 5 was obtained from Biostatus Limited (Shepshed, Leics, UK).
- FACSflow sheath fluid and CellFIX were obtained from BD Bioscience (Oxon, UK).
- 3.8% sodium citrate was obtained from Phoenix Pharmaceuticals Ltd (Gloucester, UK).
- Percoll and dextran were obtained from GE Healthcare UK Ltd (Little Chalfont, Bucks, UK).
- GM-CSF were obtained from R&D Systems Ltd (Oxon, UK).
- All other chemicals were of the finest grade available and were purchased from Sigma (Poole, Dorset, UK).
- All plastic and glassware was LPS-free and purchased from VWR (Leicestershire, UK).

3.3 Results

3.3.1 Validation of the whole blood assay

The effects of priming with physiological concentrations of GM-CSF (10 ng/ml) on neutrophils that have been purified from the peripheral blood of healthy volunteers using the gold standard LPS free plasma-percoll gradient method (Haslett et al. 1985) have been confirmed in this study as; a significant change in cell shape as denoted by an increase in forward scatter (see figure 3.1), a significant reduction in the expression of cell surface CD62L (see figure 3.2), and a significant up-regulation of cell surface CD11b expression (see figure 3.3). Similar results were obtained when using the whole blood assay to assess the same neutrophil priming markers again on peripheral blood taken from healthy volunteers. Neutrophils analysed with the whole blood assay demonstrated a 1.9 fold increase in mean forward scatter (from 229.5 +/- 35.53 AU to 427.9 +/- 77.45 AU) (see figure 3.1), a 3.0 fold decrease in mean CD62L expression (from 953.3 +/- 359.8 AU to 315.7 +/- 102.1 AU) (see figure 3.2), and a 6.4 fold increase in mean CD11b expression (from 140.4 +/- 114.5 to 904.3 +/- 161.3 AU) (see figure 3.3).

No significant differences were found in the baseline characteristics of unstimulated neutrophils using the 2 different methods, although there was a non significant trend for reduced basal cell surface CD11b expression of neutrophils analysed by the whole blood assay compared to the purified neutrophils (140.4 +/- 114.5 AU vs 416.8 +/- 217.6 respectively) suggesting that the plasma percoll prepared neutrophils may have slightly more primed compared with unmanipulated cells in whole blood. This is seen further, when looking at the effects of GM-CSF priming, where the mean CD11b expression of neutrophils was not only greater on purified neutrophils compared to neutrophils in whole blood (1411 +/- 149.3 AU vs 904.3 +/- 161.3 AU respectively, $p < 0.05$) but also underwent a lesser fold change following stimulation (3.4 fold increase vs 6.4 fold increase respectively, not significantly different) (see figure 3.3).

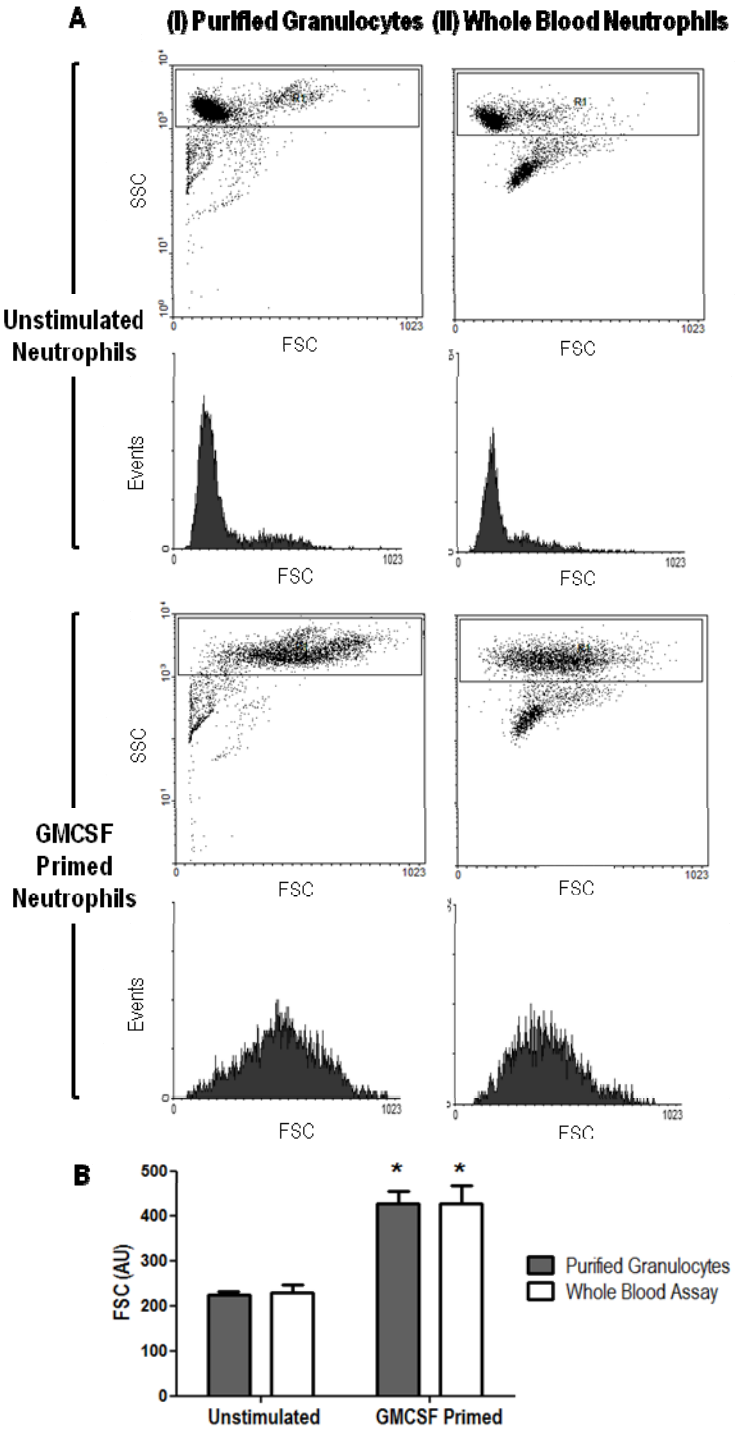

Figure 3.1 Measurement of neutrophil shape change from purified granulocytes and from whole blood samples. Panel A shows representative scatter plots of 4 independent experiments, demonstrating side scatter and forward scatter properties of (i) purified granulocytes and (ii) nucleated cells (DRAQ 5 +ve) from whole blood, unstimulated and after priming with GM-CSF 10 ng/ml. The histograms represent the forward scatter properties of these cells after gating on the neutrophil population (R1). Panel B shows the mean forward scatter of unstimulated neutrophils and GM-CSF (10 ng/ml) primed neutrophils from purified granulocytes and whole blood preparations (mean +/- SD, n=4). (* $p<0.05$ compared to unstimulated neutrophils).

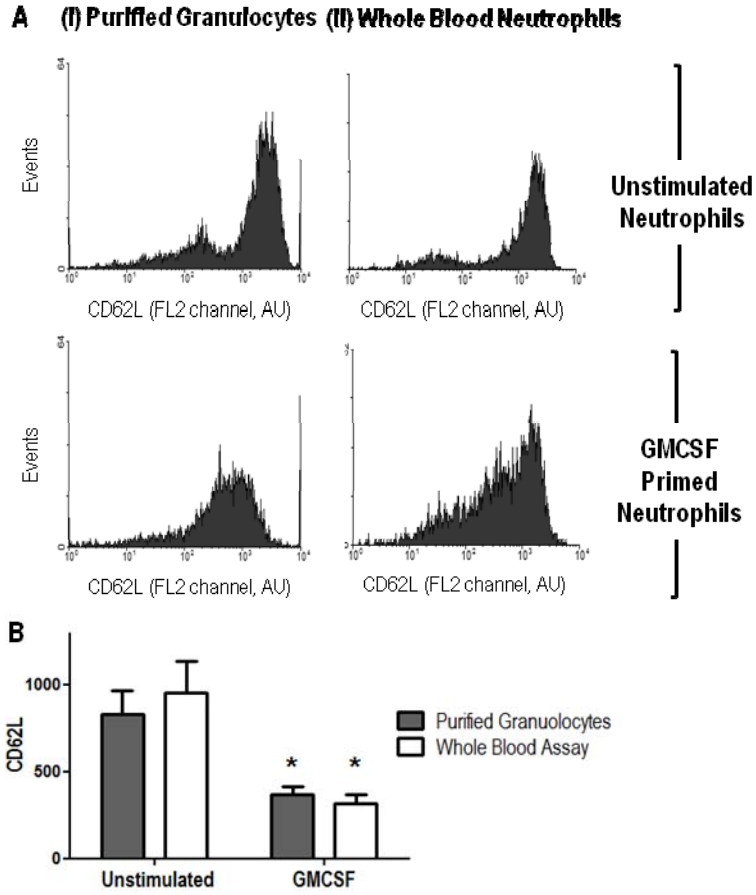

Figure 3.2 Measurement of neutrophil cell surface CD62L expression of purified granulocytes and of whole blood. Panel A shows representative histograms of 4 independent experiments, demonstrating FL2 fluorescence intensity characteristics, and hence CD62L labelling, of neutrophils from (i) purified granulocytes and (ii) nucleated (DRAQ 5 +ve) cells from whole blood, unstimulated and after priming with GM-CSF 10 ng/ml. Panel B shows the mean CD62L cell surface expression of unstimulated neutrophils and GM-CSF (10 ng/ml) primed neutrophils from purified granulocytes and whole blood preparations (mean +/- SD, n=4). (* $p<0.05$ compared to unstimulated neutrophils).

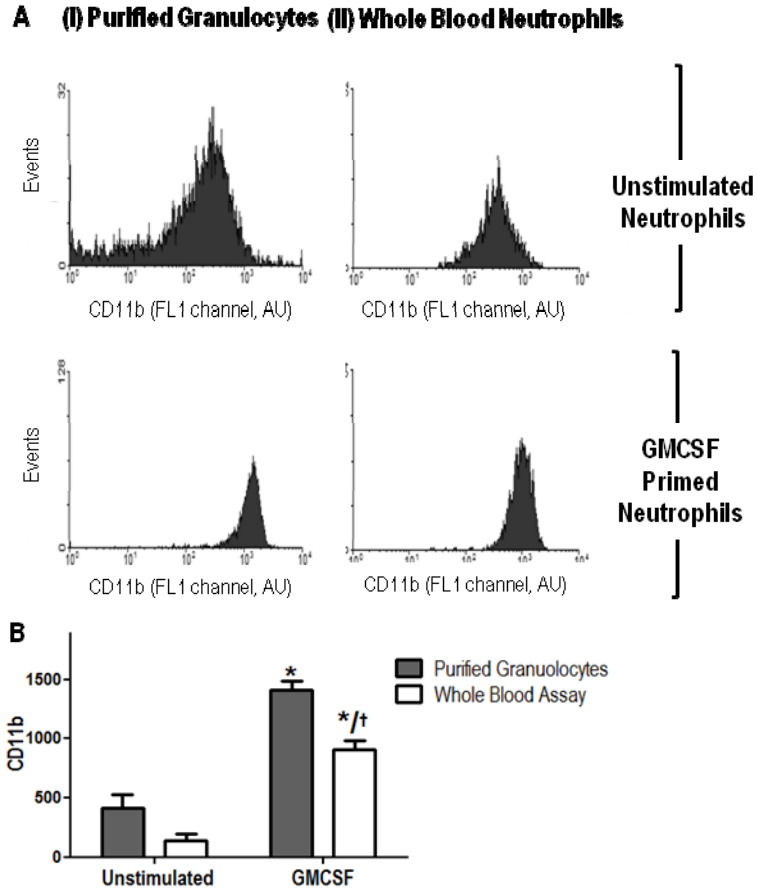

Figure 3.3 Measurement of neutrophil cell surface CD11b expression of purified granulocytes and of whole blood. Panel A shows representative histograms of 4 independent experiments, demonstrating FL1 fluorescence intensity characteristics, and hence CD11b labelling, of neutrophils from (i) purified granulocytes and (ii) nucleated (DRAQ 5 +ve) cells from whole blood, unstimulated and after priming with GM-CSF 10 ng/ml. Panel B shows the mean CD11b cell surface expression of unstimulated neutrophils and GM-CSF (10 ng/ml) primed neutrophils from purified granulocytes and whole blood preparations (mean +/- SD, n=4). (* $p<0.05$ compared to unstimulated neutrophils, † $p<0.05$ compared to GM-CSF primed neutrophils from purified granulocytes).

3.3.2 Trans-pulmonary neutrophil priming gradients in patients with systemic sepsis without lung involvement

Six patients with systemic sepsis without lung involvement were studied (mean age 64.6 years (+/- 13.9), two male). The cause of sepsis for each case was secondary to catheter related sepsis, urinary sepsis, necrotising fasciitis, biliary sepsis, and 2 cases of abdominal sepsis secondary to perforated bowel.

The absolute values obtained for neutrophil counts, shape change (as measured by mean forward scatter), mean CD11b cell surface expression and mean CD62L cell surface expression varied greatly between patients (see figure 3.4), which is reflective of the great variation in the aetiology and clinical severity of disease between patients and also to some extent reflects the general biological variance amongst individuals of varying age, ethnicity and chronic co-morbidities. Also, secondary to technical problems, the FACSort flow cytometer being used had to be changed midway through the study to a FACSCaliber, hence different blood samples have been analysed on different flow cytometers. Although a trans-pulmonary gradient in either neutrophil counts, or neutrophil priming status, can be determined by subtracting the results of the arterial sample from that of the paired venous sample, this would not allow a meaningful comparison of trans-pulmonary gradients between patients and between patient groups due to the wide variation of individual patient results as outlined above. Trans-pulmonary gradients have therefore been determined by expressing the results for neutrophils in the arterial sample as a ratio of the paired venous sample results, where a value of 1 represents no gradient; greater than 1 represents a positive gradient and less than 1 represents a negative gradient.

Figure 3.5 provides a summary of the results of paired central venous and arterial blood samples taken from patients on a day when their condition was clinically at its most severe. This was determined by looking at physiological parameters such as body temperature, heart rate, blood pressure; the need for pharmacological vasopressors or inotropes; and biochemical parameters such as C-reactive protein levels. There were no consistent trans-pulmonary gradients in terms of absolute neutrophil counts, neutrophil shape change or cell surface CD11b expression. However, there was a statistically significant positive trans-pulmonary gradient ($p=0.031$) for neutrophil CD62L expression with a mean A-V ratio of 1.122 (+/- 0.09), (see figure 3.5 and 3.8), whereby, neutrophils leaving the pulmonary circulation (in arterial blood) express 1.12 times the amount of cell surface CD62L

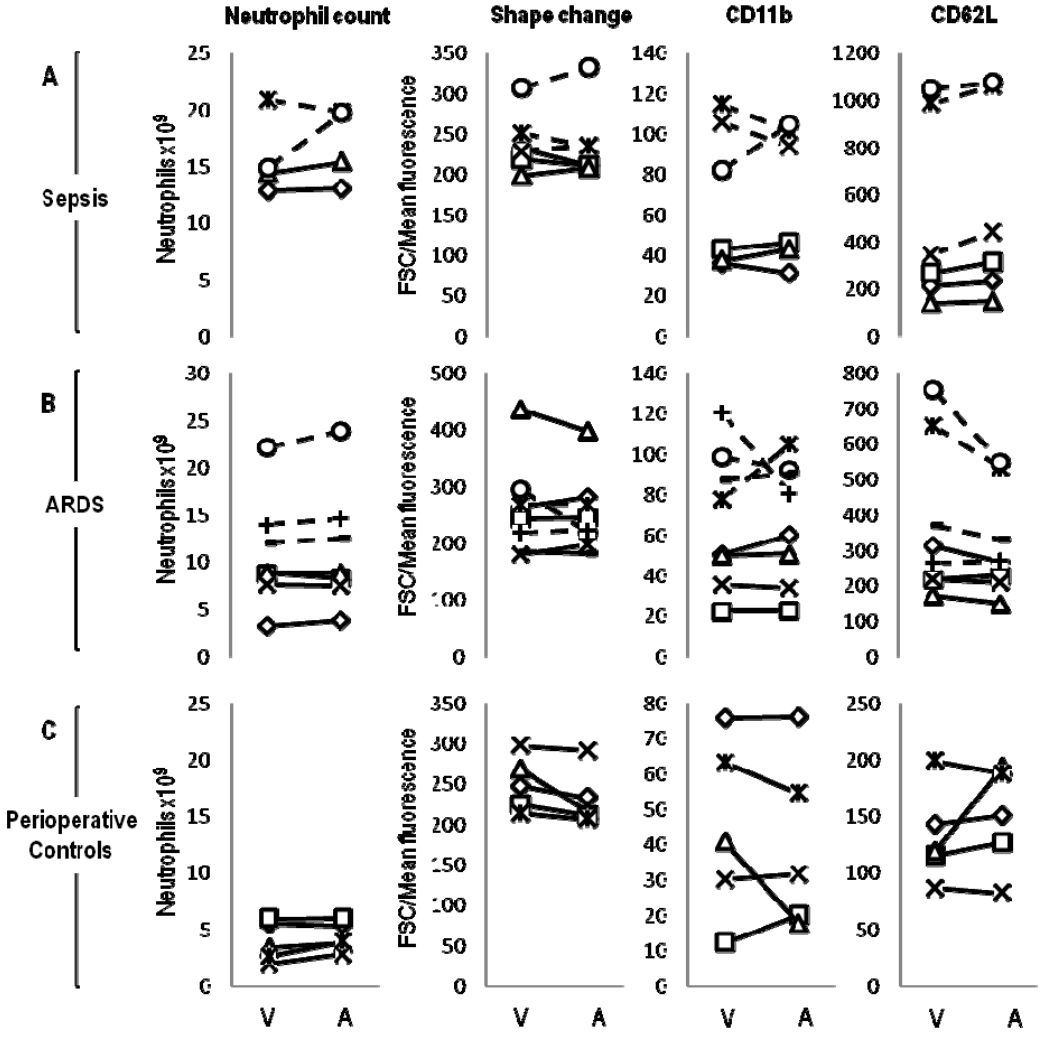

Figure 3.4 Measurement of neutrophil priming status across the lungs. Paired central venous and radial arterial blood samples obtained from (A) patients with systemic sepsis without lung involvement (n=6), (B) patients with ARDS (n=8), and (C) perioperative patients undergoing elective oesophagectomy (n=5) were assessed for absolute neutrophil count by Coulter counter; and neutrophil shape change, cell surface CD11b expression and cell surface CD62L expression by flow cytometry. Dashed lines represent blood samples analysed with a FACSort flow cytometer, whereas continuous lines represent samples analysed with a FACSCaliber flow cytometer. Symbols in each panel represent the same patient across the 4 variables measured.

than neutrophils entering the pulmonary circulation in central venous blood. No significant venous-arterial differences were found in terms of fold changes in shape change, CD11b expression or CD62L expression on GM-CSF priming (data not shown).

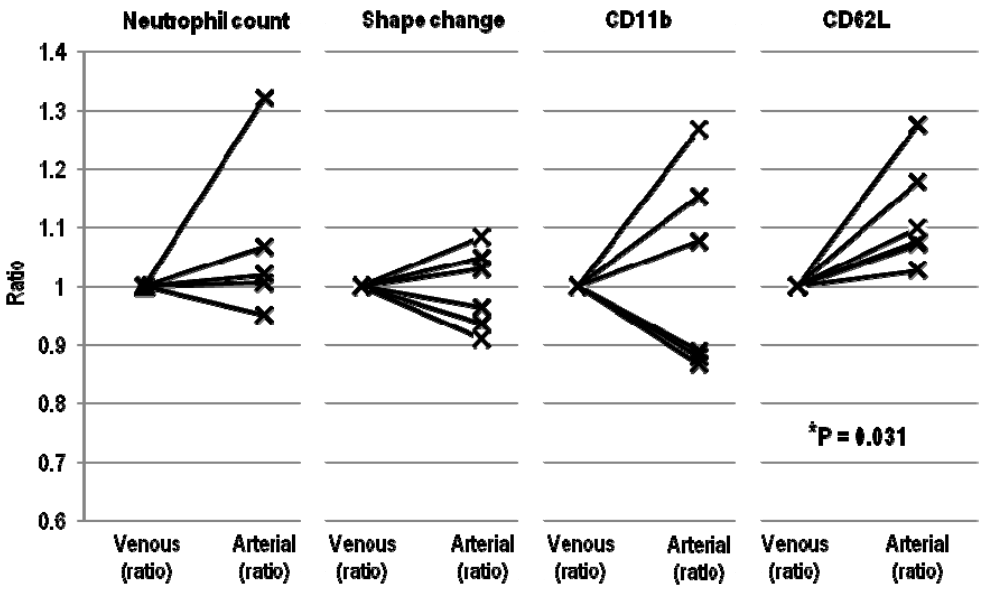

Figure 3.5 Trans-pulmonary neutrophil priming gradients in patients with systemic sepsis without lung involvement. Paired central venous and radial arterial blood samples obtained from critically ill patients with systemic sepsis without lung involvement (n=6) on the day when their clinical condition was at its most severe, were assessed for absolute neutrophil count by Coulter counter; and neutrophil shape change, cell surface CD11b expression and cell surface CD62L expression by flow cytometry. Results have been expressed as a ratio of the paired venous sample.

3.3.3 Trans-pulmonary neutrophil priming gradients in patients with ARDS

Eight patients with ARDS were studied (mean age 57.8 years (+/- 18.5), five male). The cause of ARDS for each case was massive blood transfusion, trauma, two cases of community acquired pneumonia, hospital acquired pneumonia, septic shock from an unknown source, sepsis secondary to ascending cholangitis and sepsis secondary to necrotising fasciitis. This last case was initially studied in the sepsis without lung involvement group but then quickly developed ARDS and so was re-studied as part of this group.

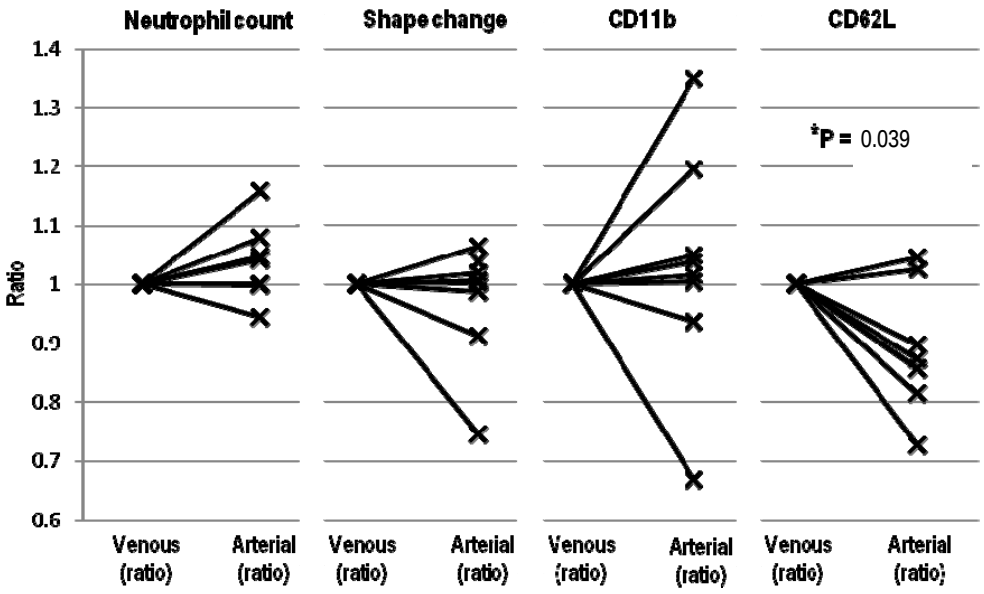

Figure 3.6 Trans-pulmonary neutrophil priming gradients in patients ARDS. Paired central venous and radial arterial blood samples obtained from critically ill patients with ARDS (n=8) on the day when their clinical condition was at its most severe, were assessed for absolute neutrophil count by Coulter counter; and neutrophil shape change, cell surface CD11b expression and cell surface CD62L expression by flow cytometry. Results have been expressed as a ratio of the arterial to paired venous sample.

Figure 3.6 summarises the measured A-V ratios for this group, again on the day their condition was clinically at its most severe (absolute numbers can be seen in figure 3.4B). This was determined by examining on a daily basis the PaO_2/FiO_2 ratios, amount of infiltration on frontal chest radiographs, level of PEEP being delivered to ventilated patients and changes in lung compliance (as measured by the ventilator). Once again, no consistent trans-pulmonary gradient was observed in terms of absolute neutrophil counts, neutrophil shape change or cell surface CD11b expression. However, there was a statistically significant negative trans-pulmonary gradient (p=0.039) for neutrophil CD62L expression with a mean A-V ratio of 0.89 (+/- 0.105), (see figure 3.6 and 3.8), whereby, neutrophils leaving the pulmonary circulation in arterial blood express 0.89 times less the amount of cell surface CD62L than neutrophils entering the pulmonary circulation in central venous blood. Again, no significant venous-arterial differences were found in terms of fold changes in shape change, CD11b expression or CD62L expression on GM-CSF priming (data not shown).

3.3.4 Trans-pulmonary neutrophil priming gradients in perioperative patients

Five perioperative patients were studied (mean age 55 years (+/- 16.7), three male). These patients were to undergo elective oesophagectomy, four for oesophageal carcinoma and the fifth for oesophageal strictures secondary to acid ingestion. All had normal spirometry and perioperative chest radiographs, and had not smoked tobacco for at least 6 months. Central venous and radial arterial catheters were only placed after induction of anaesthesia and so blood samples were taken after patients were intubated, but before single lung ventilation or surgical incision.

Figure 3.7 shows the A-V ratios seen for this control group, (absolute numbers can be seen in figure 3.4C). There were no significant trans-pulmonary gradients observed in terms of absolute neutrophil counts, neutrophil shape change, cell surface CD11b expression or cell surface CD62L expression. Further, no significant venous-arterial differences were found in terms of fold changes in shape change, CD11b expression and CD62L expression on GM-CSF priming (data not shown).

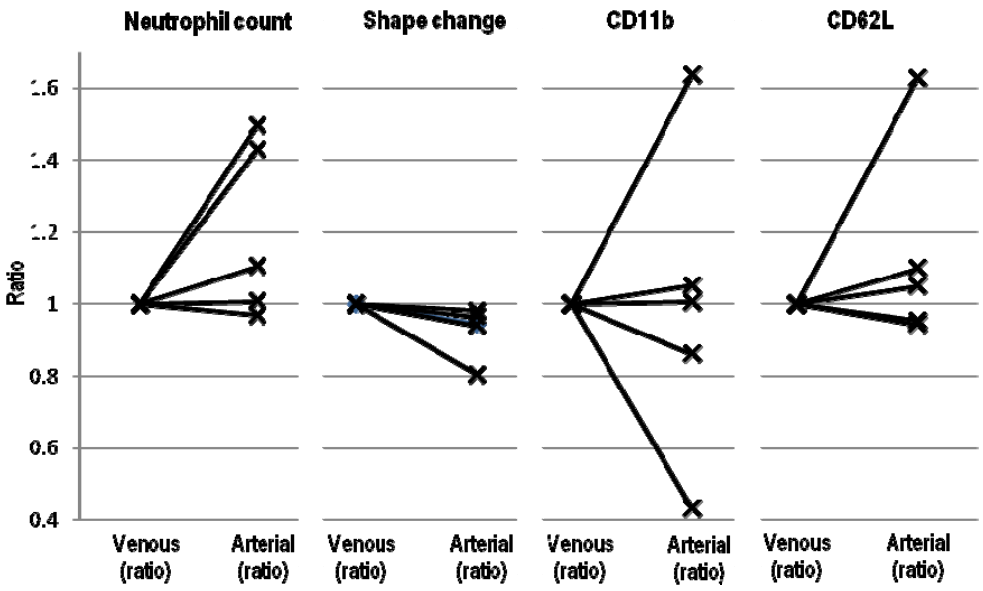

Figure 3.7 Trans-pulmonary neutrophil priming gradients in perioperative patients. Paired central venous and radial arterial blood samples obtained from patients undergoing elective oesophagectomy (n=5) were assessed for absolute neutrophil count by Coulter counter; and neutrophil shape change, cell surface CD11b expression and cell surface CD62L expression by flow cytometry. Results have been expressed as a ratio of the arterial sample compared to the paired venous sample.

3.3.5 Neutrophil CD62L A-V ratio as a marker of severity of ARDS

Figure 3.8A confirms that the positive trans-pulmonary gradient seen in patients with systemic sepsis without lung involvement is significantly different to the negative trans-pulmonary gradient seen in patients with ARDS (p=0.0043).

One of the patients studied was unique in that they were initially studied in the systemic sepsis without lung injury group and then re-studied in the ARDS group with neutrophil CD62L A-V ratios in keeping with the above results (see figure 3.8B). This was a 70 year old female who had become acutely unwell with necrotising fasciitis of the upper limb secondary to group A Streptococcus. She immediately went to theatre and had debridement of the infected arm and was then admitted to the ICU. She was enrolled into this study the following day with markers of sepsis, although improving, and a completely clear chest radiograph with a PaO_2/FiO_2 ratio of 41KPa. At this time her CD62L A-V ratio revealed a positive trans-pulmonary gradient (see figure 3.8Bii). She then developed ARDS over the next 24 hours with a rapid fall in her baseline neutrophil CD62L expression (see figure 3.8Bi) and a complete reversal in her A-V gradient (from 1.028 to 0.726). Her condition started to improve by day 4 with the finding of a persistent negative trans-pulmonary gradient but slightly less so (although her baseline neutrophil CD62L expression continued to fall).

This example supports the connection that the magnitude of the neutrophil CD62L trans-pulmonary gradient may correspond with clinical severity. Figure 3.8C shows the neutrophil CD62L A-V ratio for all paired samples taken from patients when they fulfilled ARDS criteria and confirms a significant correlation with the patients PaO_2/FiO_2 ratio, and hence the smaller the A-V ratio is (or the more negative the trans-pulmonary gradient is) the greater the degree of hypoxaemia.

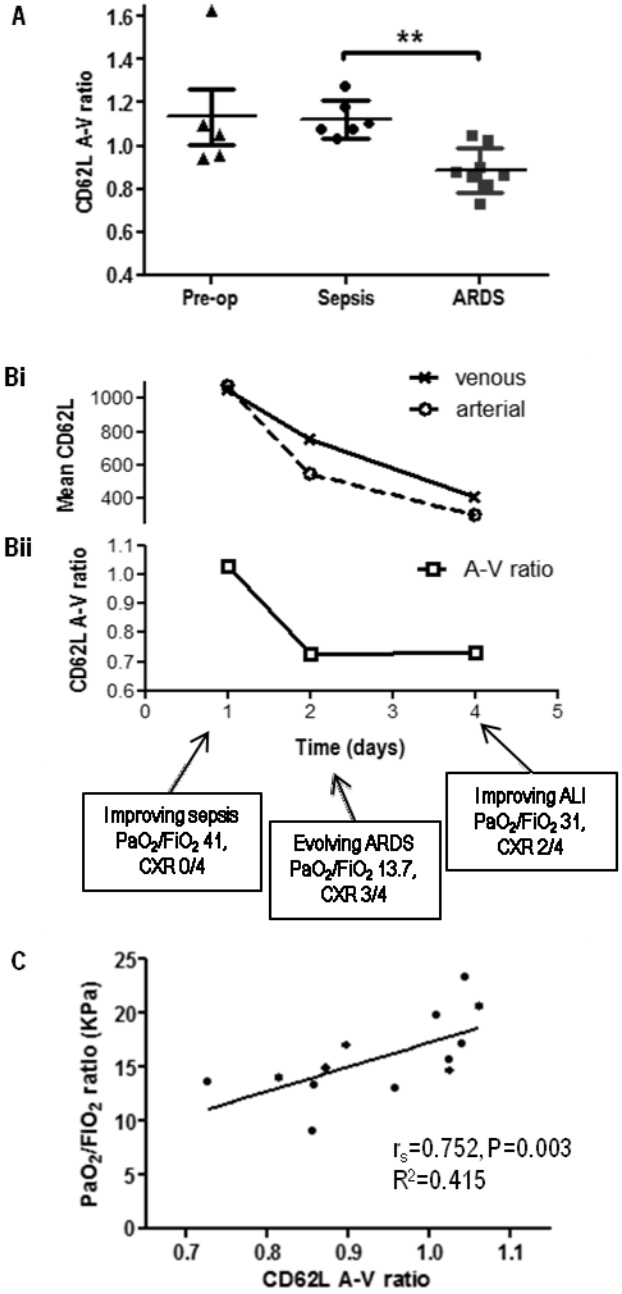

Figure 3.8 Trans-pulmonary gradients in neutrophil CD62L expression. Panel A shows a direct comparison of CD62L A-V ratios for perioperative control patients, patients with sepsis (without lung involvement) and patients with ARDS. Dots represent individual patients on the day when their clinical condition was at its most severe. Horizontal bars represent mean +/- SD. **P<0.005. Panel B shows the change in (i) neutrophil CD62L expression and (ii) neutrophil CD62L A-V ratio over time in a single subject with sepsis secondary to necrotising fasciitis who then (day 2 onwards) developed ARDS. Panel C shows the correlation between the PaO_2/FiO_2 ratio of patients with ARDS and their corresponding neutrophil CD62L A-V ratios. Data include all paired venous and arterial blood samples taken from patients when they met ARDS criteria. (r_s, Spearman's rank correlation; R^2, coefficient of determination).

3.4 Discussion

The whole blood assay, developed in the Chilvers lab (Summers 2010), which measures the priming status of neutrophils by analysis of shape change (by changes in forward scatter), mean cell surface CD11b expression and mean cell surface CD62L expression, has been shown to be non-inferior to values obtained in neutrophils prepared using the 'gold standard' LPS free plasma-percoll gradient centrifugation technique (Haslett et al. 1985). In fact there is a suggestion from my data that this assay may, unsurprisingly, be superior in that there were lower levels of basal (resting) cell surface CD11b on the neutrophils assessed by the whole blood assay compared to the neutrophils from purified granulocytes, which was also evident on GM-CSF priming. This has been observed by other authors (Alvarez-Larrán et al. 2005; Kuijpers et al. 1991) and suggests that even optimally prepared purified granulocytes are partially primed in some way. Neutrophils analysed in whole blood on the other hand are not subject to centrifugation, or any wash steps, and so are minimally manipulated and can be analysed by flow cytometry almost immediately after obtaining the sample. Further, since this technique only requires very small amounts of blood (only 2.5 ml were used compared to 40 ml required for granulocyte purification), this offers a more preferable method for investigating neutrophils in critically ill patients. Finally, I have demonstrated for the first time that this whole blood assay in sufficiently sensitive to appreciate discrete changes in the priming status of neutrophils (as reflected in alterations in CD62L shedding) across the pulmonary vasculature.

This study has shown that in perioperative 'control' patients, no trans-pulmonary neutrophil priming gradient is evident. However, patients with systemic sepsis without any lung involvement were found to have a 'positive' trans-pulmonary priming gradient. Neutrophils from central venous blood (and hence neutrophils that are about to enter the pulmonary circulation) were found to express less cell surface CD62L (and hence are more primed) than neutrophils leaving the pulmonary circulation, in radial arterial blood. A similar finding was made by Nahum et al. in a study that assessed neutrophil priming by measurement of H_2O_2 production of neutrophils activated ex-vivo by zymosan (Nahum et al. 1991). In this study, it was found that neutrophils from the pulmonary artery of patients with sepsis without lung infiltrates on chest radiography, were more primed (produced more H_2O_2 on

activation) than neutrophils taken simultaneously from the radial artery. This suggests that in patients with systemic sepsis without lung involvement and no impairment of gas exchange, neutrophils are being primed peripherally and are then either being sequestered and removed by the pulmonary circulation or are trapped, then de-primed prior to re-release. However, no trans-pulmonary gradients were seen in-terms of absolute neutrophil counts, giving evidence to support the idea of the lungs being a site of de-priming without sequestration, or at least being in a steady state of retaining and releasing neutrophils. This idea is further supported by work form Summers et al. who demonstrated only transient pulmonary retention of neutrophils that had been radio-labelled and primed ex-vivo with either GM-CSF or PAF. After a period of trapping/sequestration in the pulmonary capillary bed, these cells were released back into the systemic circulation (Summers et al. 2009).

In patients with ARDS secondary to any cause including systemic sepsis, we have found a fairly uniform reversal of this neutrophil priming gradient. In these patients neutrophils leaving the pulmonary circulation were more primed (expressed less cell surface CD62L) than neutrophils entering the lungs. Nahum et al. made a similar observation in his study, where neutrophils taken from the radial artery of patients with ALI (not just limited ARDS) with no evidence of sepsis, produced more H_2O_2 on ex-vivo activation with zymosan, than neutrophils taken from the pulmonary artery (Nahum et al. 1991). This suggests that in patients with ARDS, the lungs themselves may be acting as an inflammatory environment and switch to actively prime neutrophils.

These opposing trans-pulmonary neutrophil priming gradients were seen in a single patient over a time course, which corresponded to their clinical picture. This person, suffering from necrotising fasciitis, was observed to have a positive gradient when they were systemically unwell with sepsis but preserved lung function, suggesting that the neutrophils were being primed in the systemic circulation presumably from her infected cutaneous tissues and then being de-primed in the pulmonary circulation. However, her trans-pulmonary neutrophil priming gradient was found to reverse once she had developed ARDS, suggesting that in this case the ability of the lungs to trap and de-prime neutrophils was either lost or became overwhelmed by the pro-inflammatory influence of the ARDS lungs. As her lung injury started improving there was a very slight reduction in the size of her trans-pulmonary neutrophil priming gradients, suggesting that the magnitude of these gradients may reflect severity of disease. This was further evidenced by the finding of a positive correlation between the CD62L A-V ratio and the patients' PaO_2/FiO_2 ratio (r_s =

0.75, p=0.003, R^2=0.415), whereby the more negative the trans-pulmonary neutrophil priming gradient, the lower the observed patient PaO_2/FiO_2 and hence the more hypoxaemic they were. However, many studies use the lung injury score (Murray et al. 1988) to measure the severity of ALI, which is a scoring system based on PaO_2/FiO_2 ratios, the extent of infiltrates seen on chest radiography, the amount of PEEP a patient is receiving, and their lung compliance (see appendices, table A2). Albeit that I only studied small numbers, I found no correlation with the CD62L A-V ratio to the lung injury score (see appendices, figure A3), which may well represent the limited scale of the scoring system (0-4) and the fact that it is not a continuous scale. Hence, a score of 3 will encompass a very wide spectrum of patients. Whereas use of the PaO_2/FiO_2 ratio allows for very subtle changes. Never-the-less, the finding of a positive correlation between the magnitude of the trans-pulmonary neutrophil CD62L gradient and PaO_2/FiO_2 ratio is very interesting. Firstly, this suggests that the ability of the lungs to de-prime neutrophils, is not an all or nothing function, but instead a graduated process, which probably occurs diffusely throughout the pulmonary capillary vasculature. Secondly, the magnitude of the trans-pulmonary gradient may reflect disease severity of ARDS, and more importantly outcome, so may potentially provide a useful biomarker for interventional studies. Finally, the exact timing of when a trans-pulmonary neutrophil priming gradient is established in the natural history of sepsis or ALI has not been determined by this study, however should this occur prior to these conditions becoming clinically apparent, establishing that such a gradient exists may provide a useful marker for disease and allow for the optimal timing of therapeutic interventions.

No trans-pulmonary gradients were evident in terms of neutrophil shape change. This is perhaps surprising as Yoshida et al. demonstrated a measurable trans-pulmonary gradient of neutrophil F-actin polymerization in rats, 4 hours after the induction of experimental pneumonia with inhalation of streptococcus (Yoshida et al. 2006). In this study we measured changes in the properties of light scatter by individual neutrophils and in particular changes of forward scatter, which has previously been validated to correlate with microscopically evaluated shape change of stimulated neutrophils (Cole et al. 1995). However, this is only an indirect measurement of F-actin polymerization and neutrophil shape change has been recognised to occur in the absence of F-actin polymerization (Keller and Niggli 1993) and in one study looking at F-actin directly by F-actin staining and

subsequent flow cytometry, showed no correlation of F-actin polymerization with changes in neutrophil forward scatter (Keller et al. 1995).

No trans-pulmonary gradients were found either for neutrophil cell surface CD11b expression. This is perhaps not unexpected. Condliffe et al. for example have demonstrated the potential for dissociation of CD62L shedding from CD11b up-regulation on neutrophil priming, depending on the priming agent used (Condliffe et al. 1996). They report that whilst TNFα stimulation caused decreased expression of CD62L with up-regulation of CD11b, stimulation with low concentrations of LPS induced CD62L shedding in the absence of CD11b up-regulation, implying that CD62L is perhaps the more sensitive cell surface marker of priming. This may be relevant to this study as LPS/endotoxin is clearly important in the pathogenesis of sepsis and is the most widely used agent for inducing experimental ALI. Functionally, the up-regulation of CD11b during neutrophil priming is less important than the activation of CD11b. Schleiffenbaum et al. observed that up regulation of neutrophil cell surface CD11b by fLMP and PMA stimulation could be inhibited by incubation at 16ºC, but these neutrophils still managed to adhere to plastic tissue culture dishes. Further, this adherence could be abolished by anti-CD11b and anti-CD18 monoclonal antibodies confirming this was CD11b/CD18 dependent adhesion (Schleiffenbaum et al. 1989). It was later shown that neutrophil stimulation induces a conformational change within the ligand binding domain of CD11b, with increased adhesion capability (Diamond and Springer 1993; Oxvig et al. 1999). More recently, in-vitro studies have demonstrated that this conformational activation of CD11b can occur in the absence of its up-regulation on neutrophil stimulation by IL-8, C3a and PAF (Orr et al. 2007). Hence, a more sensitive marker may have been one directed towards determining the activation of CD11b. Finally, it has been proposed that the role of CD11b/CD18 is less important in the pulmonary circulation than in the systemic circulation. In the systemic circulation, outward migration of neutrophils occurs at a post capillary venule level and requires CD11b/CD18 interactions to mediate tight binding of the neutrophil to the endothelial surface. However, in the lung, neutrophil migration occurs in the pulmonary capillaries where neutrophils are already in constant contact with endothelial wall surfaces. Further, most neutrophil migration in the lung occurs independently of CD11b/CD18 interactions in response to various stimuli such as Streptococcus pneumonia, group B Streptococcus, Staphylococcus aureus, hydrochloric acid, hyperoxia and C5a (Doerschuk 2000).

The results of this study therefore, provide support for the hypothesis introduced in chapter 1, whereby the pulmonary circulation may provide the site for neutrophil

entrapment and de-priming, and thus protecting the body from inappropriate neutrophil mediated toxicity. However, when this defensive mechanism fails or is overwhelmed, pulmonary neutrophil inflammation ensues, such as that seen in ALI (see chapter 1, figure 1.3).

The mechanisms underlying neutrophil de-priming in the lung microcirculation are unknown. However, it has been shown that neutrophils co-incubated with monolayers of bovine pulmonary artery endothelial cells, generate 80-90% less superoxide anion in response to fMLP stimulation than neutrophils incubated alone, and have suppressed degranulation. This effect was independent of endothelial scavenging of reactive oxygen species, as inhibition of endothelial superoxide dismutase did not abrogate the effect. The inhibitory effect was also observed when neutrophils were not in direct contact with the endothelial cells, suggesting that there may be a soluble factor released by the pulmonary endothelial cells (Basford et al. 1990). A further study has implicated adenosine as the responsible agent, as the inhibitory effect was abolished with the addition of adenosine deaminase (Gunther and Herring 1991). Novel work looking at the mechanical properties of neutrophils by capturing individual neutrophils by laser beams, have provided preliminary data suggesting that primed neutrophils that have undergone a polarised change in shape revert back to a regular shape after they have been rapidly and repeatedly stretched (Ekpenyong A., Cavendish Labs, University of Cambridge - unpublished). The pulmonary circulation, receiving the entire cardiac output and having such a vast network of capillaries many of which are smaller than 5.5 μm in diameter may have the exact properties to cause such rapid, repeated cell stretching.

There are several limitations of this study. Firstly, the relatively small numbers of subjects studied raises the possibility of type 1 error; however given the level of significance found on statistical testing, the finding of opposite trans-pulmonary gradients and the significant correlation of the CD62L A-V ratio with PaO_2/FiO_2 ratio suggests that this is a true finding. Secondly, absolute neutrophil counts were measured in the main clinical haematology laboratory by Coulter counting. The machine used (Beckman Coulter DXH800 Coulter Cellular Analyser) publishes an accuracy of +/- 2% (Beckman Coulter website - https://www.beckmancoulter.com) and thus it may be possible that this not sensitive enough to measure very fine trans-pulmonary gradients in neutrophil counts. Further, central venous blood from internal jugular and subclavian catheters were used to represent blood entering the pulmonary circulation. However, these catheters sit in the superior vena cava which

mainly receives blood from the cerebral circulation. Blood from the rest of the body including the spleen and liver may be of relevance and therefore ideally a truly mixed central venous blood sample should have been used. However, this involves the use of pulmonary arterial catheters which are no longer routinely used on this ICU and was outside the ethical permissions granted for this study. Finally, patients who were suffering with systemic sepsis with a PaO_2/FiO_2 ratio of more than 40 KPa and a clear chest radiograph were often perceived to be less critically unwell than patients with fulminate ARDS, therefore the severity of disease and possibly the amount of neutrophil mediated inflammation was not equal between the two main patient groups.

Future work would be directed at:

1) Demonstrating that these findings are repeatable. Power calculations based on the data from this feasibility study reveal that in order to have a 90% power to detect a 0.1 difference in the cell surface expression of CD62L between sepsis and ARDS patients, at the 0.05 significance level, future studies will need 47 subjects in each group.
2) Study the changes in these trans-pulmonary neutrophil priming gradients over time in patients who are at risk of ARDS. Hence determine the exact time course of when these gradients occur, how they change with disease progression and resolution, and if there is any relation to patient outcomes.
3) Confirm that the positive trans-pulmonary gradients seen in patients with sepsis without lung involvement are not the result of pulmonary sequestration of CD62L low neutrophils. This could be tackled by using more sensitive measures of absolute neutrophil counts and/or cell sorting neutrophils into CD62L low and high populations, radio-labelling the CD62L low cells and imaging their journey across the lungs after venous injection.

4 ALI Post Oesophagectomy

4.1 Introduction

An oesophagectomy is a complex surgical procedure involving partial or complete resection of the oesophagus; it is indicated/ used for the treatment of mostly early oesophageal cancers (such as adenocarcinoma or squamous cell carcinoma of the oesophagus) and occasionally benign conditions such as Barrett's oesophagus with high grade dysplasia. More rarely this procedure is indicated for caustic acid ingestion complicated by severe oesophageal stricture formation.

Oesophagectomy involves mobilizing and resecting the oesophagus and dissecting and preparing the stomach for oesophageal reconstruction. The new gastric tube is then drawn up into the chest and an anastomosis formed with the remaining healthy oesophageal stump. This operation has traditionally been performed with open surgical techniques such as the Ivor Lewis and left thoraco-abdominal oesophagectomies, which involve a thoracotomy and a laporotomy, (or in the case of trans-hiatal oesphagectomies, typically for lower-third tumours, only an abdominal incision is required) (NICE 2011). With the exception of the trans-hiatal approach, during the operation one of the lungs is collapsed and the other subjected to single lung ventilation. This is termed one lung ventilation (OLV), and is used to aid access to the oesophagus.

These procedures have been associated with a significant mortality and high rates of post operative ALI/ARDS; however the actual incidence of lung injury varies quite considerably in the literature. A single centre UK study looking at 168 consecutive oesophagectomies between 1996-1999, and a single centre US study of 71 oesophagectomies between 1978-1993, reported an incidence of ARDS of 14.5% (Tandon et al. 2001) and 17% (Millikan et al. 1995) respectively. However, these publications were reliant on retrospective observations, and as per our own findings (see Chapter 2, section 2.3.3) and others (Cely et al. 2010), ARDS is often an under-recognised condition. Prospective studies have reported significantly higher rates of ARDS of 33-53%, however, these have been small studies of 18-19 cases only (Katsuta et al. 1998; Schilling et al. 1998). Further, there is very little information with regards to the incidence of less severe forms of ALI. Tandon et al. reported an incidence of 23.8% of ALI post oesophagectomy (Tandon et al. 2001), however it is not clear from the manuscript whether this figure is separate from the 14.5% ARDS cases also reported, or inclusive (as per the original AECC definition of ALI as an umbrella term), as the total incidence of respiratory failure was stated

as 23.8%. Katsuta et al. clearly reports ALI separately from ARDS, and observes an ALI incidence as high as 37% (Katsuta et al. 1998), and Morita et al. reports an incidence of 42% (Morita et al. 2008), but once again these were very small studies (n = 19 and 27 respectively).

In more recent years, there has been a trend towards performing oesophagectomies with minimally invasive surgical techniques using thoracoscopy and laparoscopy, although these approaches still require OLV and often extended operative time. Data establishing the incidence of ALI/ARDS after minimally invasive oesophagectomies is currently very limited and certainly not covered by the above studies. A recent large, multicenter, retrospective study looking at 858 oesophagectomies between 1998-2008, which included 464 minimally invasive procedures, reported an incidence of ARDS of only 1.5% (Zingg et al. 2011). However, once again this was a retrospective analysis with very limited detail about the quality of ARDS diagnosis and no reports of the incidence of ALI. The very low incidence figure in this study may also suggest significant under recognition/ reporting of ALI/ARDS.

The reason for ALI post oesophagectomy is not immediately clear; the main contenders appear to be direct trauma due to the lung being handled, ventilator associated barotrauma and ischaemia-reperfusion injury sustained during and after the period of OLV (see Chapter 1, 1.3.2). There have also been associations between ALI/ARDS and the total length of operation time (Morita et al. 2008; Tandon et al. 2001), the number of anastomotic leaks (Morita et al. 2008; Tandon et al. 2001), intra-operative fluid balance, low BMI, smoking history (Tandon et al. 2001) and respiratory co-morbidities (Zingg et al. 2011). Regardless of the cause, oesophagectomy patients appear to offer a reasonable model of ALI/ARDS with a known timing of the lung insult and so would be a useful population to support further ALI research.

Given the wide variation of the reported incidence of ARDS post oesophagectomy, the lack of specific data for ALI, and the minimal data concerning newer surgical techniques, we have undertaken an 18 month, prospective study to determine the incidence of ARDS and ALI within a large UK teaching hospital. Particular attention has been given to accurate case validation and the recognition of any associated preoperative and perioperative measures.

4.2 Method

This study was conducted within Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust. This is a 1250-bedded University Hospital with a local catchment of 350,000 people and extended referral area of c. 2 million. The upper gastro-intestinal service therefore provides a tertiary level service for the East of England, and performs up to 3 oesophagectomies per week. Addenbrooke's Hospital forms part of Cambridge University Hospital NHS Foundation Trust and has the second lowest standardised mortality rate of any hospital in the UK at 0.80 (drfosterhealth.co.uk 2008).

For this study, all patients undergoing an elective oesophagectomy between 18th May 2010 and 17th November 2011 were followed up throughout their admission. A typical admission pathway for a patient undergoing elective oesophagectomy was as follows: Patients were admitted electively on the morning of their operation. After their oesophagectomy they were immediately extubated where possible and transferred to a post-operative recovery unit where they received intensive monitoring. At this stage they had a routine frontal chest radiograph and monitoring of their arterial blood gases. If stable after 24 hours they were transferred to the HDU (or ICU if unstable). Further chest radiographs or arterial blood gas analysis were only performed if clinically indicated. Patients were then stepped down to normal ward care and eventually discharged home when clinically appropriate.

Data were collected prospectively by myself, (and I can confirm that I was not involved in routine patient care). The clinical staff involved were not aware of the aims of this study or any of the results during the period of data collection. The initial preoperative data set collected for each patient included age, gender, BMI, smoking history, list of co-morbidities, list of medications, pulmonary spirometry results and reason for elective oesophagectomy (if oesophageal cancer - then type, stage and previous treatment). Twenty-four hours post oesophagectomy, the perioperative data were collected. This data set included date of operation, type of operation, length of time of surgery (from initial incision to closure of skin – recorded to the nearest 15 minutes), length of time of OLV (recorded to the nearest 15 minutes), volume of blood replacement (if any), intra-operative fluid balance and 24 hour fluid balance. Patients were then reviewed on a daily basis throughout the rest of their hospital admission with ongoing data collected as to the chest radiograph appearance, arterial blood gas results, the development of complications

(specifically ALI or ARDS, sepsis, anastomotic leak or pneumonia), lengths of ICU, HDU and total hospital stay, and eventual hospital outcome.

ALI/ARDS was defined using the 1994 AECC criteria. These were disease of acute onset, associated with bilateral infiltrates on a frontal chest radiograph consistent with pulmonary oedema, the absence of clinical left atrial hypertension, and a PaO_2/FiO_2 ratio of less than 40 KPa for ALI and less than 27 KPa for ARDS (Bernard et al. 1994). An additional criterion was included in that the chest radiograph and PaO_2/FiO_2 analysis must be performed within 24 hours of each other. Sepsis was defined as a known or suspected infective source with 2 or more of the following: A body temperature of less than 36ºC of greater than 38 ºC; a heart rate of greater than 90 beats per minute; a respiratory rate of greater than 20 breaths per minute or a $PaCO_2$ of less than 4.3KPa; and a white cell count of less than 4 x 10^9/L or greater than 12 x 10^9/L. An anastomotic leak was determined after appropriate demonstration by a water soluble radio-contrast swallow test. Pneumonia was defined as a new infiltrates on a chest radiograph with new or increased purulent sputum production with initiation of antibiotics by the treating clinicians.

There are several reports of inter-observer variation in the interpretation of chest radiographs for the assessment of ALI (e.g. Rubenfeld et al. 1999). In the context of chest radiographs post oesophagectomy, this may be particularly true as there is direct handling of the lungs during the procedure with frequent development of post operative atelectasis, contusion and pleural effusions. This is exampled in Figure 4.1, which shows examples of chest radiographs that have appearances clearly consistent (A) or not consistent (B) with a diagnosis of ALI, and also a chest radiograph where the appearances are somewhat more difficult to interpret (C). For this reason all chest radiographs were reviewed by 2 Respiratory Physicians and a Thoracic Radiologist (all of whom were blinded to the clinical data) to confirm whether or not appearances were in keeping with ALI. All 3 observers were reviewing the chest radiographs together (and thus were not blinded to each other's opinions). A consensus as to whether the appearances may be in keeping ALI/ARDS or not, was reached when at least 2 of the 3 agreed.

4.2.1 Statistical analysis

Data were analysed using GraphPad Prism 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Categorical variables were

described using proportions and analysed using Fisher exact and chi-squared tests. Continuous variables were described using median (inter-quartile range) unless otherwise stated, and analysed using Mann-Whitney and Kruskal-Wallis tests with Dunn's post test. All tests were 2 tailed. A *P* value of <0.05 was considered statistically significant.

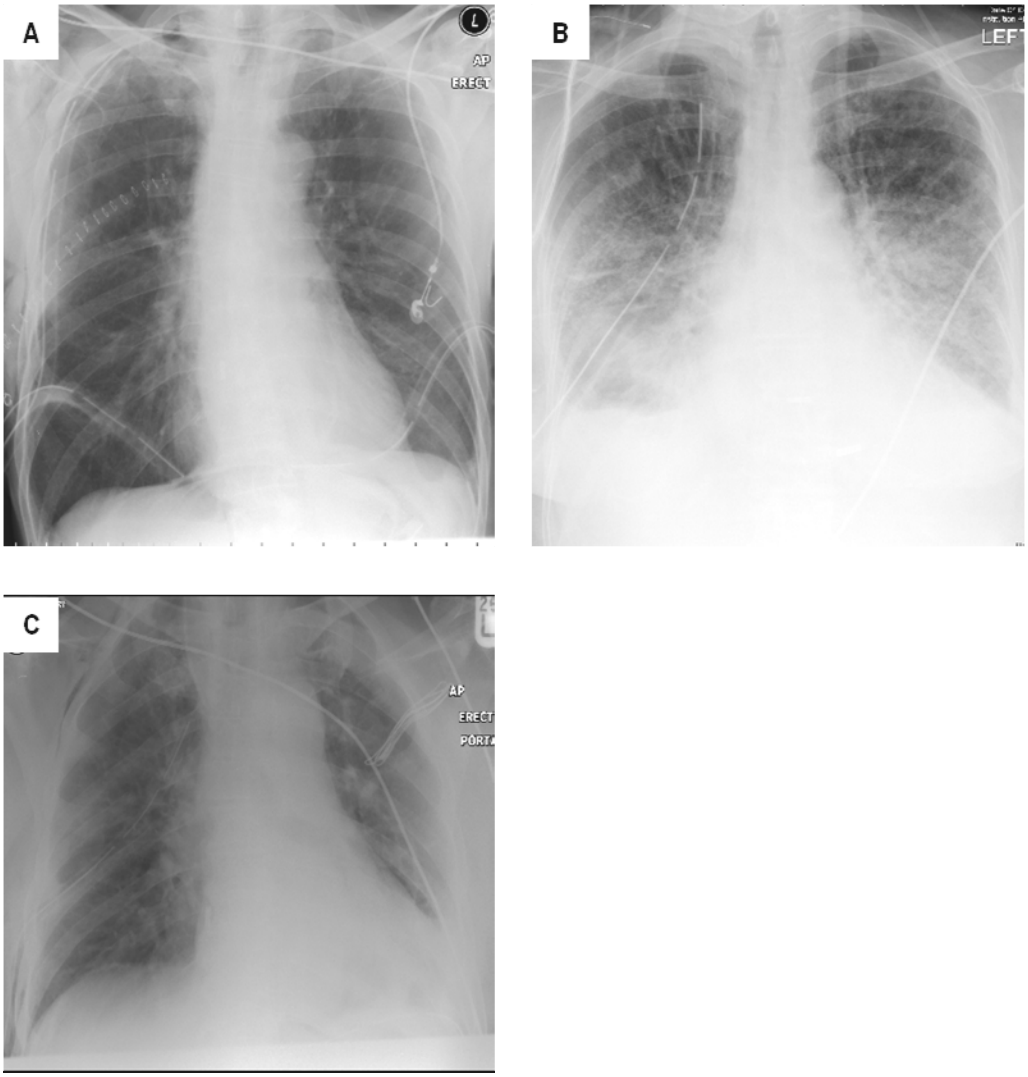

Figure 4.1 Examples of frontal chest radiographs taken in patients post oesophagectomy. Panel A shows appearances with clear lung fields and bilateral chest drains *in-situ*. Panel B shows bilateral pulmonary infiltrates consistent with pulmonary oedema. The cardiac silhouette is not enlarged. These appearances are consistent with a possible diagnosis of ALI. Panel C shows bilateral opacities over the lower zones bilaterally. On the left there is evidence of collapse and consolidation of the lower lobe with a possible pleural effusion. On the right side there is evidence of the gastric pull through and atelectasis within the lower zone.

4.3 Results

4.3.1 Incidence of ALI and ARDS

Eighty-nine patients were admitted for elective oesophagectomy over the 18 month study period. Of these 2 patients did not proceed to a full oesophagectomy due to advanced malignant disease found at the time of the operation, 1 patient did not receive a chest radiograph post operatively and therefore could not be assessed for the development of ALI/ARDS, and 1 patient was lost to follow up. Eighty-five cases were therefore reviewed (see figure 4.2). Thirteen patients (15.3%) developed ALI (but not ARDS) and a further 13 patients (15.3%) developed ARDS post operatively. The pre-operative characteristics of these patients are compared in table 4.1 and were similar to those patients who did not develop lung injury (NLI).

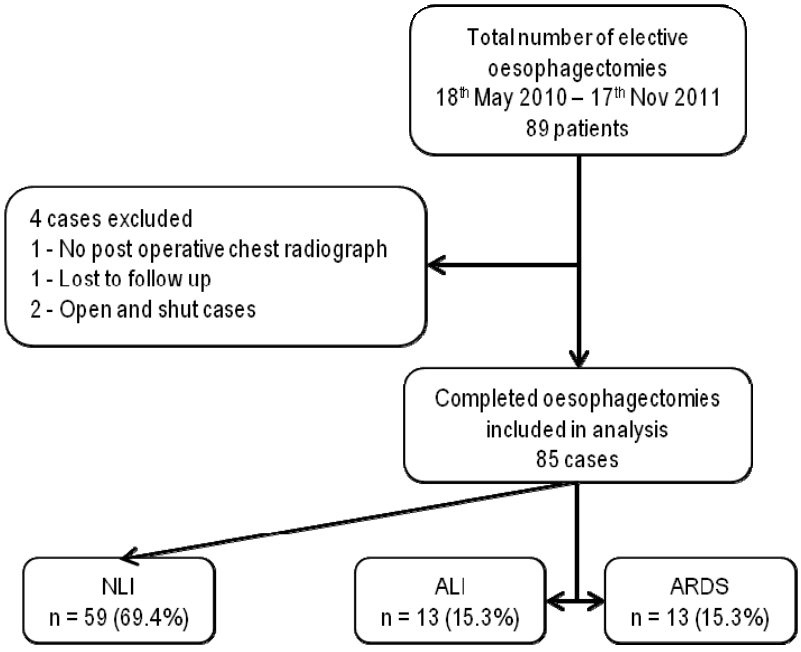

Figure 4.2 Schematic showing the number of elective oesophagectomies reviewed. NLI – no lung injury.

	NLI	ALI	ARDS
n (%)	59 (69.4)	13 (15.3)	13 (15.3)
Age (years)	63.1 (65)	69 (69)	63.5 (60)
Male (%)	84.7	61.5	76.9
BMI (Kg/m^2)	26.8 (26)	27.8 (28.8)	24.9 (26.8)
FEV_1 (% predicted)	96.1 (96.5)	97.2 (104.1)	93.5 (94)
Smoking hx (pack years)	20.9 (10)	23.1 (7.5)	32.5 (30)
Respiratory co-morbidities (%)	23.7	23.17	38.5
Cancer type			
Adenocarcinoma (%)	83	92.3	61.5
Squamous cell carcinoma (%)	8.5	0	30.8
Other cancer (%)	3.4	7.7	0
Benign (%)	5	0	7.7

Table 4.1 Baseline patient characteristics. NLI - no lung injury, BMI – body mass index, FEV_1 – forced expiratory volume in 1 second, Respiratory co-morbidities – percentage of patients who had 1 or more respiratory co-morbidity prior to their oesophagectomy. Values represent mean (median) unless otherwise indicated. No significant differences were seen between NLI, ALI and ARDS groups.

The timing of onset of ALI and ARDS post oesophagectomy is shown in figure 4.3. There appears to be 2 peaks, with ALI/ARDS occurring most frequently immediately post operatively within the first 24 hours and then again at around the 6^{th} post-operative day (see figure 4.3A). ALI accounts for almost all of the initial immediate post-operative cases (6 cases), whereas ARDS seems to occur later on (post-operative day 2-7).

4.3.2 Perioperative influences

The length of time taken to perform an oesophagectomy is variable. Operative times during this study varied between 3.25 and 7.75 hours. Patients who subsequently developed ALI had significantly longer operation times (median 6.5 (5.6-7.4) hours) than those who had NLI (median 5.5 (4.2-6.8) hours) ($p<0.01$). Patients who developed ARDS also had a trend towards longer operating times (median 5.8 (5.3-6.2) hours), but surprisingly this was non-significant when compared to NLI patients (see figure 4.4A). There remained a significant difference, however, when comparing all ALI/ARDS patients (median 6.1 (5.4-7.3) hours) with NLI patients ($p<0.01$).

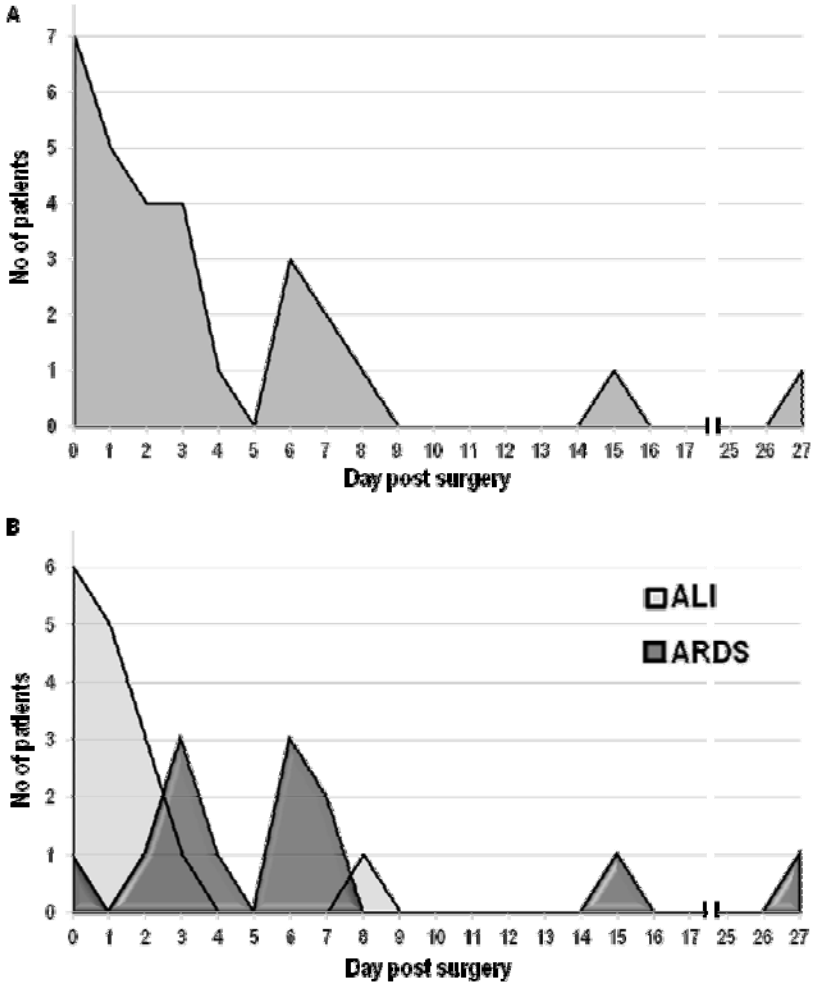

Figure 4.3 Time from oesophagectomy to the development of ALI/ARDS. Panel A – shows cumulative data for ALI and ARDS combined. Panel B – Shows the individual contribution of ALI and ARDS

All but 1 patient underwent a period of OLV during their operation. This was achieved by intubating patients with a double lumen endotracheal tube and clamping off one of the lumens, thus allowing collapse of the lung that it supplies and ongoing ventilation through the other lumen to the contra-lateral lung. Patients who developed ARDS spent significantly longer periods on one lung ventilation (median 2.3 (2.2-3.2) hours) compared those who developed NLI (median 2 (1.5-2.3) hours), (p=<0.01). There were no significant differences between patients with ALI (median 2.2 (1.8-2.5) hours) and NLI patients (see figure 4.4B). When combining ALI and ARDS patients the median time (2.3 (2-2.8) hours) was significantly different to that of NLI patients.

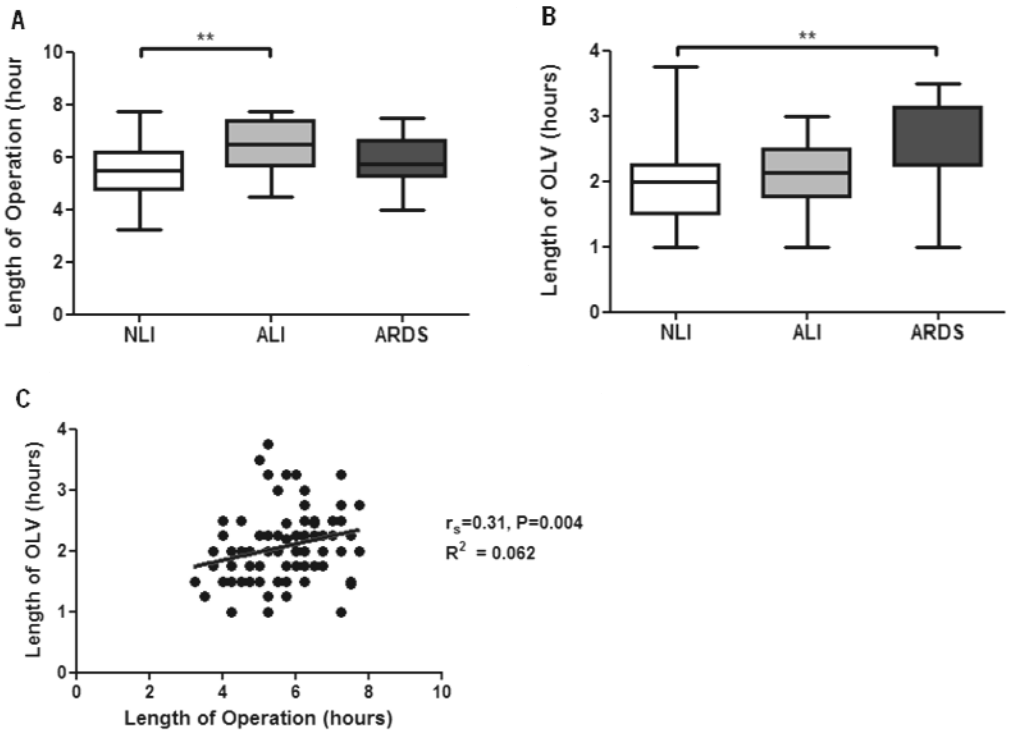

Figure 4.4 Length of operation time and one lung ventilation for patients who developed NLI, ALI and ARDS. Panel A – length of total operation time. Panel B – length of time spent on one lung ventilation. Data represent median, inter-quartile ranges and full ranges. (**p<0.01 comparing ALI or ARDS and NLI groups). Panel C – correlation between length of operation time and length of one lung ventilation. (r_s, Spearman's rank correlation; R^2, coefficient of determination).

All oesophagectomies were performed by one of 2 experienced consultant upper gastro-intestinal surgeons with the help of an assistant who varied throughout the study period. One of these surgeons performed Ivor-Lewis oesophagectmies exclusively (n=30), except in the case of 1 patient with poor pre-operative lung function, who underwent a trans-hiatal procedure. The second surgeon performed minimally invasive procedures (n=28), laparoscopically assisted Ivor Lewis oesophagectomies (n=12) or left thoraco-abdominal oesophagectomies (n=14) depending on the size and site of the oesophageal tumour. For the purposes of this study, operative techniques were grouped into procedures involving a thoracotomy (i.e. Ivor Lewis, laparoscopic assisted Ivor Lewis and thoraco-abdominal oesophagectomies) (n=56) and those involving thoracoscopy (i.e minimally invasive oesophagectomy) (n=28). Although the thorascopic procedures involved significantly longer operating times compared to thoracotomy/laparotomy based

procedures (median 6.4 (5.8-7.3) vs 5.3 (4.6-6.0) hours respectively, p<0.0001), there were no differences found in terms of lengths of one lung ventilation. Further there were no significant differences found in terms of the incidence of subsequent development of ALI/ARDS (25% and 39% post thoracotomy and thoracoscopy respectively).

Patients received intravenous fluids (and infrequently blood products) during and immediately after their operation. By deducting the volume of urine produced, an assessment of fluid balance was achieved for each patient. There were no significant differences with regards to the intra-operative and total 24 hour fluid balance status between patients who developed NLI, ALI or ARDS (see figure 4.5). No differences were seen between the NLI and combined ALI/ARDS patients.

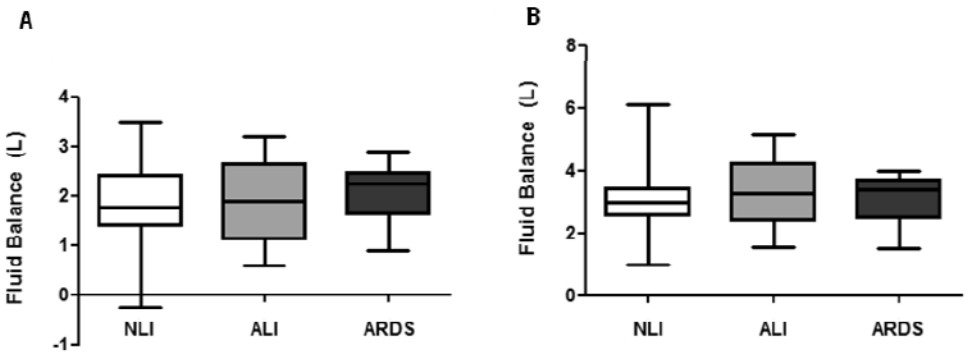

Figure 4.5 Intra-operative (Panel A) and 24 hour (Panel B) fluid balance of patients who developed NLI, ALI and ARDS. Data represent median, inter-quartile ranges and full ranges.

4.3.3 Post-operative course

The most common post-operative complications encountered post oesophagectomy other than ALI/ARDS were pneumonia (n=42 (49%)), development of a leak at the site of anastomosis (n=9 (10.6%)) and the development of a chyle leak (n=5 (5.6%). Anastomotic leaks occurred significantly more frequently in patients who developed ARDS (5/13, 38.5%) compared to patients with NLI (3/59, 5%) (p=0.0057), although were only evident in 7.7% (1/13) of patients with ALI. Of the 6 patients that had developed ALI/ARDS and an anastomotic leak, 5 of these had developed ALI/ARDS prior to the diagnosis of their leak. Pneumonia also occurred significantly more

frequently in patients with ARDS (13/13, 100%) compared to NLI patients (21/59, 35.6%), ($p<0.0001$). There were no significant differences between ALI and NLI patients in regards to post-operative complications.

Patients who had developed ARDS spent significantly more time on the ICU (median 3 days) compared to NLI patients (median 0 days), ($p<0.0001$), and had significantly longer total lengths of hospital stay (median 23 vs 12 days respectively), ($p<0.001$). ALI patients however had similar ICU and hospital lengths of stay as NLI patients (see figure 4.6).

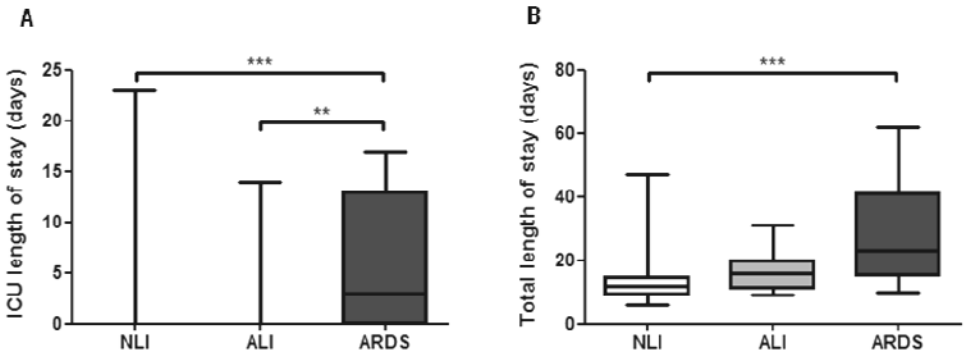

Figure 4.6 ICU (Panel A) and total hospital (Panel B) lengths of stay in patients who developed NLI, ALI and ARDS. Data represent median, inter-quartile ranges and full ranges. (**p<0.01, ***p<0.001 comparing ALI or ARDS and NLI groups).

There were 2 in-hospital deaths post oesophagectomy representing a total hospital mortality rate of 2.35%; this is below the in-hospital mortality rate of 8.8% reported in a review of 312 studies (70756 oesophagectomies) between 1990-2000 (Jamieson et al. 2004). Both of these deaths occurred in patients who had developed ALI/ARDS, giving a mortality rate of 7.7% compared to 0% for patients with NLI.

4.4 Discussion

We report a total incidence of 15.3% ALI and 15.3% ARDS in patients post elective oesophagectomy in a UK teaching hospital. This is entirely in-keeping with the incidence of 14-17% ARDS reported from retrospective studies from the UK and the US in the 1990s (Millikan et al. 1995; Tandon et al. 2001). This contrasts to some prospective studies, which have reported a significantly higher incidence of 33-53% for ARDS (Katsuta et al. 1998; Schilling et al. 1998) and 37-42% for ALI (Katsuta et al. 1998; Morita et al. 2008). However, these later studies were small (n=18-27) and at considerable risk of type 1 error. Further, 2 out of 3 of these studies were from Japan where oesophageal carcinomas are extremely prevalent and so may be influenced by differing genetic factors. Also, the somewhat subjective nature of the AECC ALI/ARDS diagnostic criteria may have influenced these findings especially in the diagnosis of the less severe ALI. Great variability has been demonstrated in the interpretation of chest radiographs in the diagnosis of ALI (Angus 2012; Meade et al. 2000; Rubenfeld et al. 1999) and it is clear that chest radiographs performed post oesophagectomy are even more difficult to interpret with certainty; the breach in the pleural cavity, direct handling of the lungs and collapse and re-expansion of the lung during the operation often lead to transient or persistent atelectasis and pleural effusions. Further there is the added complication of the overlying gastric pull through in the chest. For this reason, the primary aim of this study was the accurate diagnosis of ALI/ARDS using a consensus approach to interpreting post operative chest radiographs. Chest radiographic appearances were only deemed in keeping with a diagnosis of ALI/ARDS if at least 2 out of 3 assessors (2 Respiratory physicians and 1 Thoracic radiologist not blinded to each other's opinion) agreed. Using this approach, unanimous agreement between these three adjudicators was only reached in 79% of cases.

A number of preoperative risk factors for the development of ARDS have been reported in the literature. Tandon et al. has reported that a lower BMI increases the risk of developing ARDS post oesophagectomy (Tandon et al. 2001). This is in keeping with a study of patients with ARDS from any cause demonstrating that overweight patients benefited from lower mortality rates compared to patients with normal weights, and that patients with lower BMIs were associated with higher odds of death (O'Brien et al. 2006). Stapleton et al. has shown an inverse relationship with BMI and levels of various proinflammatory cytokines such as IL-6, IL-8 and the

surfactant protein D, and thus suggests that this is possibly due to less epithelial damage in obese patients. However, this inverse correlation of BMI with mortality in patients with ARDS has not been repeated in similar studies since (Morris et al. 2007; Stapleton et al. 2010), and in the current study there were no associations with BMI and the development of ARDS post oesophagectomy.

Smokers have also been reported to be more at risk of ARDS post oesophagectomy than non-smokers (Tandon et al. 2001; Zingg et al. 2011). This is entirely plausible as smoking is clearly associated with COPD and several other forms of airway and lung disease and is known to increase pulmonary neutrophil migration (Blidberg et al. 2012); smoking has also been found to be an independent risk factor for increased mortality in patients with ARDS (Ando et al. 2012). However, in our study, we again found no correlation between the development of ALI/ARDS and smoking history in terms of smoking pack years (see table 4.1) or smoking status (current vs ex-smoker vs life long non-smoker) (data not shown). This may represent the near normal spirometry observed for these patients (see below).

The presence of respiratory co-morbidities has also been implicated as a risk factor for the development of ARDS post oesophagectomy (Zingg et al. 2011). We, again did not find this, although of note is that the baseline lung functions for patients in this study were extremely well preserved (FEV_1 95.7 % predicted +/-20.9 (mean +/-SD)).

Perioperative factors have also been implicated. Tandon et al. reported an association of longer operative times and OLV times with the development of post oesophagectomy ARDS. However, rather unsurprisingly, we have demonstrated a positive correlation between the length of operation time and length of OLV (see figure 4.3C), and hence whether these 2 risk factors are truly independent or merely collinear variants is uncertain. This study has confirmed the finding of longer OLV times with the development of ARDS. This suggests OLV as a possible cause, or contributing factor, of ALI/ARDS, which is thought to result (at least in part) from barotrauma to the ventilated lung and ischaemia-reperfusion injury to the collapsed lung on subsequent re-inflation (see Chapter 1, section 1.3.2). The length of operation time was found to be associated with the development of ALI only and not ARDS. Given ALI/ARDS represents a spectrum of the same condition (with ARDS being at the more severe end), this is a peculiar result and suggests that this may be an anomaly. This idea is supported by the fact that no differences in the development of ALI/ARDS were observed post minimally invasive

oesophagectomies (thorascopic approach) compared to post open oesophagectomies (thoracotomy based approaches), even though the minimally invasive procedure involved significantly longer operating times (but similar OLV times).

Increased intra-operative fluid balance during lung resection surgery (Evans and Naidu 2012) and oesophagectomy (Tandon et al. 2001) has been shown to be associated with postoperative development of ARDS. In this study, although there was a trend towards increasing positive intra-operative fluid balance in patients who developed ARDS (median +2.25L (1.6-2.5)) compared to those who developed ALI (+1.90L (1.1-2.7)), compared to those with NLI (median +1.76L (1.4-2.4)) (see figure 4.4A), these differences were not statistically different. A similar finding was made when measuring 24 hour fluid balances.

This study and others (Morita et al. 2008; Tandon et al. 2001) have shown an association between the development of an anastomotic leak and the development of ARDS. However, it is not absolutely clear whether this is a cause or effect relationship. In 83% of the cases in which patients had developed both an anastomotic leak and ARDS/ALI, the anastomotic leak was diagnosed after the development of ALI/ARDS. This suggests ALI/ARDS as a cause and may represent the global effects of this syndrome with possible negative effects on wound healing. However, we cannot be certain as to the true temporal relationship, as anastomotic breakdown may occur much earlier than the clinical picture, in which case local tissue necrosis and infection may cause ALI/ARDS.

The finding of ALI occurring most frequently immediately post operatively while ARDS occurring most frequently around day 2-7 post operatively, suggests that patients who develop ARDS (rather than just ALI) sustain their injury later. This gives further support to the idea of ARDS being the result of a “2 hit” process, whereby there is the initial injury sustained intra-operatively perhaps by the barotrauma and ischaemia-reperfusion injury caused by OLV and then a subsequent 2nd insult (e.g. ongoing mechanical ventilation or local infections) resulting in neutrophil migration and tissue injury (see Chapter 1, section 1.2.4).

There are several limitations of this study. Firstly, these results are from a single centre, and therefore may not be representative where local differences in pre-operative patient optimisation, surgical techniques and post-operative management may be apparent. Secondly, there were only 2 main surgeons operating during this study, minimally invasive oesophagectomies were performed exclusively by one

surgeon and open oesophagectomies mainly by the other. Therefore any differences, or lack of differences seen, may be the result of the different surgeons rather than the different procedures. Thirdly, we are assuming that patients are ventilated in the same way during their operation, however different anaesthetists may have different management strategies resulting in different levels of oxygenation and peak airway pressures and tidal volumes, all of which may have a bearing on subsequent development of ALI/ARDS. Finally, multivariable logistic regression would have been a more appropriate method of statistically evaluating factors that may have independently increased the risk of ALI/ARDS, but the sample size in this study is inadequate for this analysis.

In conclusion, this study confirms the incidence of ALI/ARDS post elective oesophagectomy as 30.6% and strengthens the hypothesis that OLV is an important causative factor. For these reasons, together with the elective nature of this operation, makes patients undergoing oesophagectomy present an attractive model for future ALI/ARDS research.

5 Establishing Methodology to Allow Assessment of Neutrophil Pulmonary Transit Times Pre and Post-Oesophagectomy

5.1 Introduction

Under normal physiological conditions neutrophils are thought to pass through the pulmonary circulation rapidly with minimal retention. This has been demonstrated by the Chilvers lab who have developed a novel out-flow tract sampling method to measure transit times of neutrophils across the pulmonary circulation (Summers et al. 2009). The original experiments involved injecting ^{111}In-radiolabeled autologous neutrophils and ^{99m}Tc-radiolabelled erythrocytes as a mixed bolus into the right internal jugular catheter of anaesthetised adults, with simultaneous rapid sampling of radial arterial blood. By measuring the ^{111}In and ^{99m}Tc activity in the arterial samples collected, correcting for background and cross-talk radioactivity as well as radionuclide decay, it was possible to assess the transit time of the two tracers (and hence neutrophils and erythrocytes) across the pulmonary circulation. The contamination of the 'first pass' data due to re-circulation of the radiolabelled cells was handled using gamma variate mathematical modelling. In 6 non-smoking subjects with normal spirometry and thoracic radiology, neutrophils transited the pulmonary circulation only marginally slower than erythrocytes (2.7 +/-1.0 seconds slower, n=6), with only 4.4+/-0.8 (SD) % of the neutrophils retained on first pass (Summers et al. 2009). Since it is established that erythrocytes do not undergo pulmonary retention, this represents rapid neutrophil pulmonary transit. However, neutrophils are clearly important in the pathogenesis of ALI and their retention within the lungs during ALI have been shown in several human observational studies (Bachofen and Weibel 1982; Bachofen and Weibel 1977; Lee and Downey 2001; Matute-Bello et al. 2011; Parsons et al. 1985; Rinaldo and Borovetz 1985; Steinberg et al. 1994) (this has been discussed previously, see 1.2.2).

To my knowledge there have been no studies looking at the trans-pulmonary neutrophil trafficking in ALI in humans. Having now established the incidence of ALI post oesophagectomy and confirming the utility of oesophagectomy patients as a model of ALI, I undertook to modify the above technique to allow assessment of neutrophil transit times pre and 24 hours post elective oesophagectomy, with the hypothesis that delayed neutrophil transit across the lung, and/or pulmonary neutrophil retention, would predict the presence or risk of ALI/ARDS in this high risk population. Hence, in patients with nascent ALI/ARDS, the postoperative transit time would be longer compared to the pre-operative values.

5.2 Method

This study was approved by the Cambridgeshire Ethics Committee (03/385) and by the Administration of Radioactive Substances Advisory Committee (ARSAC). Adult subjects were invited to participate if they had fulfilled the following criteria:

- Undergoing elective oesophagectomy involving one lung ventilation
- Abstinent from smoking for preceding 6 months
- Have an FEV_1 and FVC of greater than 75% predicted and less than 125% predicted on spirometric lung functions
- Have no prior lung pathology
- Age >25 years for males, and >35 years for females

Direct measurements of first pass neutrophil transit were undertaken using arterial outflow detection as previously developed by the Chilvers lab (Summers et al. 2009).

5.2.1 Neutrophil Isolation and radiolabelling

Venepuncture was undertaken using a 19 gauge butterfly needle and two 50 ml Plastipak syringes; each syringe contained 5 ml acid-citrate-dextrose (NIH formula A) and blood was collected to a total volume 50 ml in each syringe. All processing from this point onwards was undertaken in a blood cell labelling isolator (Amercare Limited; serial number A339) by professional radio-pharmacists in the Department of Nuclear Medicine, Addenbrooke's Hospital. Twenty milliletres of blood were transferred to a sterile tube and centrifuged at 1500 *g* for 15 min and the supernatant (cell-free plasma, CFP) aspirated and retained in a sterile tube. The remaining blood was dispensed into 2 sterile tubes, each containing 5 ml 6% hydroxyethyl starch and allowed to sediment at room temperature for up to 60 minutes. Once sedimented, the supernatant was transferred into sterile 20 ml tubes and centrifuged at 150 *g* for 5 minutes to pellet the mixed leucocytes. The supernatant was again removed and centrifuged at 1500 *g* for 5 minutes to produced cell-free plasma with starch (CFP-S).

Iso-osmotic Percoll (IOP) was prepared by mixing 9 ml Percoll with 1 ml sterile 1.5 M sodium chloride solution. The IOP was diluted with CFP in sterile 10 ml tubes to obtain 65%, 60% and 50% solutions of Percoll in plasma. The 3-step discontinuous density gradients were carefully overlayed in order of decreasing Percoll density in a

sterile 10 ml tube. The mixed leucocyte pellet was re-suspended in 2 ml CFP and carefully overlayed onto the gradients and centrifuged at 150 *g* for 10 minutes, before the plasma layer and cells at the plasma/50% interface (platelets and mononuclear cells) were drawn off and discarded. The granulocytes from the 50%/60% interface were transferred to a sterile 10 ml tube and washed twice with 5 ml CFP-S (spun at 150 *g* for 5 min) and the supernatant aspirated. The granulocytes were then radiolabelled with ^{111}Indium tropolonate using previously published methods (Peters et al. 1983). An ^{111}Indium chloride stock solution containing 1.2 MBq of activity in 0.5 ml was prepared. 0.1 ml of tropolone solution was added to the cell pellet, followed by 0.4 ml of the stock indium chloride solution. The cells were then gently mixed and incubated for 15 minutes at room temperature. The remainder of the indium chloride solution (0.1 ml) was used as a standard for scintillation counting. After radio-labelling, the cell pellet was washed twice in CFP (150 *g*, 5 min) before re-suspension in 4 ml CFP ready for injection.

Radio-labelled red cells were prepared by the addition of 0.1 ml dilute stannous medreonate (tin augmentation solution, 6.6 µg/ml) to 2 ml of acid citrate dextrose (NIH formula A) anti-coagulated blood and incubating for 10 minutes at room temperature. 5 ml of 0.9% sodium chloride solution was added, the sample centrifuged (500 *g*, 5 minutes) and the supernatant discarded. ^{99m}Technetium pertechnetate was added to the packed red cells and incubated for 15 min at room temperature. The cells were then washed twice with 5 ml 0.9% saline (500 *g*, 5 minutes) and the radioactivity measured. 10Mbq of activity was then withdrawn.

Both erythrocytes and granulocytes were re-suspended in 4 ml autologous plasma for ready for re-injection. The total radioactive activity was therefore 10MBq of ^{99m}Tc and 1Mbq of ^{111}In making an effective dose of 0.6 mSv.

5.2.2 Injection of radiolabelled Cells

After induction of general anaesthesia, subjects had right internal jugular vein and right radial arterial catheters placed as part of their routine clinical management. The pre-prepared radiolabelled cells were mixed and re-injected, along with 0.3mmol lithium chloride as a single bolus, into the right internal jugular vein catheter immediately prior to their surgery. Using a high fidelity peristaltic pump

(LiDCO, UK) and a fraction counter (Pharmacia LKB FRAC-100), blood samples were taken at 3.6 second intervals from the radial arterial line see figure 5.1 and collected into pre-weighed scintillation tubes. The collection of these blood samples started just prior to the initial bolus injection and continued over a 7 minute period. The 3.6 second sampling interval had previously been established as the shortest sampling interval at which the fraction counters could accurately collect a sufficient blood sample to allow scintillation counting. Blood ^{111}In and ^{99m}Tc activity were measured in the collected fractions using liquid scintillation counting. The subjects then went on to have their operation as normal.

Twenty four hours post operatively, the whole procedure was repeated while the subject was in post-operative recovery but fully awake.

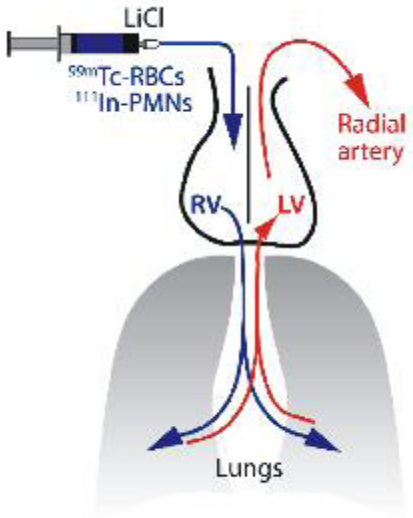

Figure 5.1 Schematic representation of the experimental set-up. Autologous ^{99m}Tc-labelled erythrocytes and ^{111}In-labelled neutrophils were mixed with 0.3 mmol lithium chloride and injected as a single bolus into the right internal jugular veins of patients just prior to and 24 hours post elective oesophagectomy. Simultaneous radial arterial blood samples were taken every 3.6 sec using a peristaltic pump and fraction counter. Reproduced with kind permission from Dr C Summers (Summers et al. 2009).

5.2.3 Scintillation counting

The scintillation tubes were then re-weighed to determine the exact mass of blood aspirated into each tube and the samples were then counted for 200 sec per sample in a liquid scintillation counter (Wallac Wizard 3 - 1480 Automatic Gamma Counter, serial number 4800317), along with a pre-injection sample of blood to determine the level of background radiation, and ^{111}In and ^{99m}Tc standards. Samples were recounted after 10 Tc half-lives had elapsed (t½ Tc=6.02 h) to ensure accuracy of the indium counting (t½ In=67.4 h). Measured activity values were corrected for background radiation, radio-isotope decay (based on values quoted above) and cross-talk, before being expressed as a fraction of the injected dose.

5.2.4 Statistical analysis

Data were analysed using Microsoft Office Excel 2007.

5.3 Results

Seven subjects were studied. However, I was unable to obtain a complete dataset (i.e. pre operative and 24 post operative results) for any one subject owing to several failures in each experiment. Further, a successful bolus injection followed by complete blood sampling was only managed pre-operatively during one study and postoperatively during another study.

Figure 5.2 shows the data set for the only successful pre-operative study. In this 58 year old male subject, ^{111}In radio-labelled neutrophils transited between the right internal jugular venous catheter and the right radial artery (and hence the pulmonary circulation) at the same rate as the ^{99m}Tc radio-labelled erythrocytes.

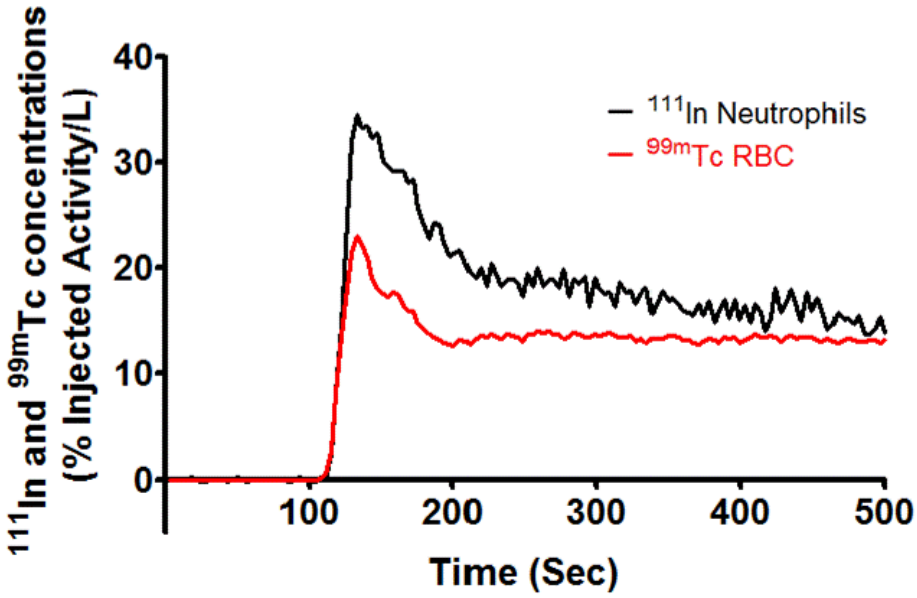

Figure 5.2 Pre-oesophagectomy study. Graph to show the concentrations of ^{111}In activity (black plot) of radial arterial blood over time in relation to ^{99m}Tc activity (red plot) after correction for background and cross talk radioactivity as well as radionuclide decay in a subject prior to their oesophagectomy.

Figure 5.3 shows the data set for the only successful 24 hour post oesophagectomy study. This 29 year old male who had severe oesophageal strictures secondary to caustic acid ingestion. He underwent a thorascopic assisted oesophagectomy lasting 4.25 hours, with a one lung ventilation time of 1 hour. He did not develop

ALI post operatively. In this study, ^{111}In radio-labelled neutrophils transited between the pulmonary circulation 3.6 sec slower than the ^{99m}Tc radio-labelled erythrocytes.

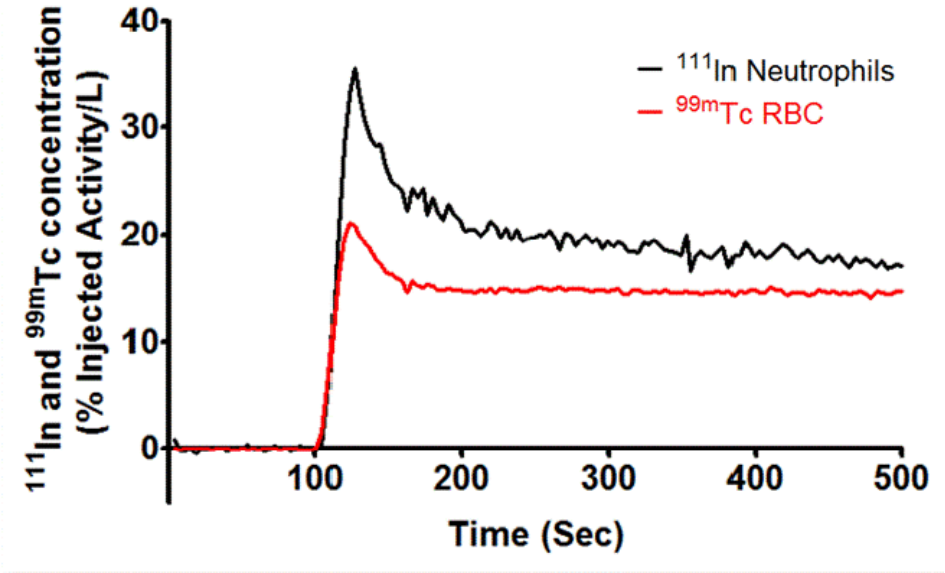

Figure 5.3 24 hour post-oesophagectomy study. Graph to show the concentrations of ^{111}In activity (black plot) of radial arterial blood over time in relation to ^{99m}Tc activity (red plot) after correction for background and cross talk radioactivity as well as radionuclide decay in a subject 24 post oesophagectomy.

For the failed studies, several difficulties were encountered at each stage of the experiment. These included:

- Delay in the start time of elective surgery causing the pre-prepared radio-labelled autologous neutrophils and erythrocytes to be unsuitable for re-injection.
- Incorrect placement of the central venous catheter (one subclavian catheter was retrospectively found to be directed caudally along the internal jugular vein). This made it unsuitable to re-inject radioactive material due to safety concerns and also made delivery of a bolus injection impossible.
- The delivery of true bolus injection of the mixed radioactive cells is crucial to obtaining useful data. On 4 occasions, this initial bolus injection was missed timed. On 2 of the occasions the initial injection was given more as a slow push meaning that some of the cells would have already been re-circulating before all of the remaining cells had been administered, and hence this

makes it impossible to differentiate cells that are transiting the lungs on first pass and which ones are re-circulating. On the other 2 occasions, the re-injection of cells were given as a bi-bolus injection again making it impossible to differentiate cells that are transiting the lungs on first pass and which ones are re-circulating.

- Power failure of the peristaltic pump responsible for continuous arterial blood sampling. The peristaltic pump is powered by a 9v battery which failed during a study. New batteries were fitted prior to every subsequent study.
- Malfunction of the fraction collector responsible for time and volume specific collection of arterial blood samples. Radial arterial blood samples are collected continuously and deposited into scintillation tubes. A different scintillation tube is presented for blood collection every 3.6 sec by the rotating fraction collector. On one occasion, the fraction collector missed several tubes at random intervals for random lengths of time. During a separate study some of the scintillation tubes were misplaced causing a mechanical obstruction to further blood collection.
- Failure of the radial arterial line 24 hours post operatively. On 4 separate occasions the radial arterial line failed to allow continuous and uniform blood aspiration. I was unable to replace the line as I did not have ethical approval to cover additional arterial line insertion for study purposes alone.

5.4 Discussion

As each of the above problems arose at almost every step of this study they were solved apart from:

(i) Being able to consistently deliver the mixed radiolabelled neutrophils and red cells as a true bolus. Future attempts should look into taking away human error and automating this step perhaps by using a syringe driver. The cells ready for injection could also be already in the lumen of a sufficiently long central venous catheter awaiting injection.

(ii) The repeated failure of the radial arterial catheter 24 hours post procedure. This occurred despite the use of longer catheters. Future studies would need to seek amendments in the ethics application so that a new arterial catheter can be replaced just prior to the post-op study.

The successful pre-operative study shows ^{111}In labelled granulocytes transiting the pulmonary circulation at a similar rate as the ^{99m}Tc labelled erythrocytes which is in keeping with previous data (Summers et al. 2009). The original hypothesis is that neutrophils would have a delayed pulmonary transit time post oesophagectomy particularly in those that went onto develop ALI/ARDS In the only successful post-operative study however, neutrophils were not shown to have a significantly prolonged transit time 24 hour post elective oesophagectomy. This may well be type II error or represent the fact that this particular individual did not go onto develop ALI/ARDS. Clearly, however, the results of this experiment are insufficient in number to draw any conclusions, but do demonstrate that with possibly some minor adjustments to the method this experiment would be capable of measuring neutrophil pulmonary transit times pre- and post-oesophagectomy.

6 Summary and Conclusions

One of the ambitions of this research was to establish methodology to allow assessment of neutrophil transit times pre and post-oesophagectomy, and thus study trans-pulmonary neutrophil trafficking in ALI. The underlying hypothesis for this work was that delayed neutrophil transit across the lung, and/or neutrophil retention within the lung, would predict the presence or risk of ALI/ARDS in this high risk group. However, despite the challenges encountered in answering this, the work contained within this thesis raises relevant results and interesting discussion points.

Some believe that the incidence and mortality rates of ALI/ARDS are falling owing to better supportive ICU care and the introduction of lung-protective ventilation i.e. low tidal volumes (Li et al. 2011). However, I provide evidence that ALI/ARDS (at least in a UK tertiary hospital ICU setting) is still a common condition associated with high ICU, hospital and 2 year mortality rates, and greater use of ICU resources. Of interest, the less severe form of ALI had a similar mortality rate to fully developed ARDS reiterating the severe nature of the whole disease spectrum. The relatively high mortality rates encountered may also be contributed by the case mix seen within the Addenbrooke's general ICU with large numbers of patients with sepsis (perhaps indicative of the high proportion of immunosuppressed patients due to the large volume of transplant services) and low numbers of patients with trauma (reflective of a separate neuro-trauma intensive care unit not captured in this study).

Also highlighted by this study, is the significant under recognition and under reporting of ALI/ARDS. This is caused in part by the difficulty that still exists in making an accurate diagnosis of ALI/ARDS with the part-subjective nature of the AECC definition, inability of the ICNARC case mix programme to accurately highlight ARDS/ALI cases, and the fact that the UK national coding system only codes for ARDS (and does not recognise ALI). Surprisingly, there was little change in the tariff generated for patient episodes when a diagnosis of ALI/ARDS was included retrospectively (personal communication, Medical Coding Department, Addenbrooke's Hospital, CUHNHSFT). Further, ARDS often results in multi-organ failure, which was the predominant cause of death assignment on the death certificates in patients who died in hospital with ARDS. This under recognition has huge implications for the awareness of this condition, treatment planning, and the future direction of research in this field.

The finding of a measurable trans-pulmonary gradient in neutrophil cell surface expression of CD62L in patients with systemic sepsis (with no lung involvement),

where blood entering the pulmonary circulation were found to be CD62L low (indicative of neutrophil priming) and blood leaving the pulmonary circulation CD62L high (indicative of the cells being less primed or un-primed), in the absence of a demonstrable gradient in-terms of neutrophil counts, supports the hypothesis that the pulmonary circulation is behaving in a protective manner in selecting out primed neutrophils (CD62L low) and de-priming them before being released back into the systemic circulation. Whether this de-priming is a passive (i.e. time-dependent process) or active process (i.e. a specific interaction between the pulmonary endothelial surface and the neutrophil) is, at this stage, uncertain. This process may explain why patients who are at risk of developing ALI due to enhanced neutrophil priming in the circulation and enhanced neutrophil retention in the pulmonary circulation (e.g. in the context of systemic sepsis or pancreatitis etc) do not always develop lung injury. The finding of a reversal of this gradient in subjects who had developed ARDS and further, that the magnitude of this gradient correlates with levels of hypoxaemia, strengthens this hypothesis, and suggests that when this protective mechanism fails lung injury ensues. There are several questions left to be addressed however:

1) How do 'de-primed' neutrophils re-express cell surface CD62L? To my knowledge there are no examples CD62L recovery in the literature. In in-vitro experiments where neutrophils were reversibly primed with PAF, initial changes in cell shape and cell surface CD11b expression reverted back to baseline. However, the cell surface expression of CD62L failed to repopulate after initial shedding over a 4 hour time period (Summers, University of Cambridge, un-published). Further, direct evidence of CD62L recovery in-vivo is needed. Future experiments could utilise radio-labelling of autologous CD62L low neutrophils, re-injecting them and radioisotope scanning over the chest as well as arterial blood sampling. This will demonstrate whether or not these CD62L low neutrophils are retained within the lungs and whether they are released back into circulation, and over what time course. Use of an ex-vivo perfused human donor lung model of ALI could also be used. Again CD62L low neutrophils could be injected into the system and the effluent sampled to see if the neutrophils have recovered their CD62L expression.
2) Where exactly does 'de-priming' occur? The finding that the magnitude of the neutrophil CD62L trans-pulmonary gradient correlates with the degree of hypoxaemia in patients with ARDS suggests that failure of this protective de-

priming mechanism is a graduated process and perhaps occurs diffusely throughout the capillary circulation. However, more detailed examination is required to see where and how neutrophils are entrapped and released. Two photon excitation microscopy could be considered to follow individual cells in animal models.

3) When in the evolution of ARDS is a neutrophil CD62L trans-pulmonary gradient evident? It would be extremely useful to measure neutrophil CD62L trans-pulmonary gradients in patients at risk of ALI/ARDS (e.g. patients undergoing oesophagectomy) over a detailed time course to see when and how changes take place in relation to disease progression and clinical outcome. Given the relatively small volumes of blood required to make these measurements, any positive findings in this regard may make neutrophil CD62L trans-pulmonary gradients a useful clinical tool.

The study looking at the incidence of ALI/ARDS post oesophagectomy focused on accurate diagnosis of ALI/ARDS and highlighted the difficulties of radiological interpretation. Chest radiographs showing pulmonary infiltrates were often reported by the standard reporting radiologist as showing ‘consolidation’, which immediately suggests pulmonary infection to the clinician, however this radiographic sign has a very different connotation to a radiologist and may have numerous aetiologies including ALI. Further difficulties in chest radiograph interpretation were seen in differentiating atelectasis (a common post-operative finding) and opacities caused by the gastric pull through from pulmonary infiltrates. Clearly CT evaluation would aid diagnosis further. Similar conclusions were made in the recent Berlin definition of ARDS (Ranieri et al. 2012).

A positive association was found between the duration of OLV and the development of ALI/ARDS, strengthening the idea that this is a causative factor. However, the finding that the majority of patients who went on to develop ARDS, did so between 48 hours and 7 days post operatively, suggests that OLV is not the only cause and supports the hypothesis of ALI/ARDS being the result of a “2 hit” process where only after a second insult such as an anastomotic leak following the primary insult of barotrauma / ischaemia-reperfusion injury sustained during OLV, does ALI/ARDS develop.

The initial assumptions of the operating surgeons prior to this study were that very few of their patients develop ALI (less than 10%); the finding therefore of an

incidence of ALI/ARDS post oesophagectomy of 31% further highlights the significant under recognition and under reporting of this condition. Further, this confirms that patients undergoing oesophagectomy present an attractive model for ALI/ARDS research and validates one of the original aims of using this model to study neutrophil trafficking in ALI.

Appendices

A1 Publications arising from this thesis

Singh NR, Johnson A, Peters AM, Babar J, Chilvers ER, Summers C. Acute lung injury results from failure of neutrophil de-priming: a new hypothesis. Eur J Clin Invest. 2012; 42:1342-1349.

Summers C, **Singh NR**, White JF, Mackenzie IM, Johnston A, Solanki C, Balan KK, Peters AM, Chilvers ER. Establishing the transit time of primed and un-primed neutrophils across the human lung. *Sci. Transl. Med., submitted.*

Singh NR, Summers C, Worple L, Simmonds R, Johnston A, Gunning KE, Condliffe AM, Babar J, Chilvers ER. Incidence and impact of acute lung injury in a UK teaching hospital intensive care unit. Br J Anaesth., submitted.

Singh NR, Summers C, Johnston A, Peters AM, Chilvers ER. Differential effects of sepsis & ARDS on CD62L expression in neutrophils entering and leaving the lung. Am J Respir Crit Care Med 2012; 185:A17 (Abstract).

A2 Calculation of the lung injury score

Chest Radiograph score	
No alveolar consolidation	0
Alveolar consolidation confined to 1 quadrant	1
Alveolar consolidation confined to 2 quadrant	2
Alveolar consolidation confined to 3 quadrant	3
Alveolar consolidation confined to 4 quadrant	4
Hypoxaemia score	
$PaO_2/FiO_2 \geq 300$ (mmHg)	0
PaO_2/FiO_2 225-299 (mmHg)	1
PaO_2/FiO_2 175-224 (mmHg)	2
PaO_2/FiO_2 100-174 (mmHg)	3
$PaO_2/FiO_2 < 100$ (mmHg)	4
PEEP score	
≤ 5 cmH_2O	0
6-8 cmH_2O	1
9-11 cmH_2O	2
12-14 cmH_2O	3
≥ 15 cmH_2O	4
Respiratory compliance score (when available)	
≥ 80 ml/cmH_2O	0
60-79 ml/cmH_2O	1
40-59 ml/cmH_2O	2
20-39 ml/cmH_2O	3
≤ 19 ml/cmH_2O	4

Table A2 Calculation of the lung injury score. The score is calculated by adding the sum of each component and dividing by the number of components used. A score of 0 relates to no lung injury; 0.1-2 is mild to moderate lung injury; and >2.5 is severe lung injury. Adapted from Atabai et al. (Atabai and Matthay 2002).

A3 Poor correlation between the lung injury score and neutrophil CD62L A-V ratio

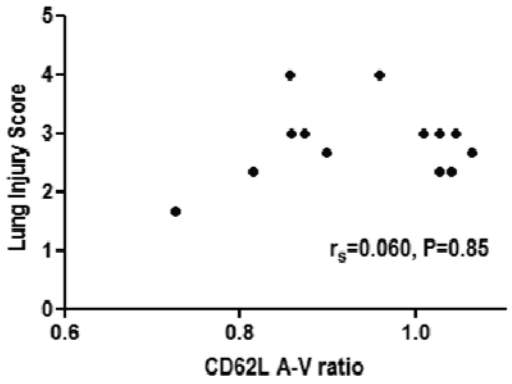

Figure A3 Poor correlation between the lung injury score of patients with ARDS and their corresponding neutrophil CD62L A-V ratios. Data includes all paired venous and arterial blood samples taken from patients when they met AECC ARDS criteria. (r_s, Spearman's rank correlation)

List of Abbreviations

^{111}In	Indium-111
^{99m}Tc	Technetium-99m
AECC	American-European Consensus Conference
ALI	Acute Lung Injury
APACHE II	Acute Physiology and Chronic Health Evaluation score
ARDS	Acute Respiratory distress Syndrome
ATS	American Thoracic Society
AU	Arbitrary units
A-V	Arterial-venous
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
C5a	Complement component C5
CD11b	β_2 Integrin molecule
CD16	Fc receptor FCγ RIII
CD62L	L-Selectin
cmH_20	Centimetres of water
COPD	Chronic obstructive pulmonary disease
CT	Computed Tomography
CXR	Chest radiograph
DNA	Deoxyribonucleic acid
ECMO	Extracorporeal membrane oxygenation
EDTA	Ethylenediaminetetraacetic acid
ESICM	European Society of Intensive Care Medicine
FEV_1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionine-leucine-phenylalanine
GI	Gastro-intestinal
GM-CSF	Granulocyte macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
HDU	High Dependency Unit
hx	History
ICNARC	Intensive Care National Audit and Research Centre
ICU	Intensive Care Unit
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
kg	Kilogram
LAD-1	Leukocyte adhesion deficiency syndrome 1
LAD-1	leukocyte adhesion deficiency syndrome 1

LFA-1	lymphocyte function-associated antigen-1
LIS	Lung Injury Score
LPS	Lipopolysaccharide
m	Meter
mdyne	Millidyne - (unit of force, equivalent to 10^{-8} Newtons)
MIP2	Macrophage inflammatory protein
ml	Millilitres
mmHg	Millimetres of mercury
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NHLBI	National Heart, Lung and Blood Institute
NHS	National Health Service
NLI	No lung injury
OLV	One lung ventilation
PAF	Platelet activating factor
PaO_2/FiO_2	Arterial oxygen tension / fraction of inspired oxygen
PBS-/-	Phosphate buffered saline without calcium and magnesium
PBS+/+	Phosphate buffered saline with calcium and magnesium
PEEP	Positive End-Expiratory Pressure
PMA	Phorbol Myristate Acetate
PPP	platelet poor plasma
PRP	platelet-rich plasma
ROS	Reactive oxygen species
SD	Standard deviation
TACE	TNFα converting enzyme
TNFα	Tumour necrosis factor – alpha
TRALI	Transfusion related acute lung injury
µL	Microlitre
µm	Micrometer

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