11β-hydroxysteroid dehydrogenase glucocorticoid metabolism within the lung and its influence on macrophage function in the acute respiratory distress syndrome

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This thesis is dedicated to Jo, Jenny and Lucy

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No part of this thesis has previously been submitted for the award of any degree at Warwick University, or any other institution. Clinical samples used in some chapters were archived samples from patients recruited by other investigators within the research group, Dr Gavin Perkins having collected samples from the BALTI-1 investigation, and Dr Daniel Park collecting samples from the BALTI-prevention study. Nevertheless, the work contained in this thesis is the work of Dr Christopher Bassford. I am grateful for the technical support provided by Dr. Sian Lax who undertook the qPCR experiments, and by Dr. Angela Taylor, who carried out the mass spectrometry experiments.

Abstract

The acute respiratory distress syndrome (ARDS) is an important cause of respiratory failure in critically ill patients characterised by severe inflammation within the lungs. This inflammation is limited by anti-inflammatory glucocorticoid hormones released from the hypothalamus-pituitary-adrenal (HPA) system. This thesis reports a series of investigations into glucocorticoid concentrations and glucocorticoid metabolism within the lungs of patients with ARDS. It also contains an investigation into a potential biomarker for ARDS.

Our study of glucocorticoid concentrations in alveolar epithelial lining fluid showed increased cortisol concentrations within the lungs at onset of ARDS. These concentrations have a positive relationship with critical illness severity indices, but negative relationships with alveolar permeability and alveolar neutrophil counts.

In peripheral tissues cortisone and cortisol are inter-converted by iso-enzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD). We have shown that healthy primary resident alveolar macrophages increase their production of active cortisol by the oxo-reduction of inactive cortisone in response to inflammatory stimuli.

Alveolar macrophages are responsible for the removal of spent and apoptotic inflammatory cells, failure of this process causes further inflammation. We have shown that glucocorticoids increase the rate of uptake of apoptotic cells by alveolar macrophages, and that macrophage 11β-HSD production of cortisol increases this process.

We have shown however that alveolar macrophages extracted from patients with established ARDS have decreased 11β-HSD oxo-reductase activity. This decreased conversion of cortisone to cortisol will cause a diminished response to the anti-inflammatory signal of the HPA system. The implications of this are that they will have a limited capacity to up-regulate efferocytosis and a diminished anti-inflammatory potential.

The receptor for advanced glycation end-products (RAGE) is a potential biomarker in ARDS. We have shown that RAGE concentrations in plasma and BALF had excellent diagnostic compatibility with ARDS diagnostic criteria. The use of a threshold RAGE concentration could assure pulmonary inflammation in future investigations.

1 Introduction

1.1 The acute respiratory distress syndrome

Acute respiratory distress syndrome (ARDS) is an inflammatory disease of the lung that can develop in response to a number of different insults. ARDS was first described by Ashbaugh in 1967. Ashbaugh described a syndrome of poor oxygenation in 12 patients with decreased lung compliance, and diffuse infiltrates on chest X-ray.[1] This constellation of features was originally termed the adult respiratory distress syndrome, but as it became apparent that paediatric patients were suffering with a similar process, this was modified to the acute respiratory distress syndrome.[2]

1.1.1 Diagnosis

The diagnosis of ARDS is made using the criteria suggested by the American-European consensus conference (AECC) on ARDS. In 1994 this body recommended diagnostic criteria in the hope that standardised diagnosis would lead to better research into the causes and potential therapies for the condition. This conference recognised that the syndrome was not uniform in its severity, and that a spectrum of disease existed. It was with this in mind that the conference put forward the concepts of acute lung injury (ALI) as the less severe form of the condition, with ARDS representing the more severe manifestation.[3] *See table 1.1*.

Table 1.1 The American European consensus conference criteria for the diagnosis of ARDS

Acute respiratory distress diagnostic criteria
Acute onset
Bilateral pulmonary infiltrates consistent with pulmonary oedema on chest radiogram
Absence of cardiogenic pulmonary oedema (PAOP< 18mmHg
Defect of oxygenation (P:F ratio <24.7kPa)

1.1.2 Epidemiology

The incidence of ARDS has been estimated in a North American population to be 78.9 per 100,000 person years. The in-hospital mortality in this same study was calculated at 38.5%. A relationship was also seen between mortality and the age of the patients, with older patients suffering a higher mortality. This study estimated that the United States has 74,500 deaths per annum and 3.6 million hospital days.[4] Another study observed a incidence of 4.8 to 8.3 cases per 100,000 people per year in the American state of Utah.[5] Most studies focus on patients admitted to intensive care units, but patients on general wards may also have this condition. In one study 9% of patients admitted to respiratory isolation rooms would have met criteria for ALI, and 2% met criteria for the diagnosis of ARDS.[6]

Although the death rate of patients suffering from ARDS is high, the majority of mortality is not due to the physiological changes associated with ARDS itself. Montgomery and colleagues found in 1985 that only 16% of patients with ARDS died from respiratory failure; the majority of patients dying from multi-organ failure and sepsis. Indeed patients tend to die “with”, rather than “from” ARDS.[7]

1.1.3 Pathology of ARDS

ARDS can result from a number of different insults, either direct to the lung, such as pneumonia or chest trauma, or indirect, such as systemic sepsis or pancreatitis. The inflammation in both direct and indirect causes of ARDS is thought to be mediated through pro-inflammatory mediators in the systemic and pulmonary circulation.[8] Pro-inflammatory mediators culpable in ARDS include lipopolysaccharide (LPS), TNF-α, Interleukins (IL-s) IL-1β, IL-6, and IL-8.[9-13] Elevated and persistent concentrations of IL1β,

IL-6 and TNF-α are associated with worse clinical outcomes and more severe lung injury.[10, 13]

Inflammation in ARDS is mediated through activation of the nuclear factor–$\kappa\beta$ (NF-$\kappa\beta$) system, the cell's chief pro-inflammatory transcription factor.[14] NF-$\kappa\beta$ is a cytoplasmic protein that is bound by inhibitory proteins (I-$\kappa\beta$) which prevent translocation of NF-$\kappa\beta$ to the nucleus and its binding to DNA. Under the influence of pro-inflammatory stimuli, such as LPS and TNF-α, I-$\kappa\beta$ proteins are phosphorylated and disassociate from NF-$\kappa\beta$, allowing it to become active. NF-$\kappa\beta$ activation is increased in ARDS patients compared to ventilated patients without ARDS, and is thought to play a role in the early production of pro-inflammatory cytokines.[15] Healthy cells exposed to plasma from patients with ARDS increase their production of NF-$\kappa\beta$ and its components.[16] Polymorphisms in the NF-$\kappa\beta$ gene are associated with increased susceptibility to ARDS,[17] and NF-$\kappa\beta$ activation is seen to be increased in critically ill patients who die compared to those who survive.[18]

Inflammation in ARDS is disorganised and does not follow an orderly sequence,[19] with geographical and temporal variations in the extent and phase of disease.[20] In ARDS lung inflammation progresses through three overlapping and indistinct phases. Firstly an exudative phase, characterised by increased alveolar permeability, activation of macrophages and neutrophil infiltration into the lung. Neutrophils enter the lung in large numbers and their concentrations reflect the severity of the patient's clinical condition.[19] An increase in extra-vascular lung water is also seen during this phase, as pulmonary capillary permeability increases.[21, 22] This is followed by the proliferative phase, in which pneumocytes, primary inflammatory cells and fibroblasts grow in number, obliterating the airspaces. Later a fibrotic phase predominates, and fibrous remodelling of the lung occurs.[23, 24] Histologically the lung exhibits characteristic

changes, described as diffuse alveolar damage (DAD), that are characterised by septal thickening, hyperplasia of type II alveolar cells, and the formation of hyaline membranes. This progresses in later disease with proliferation of fibroblasts and myofibroblasts.[23]

1.2 The relevance of glucocorticoids to ARDS

1.2.1 ARDS and the host defence response

ARDS is caused by an inflammatory response in the lungs to systemic inflammatory markers. In response to an inflammatory stimulus the body activates a number of interconnected and mutually regulating systems.[8] These systems comprise the host defence response. They include the acute phase reaction,[25] the tissue host defence response,[26] sickness syndrome,[27] the coagulation system, the immune system[28] and the hypothalamic-pituitary-adrenal (HPA) system.[26, 29, 30] The individual effects of these systems is summarised in table 1.2. Of these systems, the HPA system is the principle anti-inflammatory component and its actions limit the extent and duration of the host defence response.

Table 1.2 Abridged components of the host defence response[25-28]

Component system	Physiological effects
Tissue host response	Increased vascular permeability Vasodilatation Leucocyte migration Leucocyte activation Adhesion molecule expression
Sympathetic nervous system	Release of adrenaline and noradrenaline
Coagulation	Increased coagulation Decreased fibrinolysis Extravascular fibrin deposition
Acute phase response	Increased production of C-reactive protein Increased production of amyloid-A protein Increased production of fibrinogen Decreased production of albumin
Immune system modulation	Neutrophil release from bone marrow T-lymphocyte proliferation antibody production pyrexia
Sickness syndrome	Recovery prioritisation behaviour
Hypothalamus-pituitary-adrenal system (HPA)	Production of anti-inflammatory glucocorticoid steroid hormones

1.2.2 The HPA system and glucocorticoid hormones

Glucocorticoids (GCs) are endogenous anti-inflammatory steroid hormones produced by the cortex of the adrenal gland. Production of these hormones is controlled by the hypothalamic-pituitary-adrenal (HPA) axis, which is an essential component of the host defence response.[29, 31] The hypothalamus produces corticotrophin releasing hormone (CRH), which acts on the pituitary gland, inducing an increase in its production of adrenocorticotrophic hormone (ACTH). This peptide is transported via the systemic circulation to the adrenal glands, where it provokes these glands to produce and release glucocorticoids from the adrenal cortex. Cortisol is the active glucocorticoid secreted after activation of the HPA axis, and its concentrations in serum are increased in response to critical illness.[32]

1.2.2.1 Glucocorticoid transport

Cortisol is transported in the blood in both a free active form, and bound to plasma proteins. This protein binding is principally by albumin and corticosteroid binding globulin (CBG). Albumin has a low affinity for binding steroids, but a high capacity for its transport, thus providing a large reserve of the steroid in the circulation, whereas CBG has a high affinity for corticosteroids, but a low binding capacity.[33] In critical illness patients often become hypo-proteinaemic, and have lower circulating concentrations of both albumin and CBG. Despite this the free cortisol concentrations remain high, an effect attributed to increased corticosteroid production.[34] CBG is a member of the serine proteinase superfamily of proteins. These proteins have the biologically useful property of increasing the availability of their respective hormones in times and places of stress. They accomplish this by a conformational change, mediated by neutrophil elastase as they pass through areas where this enzyme has been released by activated neutrophils.[35] Due to the increased permeability of the alveolar membrane in ARDS increased protein concentrations are present in the alveolar space. it is therefore possible that increased concentrations of glucocorticoids are present in the alveolus as a result of the increased protein concentration and increased protein binding in this space. However no data is available to inform this argument.

1.2.2.2 The glucocorticoid receptor (GR)

Cortisol acts in target cells through interaction with the glucocorticoid receptor, and the GC:GR interaction plays an important role in the regulation of acute inflammation, impeding several key inflammatory pathways, increasing the production of anti-inflammatory gene products, and decreasing the production of pro-inflammatory gene products.[30] This intracellular protein has three domains: a steroid binding domain, a

central DNA binding domain and a transcription activation domain. Heat shock proteins disassociate from the receptor as cortisol binds to it, revealing nuclear localisation regions. These regions facilitate translocation of the GC:GR complex to the nucleus. Cofactors also modulate the GR transcriptional activity, by interacting with regions such as the AF-2 region of the ligand binding domain. The GR binds to DNA as a homo-dimer to specific palindromic DNA sequences in the regulatory regions of target genes.[8, 31]

NF-κβ also interacts with the glucocorticoid induced suppression of pro-inflammatory genes. NF-κβ up-regulates the transcription of genes for pro-inflammatory cytokines, and is the key inflammatory pathway in ARDS.[15-17] The glucocorticoid receptor binds to the same DNA binding area as NF-κβ and thus prevents its transcription of pro-inflammatory genes. Activation of the glucocorticoid receptor also causes increased production of I-κβ, and in so doing prevents the translocation and inflammatory action of NF-κβ. By these mechanisms NF-κβ and GC mutually impede each others activity, and the balance between activation of these two transcription factors determines the extent and persistence of inflammation.[8]

1.2.2.3 Glucocorticoid resistance

In systemic inflammation such as that seen in ARDS a relative deficiency in the HPA function is well recognised.[16, 29, 31, 36, 37] Although absolute plasma concentrations of cortisol are increased in systemic inflammation, the ability of the body to respond to the HPA system can be degraded, and a relatively unopposed pro-inflammatory state exist. Previous research has focussed on whether the capacity of the HPA axis itself is overwhelmed in systemic inflammation, but more recently tissue resistance to glucocorticoids has also been described.[38] A resistance to the anti-inflammatory effects of glucocorticoids is recognised in

many inflammatory diseases, such as asthma,[39] rheumatoid arthritis,[40] and inflammatory bowel disease.[41]

A cell's response to a hormone, such as a glucocorticoid, will depend on a number of factors: firstly the availability of the active hormone, secondly the hormones affinity to bind to its receptor, thirdly the potency with which it activate that receptor, and finally the ability of the receptor to transduce the signal to other cellular apparatus.[42] Under inflammatory conditions the concentration of glucocorticoids and the ability of the GR to transduce the anti-inflammatory signal are altered, and represent mechanisms of glucocorticoid resistance.

1.2.2.3.1 Glucocorticoid receptor mediated glucocorticoid resistance

The ability of the GR to bind hormone and trans-locate to the nucleus is compromised in inflammation. Modification of the GR by phosphorylation, binding of ubiquitin (which allows the GR to be degraded by proteasomes), and nitrosylation by nitric oxide adversely effect its function. Defective histone acetylation, as well as increased NF-$\kappa\beta$ activation as described above also decrease the ability of the GR to bind GC, translocate to the nucleus and carry out its anti-inflammatory role.[36] The cellular signals responsible for many of these processes are pertinent in ARDS: The affinity of the GR to bind GC is decreased by the cytokines IL-2 and IL-4.[43] IL-1α also decreases the trans-location of the GR to the nucleus. Specifically for lung injury, in cells exposed to plasma from patients with ARDS the GR failed to translocate to the nucleus despite adequate cortisol concentrations in the plasma.[37] In a sheep model of LPS induced lung injury, although plasma cortisol concentrations rose during lung injury, the capacity of the GR to bind GC declined markedly.[44]

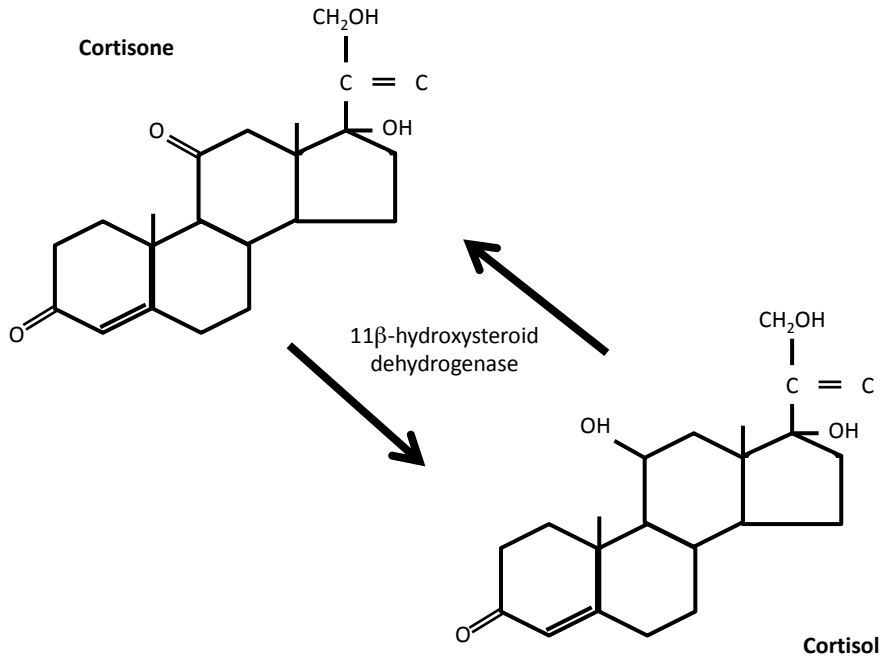
1.2.2.3.2 Glucocorticoid bioavailability mediated glucocorticoid resistance

The bioavailability of cortisol in tissues is determined by the amount released from the adrenal gland, the transport in circulating blood, and its metabolism in the tissues. Cortisol production from the adrenal gland is increased in systemic inflammation and critically ill patients,[32] but it is not merely circulating concentrations that are responsible for end-organ concentration. Metabolism of corticosteroids within tissues maintains concentrations of active steroids that influence tissue function.[45, 46] Previous investigations in burns patients have shown no link between tissue concentration of glucocorticoids and plasma concentrations, again suggesting that local metabolism plays a key role.[47] Little is known of the intracellular metabolism of glucocorticoids within lung tissue in ARDS, and aberrations in this metabolism may contribute to steroid resistance.

1.2.3 11β-hydroxysteroid dehydrogenase

After synthesis in the adrenal gland cortisol metabolism was previously thought to be accomplished primarily in the liver. However it is now clear that cortisol metabolism is carried out in many tissues. The inter-conversion of the active hormone cortisol and its inactive metabolite cortisone is catalysed in peripheral tissues by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). The activity of this enzyme therefore directly influences the concentrations of active cortisol that are available to the GR.[48]

Figure 1.1 Inter-conversion of cortisol and cortisone by 11β-hydroxysteroid dehydrogenase



Cortisone has a ketone group at position 11, which is reduced to a hydroxy- group by 11β-HSD to form cortisol. A dehydrogenation reaction reverses this process.

1.2.3.1 11β-hydroxysteroid dehydrogenase isoenzymes

11β-HSD enzymes are members of the short-chain alcohol dehydrogenase superfamily. Two iso-enzymes have been identified, 11β-HSD type 1 and type 2. The reactions of these iso-enzymes are summarised in figure 1.2.

1.2.3.1.1 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1)

11β-HSD1 is coded for on chromosome 1, and in vivo acts primarily as an oxo-reductase, converting the inactive keto-steroid cortisone, to its active dehydroxy-form: cortisol. However, in tissue homogenates it catalyses the reverse reaction, acting as a dehydrogenase to convert cortisol to cortisone.[48] Purified samples of 11β-HSD1 have an equilibrium coefficient at pH=7.0 of 0.03.[49] This implies that the reaction will preferentially move towards

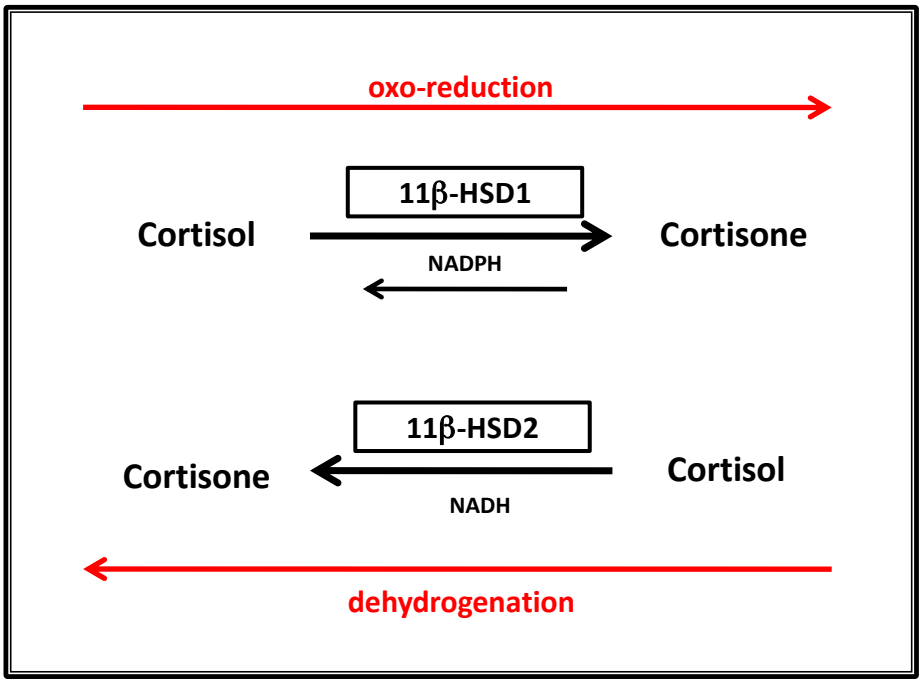
cortisone. The reasons for this enzymes difference between in-vivo and in-vitro activity are due to the availability of co-factors. The oxo-reductase activity of this enzyme is dependent on NADP(H); This co-factor is produced by the enzyme hexose-6-phosphate dehydrogenase, with which 11β-HSD1 is closely associated in the lumen of the endoplasmic reticulum. It is the activity of hexose-6-phosphate dehydrogenase and production of NADP(H) that determines the direction of reaction and activity of 11β-HSD1.[50] To date 11β-HSD1 has been seen to govern the local concentration of active steroid available and function of osteoblasts,[51] hepatocytes,[52] ocular cells, [53] neuronal tissue,[45] fibroblasts,[54] adipocytes[50, 55, 56] and in developing lung tissue.[57]

1.2.3.1.2 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2)

11β-HSD2 is coded for by a gene on chromosome 16. It is distinct from 11β-HSD1 and the iso-enzymes share only 21% sequence homology.[48] 11β-HSD2 acts solely as an NAD dependent dehydrogenase, inactivating cortisol by conversion to cortisone[48] It is membrane bound on the external surface of the endoplasmic reticulum. Although present in many sites during development, in adults 11β-HSD2 is expressed only in mineralocorticoid sensitive tissues such as the kidney, colon and salivary gland. In these sites it lies in close approximation to the mineralocorticoid receptor, and prevents this receptor from being activated by glucocorticoid hormones, thus providing mineralocorticoid specificity to this receptor.[58] Defects in 11β-HSD2 genetics prevent adequate functioning of this enzyme, allowing these receptors to be stimulated by both mineralocorticoids and glucocorticoids in hypertension due to the "apparent mineralocorticoid excess" syndrome.[59] Similarly the blockade of 11β-HSD2 by liquorice removes the glucocorticoid deactivation at the mineralocorticoid receptor, and liquorice therefore induces a hypertensive reaction.[60] 11β-HSD2 in-vivo antagonises the

effects of 11β-HSD1, and its activity can protect against the harmful effects of glucocorticoids.[61]

Figure 1.2 Schematic summarising the principle in-vivo reactions of the iso-enzymes of 11β-HSD, their substrates, products and co-factors



1.2.3.2 11β-hydroxysteroid dehydrogenase activity in the host defence response

11β-HSD1 has a role in regulating the tissue concentration of active corticosteroids under inflammatory conditions. The HPA system limits the duration and severity of the host defence response, through the actions of anti-inflammatory glucocorticoid hormones. Increased bioavailability of cortisol by up-regulation of 11β-HSD1 or down-regulation of 11β-HSD2 activity is therefore an extension of this system into local tissue metabolism.[46]

1.2.3.2.1 11β-hydroxysteroid dehydrogenase in inflammation

11β-HSD1 expression and activity has been seen to be increased in numerous cell lines by pro-inflammatory stimulation. TNF-α and IL-1β are known to increase 11β-HSD1 activity in adipocytes,[62] fibroblasts,[54, 63] osteoblasts,[64] and vascular smooth muscle.[65] RNA message for this enzyme is also increased in biopsy samples from inflamed bowel.[66] The activity of 11β-HSD activity is also altered in the acute inflammation associated with acute burns. The cortisol levels in the damaged tissues of patients with severe burns shows no relationship with plasma cortisol levels, further supporting local tissue metabolism of cortisol as an important factor in the local response to injury.[47] In critically ill patients with severe sepsis there is a global increase in 11β-HSD production of cortisol.[67]

1.2.3.2.2 11β-hydroxysteroid dehydrogenase in ARDS

Many cell types within the lung are steroid sensitive, and as ARDS causes dysfunction in many aspects of lung metabolism and mechanics 11β-HSD activity is likely to influence these processes. One postulated explanation for persistent inflammation and steroid insensitivity in ARDS is increased deactivation of cortisol by 11β-HSD2 or decreased production of cortisol by 11β-HSD1 within the lung. 11β-HSD2 is present in the airways,[68] and Immunohistochemistry of lung samples taken from patients who died with ARDS revealed high expression of 11β-HSD2 in their lungs.[69] This study by Suzuki et al localised this increase in 11β-HSD2 expression to both the alveolar wall and CD68 positive cells in the alveolar lumen. CD68 identifies these cells as those of a monocyte lineage, such as alveolar macrophages.[70] Similarly, a mouse model of pulmonary granulomatous disease shows increased 11β-HSD2 RNA and decreased 11β-HSD1 RNA in the lungs of these animals, both changes that would result in decreased cortisol

production.[71] Blockade of the action of 11β-HSD2 by carbenoxolone in animal models of ARDS produces similar physiological effects to the administration of exogenous glucocorticoids; further strengthening the case for 11β-HSD isoenzymes as important factors in lung metabolism of glucocorticoids during ARDS.[72]

Surfactant is responsible for decreasing alveolar surface tension and is essential for normal lung mechanics. Surfactant production is defective in ARDS, with altered constituents and levels of production.[73, 74] Cortisol enhances surfactant production and neonatal 11β-HSD knockout mice have delayed lung maturation due to decreased surfactant production.[57]

The inflammation of ARDS poses particular problems for resolution processes. The recruitment of large numbers of inflammatory cells to the lungs in ARDS must be followed by their safe disposal by phagocytosis. This task is undertaken by lung macrophages, in which the phagocytosis of apoptotic cells is promoted by glucocorticoids. 11β-HSD oxo-reduction of steroids is part of the phagocytosis apparatus and this enzyme together with the ability to take up spent inflammatory cells develop simultaneously: Macrophages develop 11β-HSD oxo-reductase activity only at the point of differentiation from monocytes to macrophages.[75, 76]

1.2.3.3 The cortisol: cortisone ratio

As well as the absolute concentrations of cortisol and cortisone, the relationship between these two steroids is important in determining how a patient is responding to both systemic and local inflammation. The cortisol: cortisone ratio reflects the balance between cortisol synthesis and breakdown, and as such is a marker of 11β-HSD activity. The plasma cortisol: cortisone ratio is chiefly influenced by 11β-HSD1 activity in the liver

and 11β-HSD2 activity in the kidney.[77] During periods of physiological stress increases in 11β-HSD1 activity promote active cortisol. Increases in the plasma cortisol: cortisone ratio are present in stable surgical patients in the immediate post operative days.[78] In critically ill patients with severe sepsis and therefore an exaggerated host defence response, an increased plasma cortisol: cortisone ratio is also present. consistent with systemic increases in 11β-HSD1 production of cortisol.[67] Although the plasma cortisol: cortisone provides information on global 11β-HSD activity, the metabolism within specific tissues cannot be determined from plasma data.

Some information on the cortisol: cortisone ratio within the lung in models of other disease is available. The cortisol: cortisone ratio in the lungs of patients with tuberculosis is increased, suggesting that 11β-HSD metabolism shifts towards the oxo-reductase reaction and production of cortisol in this condition.[79] The authors of this study speculated that altered lung 11β-HSD activity is responsible for the immune-paresis suffered by patients with pulmonary TB. However these findings are at odds with the RNA findings discussed earlier, which suggest a shift to cortisol degradation in granulomatous disease.[71] No information is as yet available on the cortisol: cortisone ratio in the plasma or the alveolar space of patients with ARDS.

1.2.4 Exogenous glucocorticoids in the treatment of ARDS

Numerous disease modifying therapies have been proposed and tested for the management of ALI and ARDS, including exogenous corticosteroids,[80-85] the calcitonin gene related peptide adrenomedulin,[86] surfactant,[87, 88] inhaled nitric oxide,[89] N-acetyl cysteine[90-92], prostoglandins,[93] and β-adrenoceptor agonists (β-agonists).[94, 95] However

none of these therapies have consistently been shown to impact favourably on patient outcomes, and Cochrane reviews and guidelines have been unable to recommend them.[96] The only intervention accepted to be beneficial in improving mortality of ARDS is an improvement in the supportive care of these patients. A low tidal volume, high PEEP mechanical ventilatory strategy giving an 8% drop in mortality, when compared to a high tidal volume strategy.[97]

The use of exogenous glucocorticoids as an anti-inflammatory agent in ARDS has received much interest over many decades of research. Relative steroid insufficiency and glucocorticoid resistance provides a rationale for their use, as even the increased concentrations of endogenous corticosteroids seen in ARDS do not sufficiently control the inflammatory response. However their use is still not generally accepted, as doubts over the evidence for clinical benefit and concerns over potential harms persist. Two meta-analyses have recently returned different conclusions. The first, by Peter et al in 2008 could merely find a suggestion of benefit in view of an increase in ventilator free days and a trend towards decreased mortality, but concluded that their role was not established.[98] A second meta-analysis from Tang et al in 2009 found an improvement in overall survival with glucocorticoids treatment in ARDS, especially if started early in the course of ARDS. The Tang study included cohort studies to maximise data available, although this could be criticised due to flawed methodology.[99, 100]

1.2.4.1 Anti-inflammatory effects of exogenous glucocorticoids in ARDS

Glucocorticoid therapy has been shown to decrease the cellular inflammation of ARDS. In clinical trials of prolonged courses of steroids plasma concentrations of the pro-inflammatory mediators TNF-α, IL-1β, IL-6, IL-8, and soluble TNF receptor 1 and 2 are

decreased. Increases in concentrations of the anti-inflammatory cytokine IL-10 are also seen.[9, 10, 16] The fibro-proliferation of ARDS is also modulated by the action of glucocorticoids. Pro-collagen amino-terminal peptides are proteins that are cleaved from collagen as it is laid down in the extra-cellular matrix during fibrosis, and are established as a marker of collagen deposition. The concentrations of these peptides are seen to be increased in ARDS, and persistant elevation is associated with worse outcome. Pro-collagen amino-terminal peptide levels in blood and broncho-alveolar lavage fluid are decreased rapidly by the administration of methylprednisolone. These concentration changes are followed by corresponding improvements in lung injury and organ dysfunction scores.[101] Neutrophils that are exposed to plasma from glucocorticoid treated ARDS patients produced less inflammatory mediators and had decreased activation of NF-κβ pathways than cells exposed to plasma from placebo treated ARDS patients.[16]

1.2.4.2 Potential adverse effects of exogenous glucocorticoids in ARDS

Considerable side effects exist for the therapeutic use of glucocorticoids that are potentially more serious in the setting of the critically ill patient. Not least of these is the potential increase in serious infection due to the immunosuppressive actions of steroids. Other potential risks also involve hyperglycaemia, neuromyopathies, poor wound healing, neuro-psychiatric disease and pancreatitis[30] Surveillance and prevention measures may decrease the incidence of these complications however when used as part of steroid therapy.[100]

1.2.4.3 Evidence for the therapeutic use of exogenous steroids in ARDS

Trials of therapeutic glucocorticoids for the treatment of patients with established ARDS have used two strategies; high dose steroids of short duration, or longer courses of moderate dose steroids. The evidence for both of these modalities is summarised below.

1.2.4.3.1 High dose steroids in the management of ARDS

1.2.4.3.1.1 Weigelt et al 1985

In this early study 81 mechanically ventilated patients at high risk of ARDS were recruited into this trial of early treatment with high does steroids vs. placebo to decrease the development of ARDS. Treatment arm patients received 30mg/kg of methylprednisolone 6 hourly for 48 hours. 64% of the steroid treated group went on to develop ARDS compared to 33% of the placebo treated group, and an increase in the incidence of secondary infection was also seen in the steroid group.[102]

1.2.4.3.1.2 Bone et al 1987

304 patients were enrolled into this placebo controlled randomised study of the use of steroids in sepsis and ARDS. The treatment arm received 30mg/kg intravenous methylprednisolone 6-hourly for 4 doses, starting within 2 hours of the diagnosis of sepsis being made. No difference in the incidence of ARDS or the reversal of ARDS was present between groups. Patients who developed ARDS in the steroid treated arm had increased mortality rate and increased mortality from secondary infection[82]

1.2.4.3.1.3 Bernard et al, 1987

This trial recruited 99 patients and randomised them to either placebo or 30mg/kg methylprednisolone 6-hourly for 24 hours. There was no difference between the groups in mortality, pulmonary shunting, PaO_2: FiO_2 ratio, thoracic compliance, pulmonary artery pressure or radiological appearance.[81]

1.2.4.3.2 Moderate dose steroids as treatment of ARDS

Improved understanding of steroid biology in inflammation, and the failure of high dose exogenous glucocorticoids to show an improvement in the treatment or prevention of ARDS provoked changes in the dosages and duration of administration of steroids used in trials. Research moved away from high dose therapy to strategies using more prolonged treatments using moderate dosages.

1.2.4.3.2.1 Hooper and Kearl 1990

Citing personal clinical experience, where patients suffering from ARDS deteriorated after stopping short course high dose steroids, but improved on recommencing this treatment; these investigators used a longer duration, lower dose steroid therapy than had been previously tested. This was a non-controlled prospective study of 10 patients over an 18 month period. The investigation performed Gallium-67 citrate scans on patients to quantify inflammatory activity within the lung, and to guide steroid therapy. The dose of steroid used was variable and lasted for >21 days in all patients who survived that period. Although the numbers were small, and the methodology imperfect, the authors' conclusions that a prolonged course of steroid may be more

effective than shorter, high dose therapy and that this warranted further investigation was valid .[103]

1.2.4.3.2.2 Meduri et al 1998

In 1998 Meduri et al reported a prospective, randomised, double-blind, placebo controlled trial of steroid therapy in 24 patients with ARDS after 7days of mechanical ventilation. Patients in the treatment arm received a loading dose of 2mg/kg followed by 2mg/kg intravenous methylprednisolone daily in 4 x 6-hourly doses. This was followed by a single enteral dose for 14 days when tolerated, and a tapering dose over the subsequent 14 days. A crossover point was built into the protocol, so that patients failing to improve would join the alterative arm of the trial. An improvement was observed in the steroid treated group in terms of lung compliance, PaO_2:FiO_2 ratio, severity scores, and mean pulmonary artery pressure. None of the treatment group crossed over to the placebo arm, while 4 patients crossed over from the placebo to the treatment arm. All steroid treated patients survived their ICU stay (N=16 100%) whereas only 37% of placebo treated patients survived (N=3, p=0.002). Steroid treated patients also had an increased chance of surviving their hospital admission (87% vs. 37%, p=0.03). No significant increase in the rate of infection or hypoglycaemia was observed.[83]

In a precedent that may have foretold the results of the ARDSnet study[85] those patients that received steroid after failure of therapy in the placebo arm did significantly worse than the patients who were given steroids at the time of initial randomisation (p=0.04). The authors of this paper concluded that steroids should be given prior to the fibrotic phase of ARDS.[83]

1.2.4.3.2.3 Steinberg et al (ARDSnet) 2006

The ARDSnet study, which reported in 2006, enrolled 180 patients between 7 and 28 days after the onset of ARDS. They were then randomised to either treatment or placebo arms. The treatment group received a single dose of 2mg/kg predicted body weight and then 0.5mg/kg 6hourly for 14 days, followed by 0.5mg/kg 12 hourly for 7 days, then a tapering dose until stopped. Their results failed to show a difference in the 60 day mortality between the two groups. However, patients started on the drug more than 13 days after the diagnosis of ARDS, had an increase in mortality from 12% to 44% (p=0.01). Steroid treated patients did have increased ventilator free days, and an improvement in plateau airway pressure, lung compliance and time until they were able to breathe un-assisted. Unfortunately steroid treated patients had an increased requirement to return to assisted ventilation compared to the placebo treated group.[85] Steroid treated patients had an increase in the incidence of the steroid side-effects hyperglycaemia and neuromyopathy, but there was no increase in the incidence of severe infection.[104]

This study does have some limitations: with over 3000 patients found to be eligible for enrolment into the trial, only 5% were randomised, the sample may therefore not be representative. A significant proportion of the glucocorticoid treated patients required the reintroduction of ventilatory support, and it has been suggested that this is due to the abrupt weaning of patients on extubation.[105] Of the randomised patients, it has also been speculated that baseline differences between the control and treatment arms in terms of their gender mix may have impacted upon outcome. Sex differences in hepatic cytochrome p450 metabolism have affected steroid metabolism in other studies.[106] Also the findings in patients recruited after day 13 of ARDS may be flawed by differences in disease severity between groups in these patients, and thereby skew the overall results.[100] Differences between the

findings in this investigation and those by Meduri et al have been attributed to patient management differences, with measures to prevent the complications of steroid therapy in Meduri's investigations altering the outcomes between these trials.[100]

1.2.4.3.2.4 Annane et al 2006

This investigation was a sub-study of patients with ARDS conducted as part of a large investigation of steroid therapy in sepsis.[107] 300 patients were enrolled into the larger study, and of these 177 patients met the AECC criteria for ARDS. A short synacthen test was performed on all patients and subjects classified as either responders (patients with an adequate increase in serum cortisol levels) or non-responders (those with an inadequate response in serum cortisol levels). Patients received either placebo or hydrocortisone 50mg qds and fludrocortisone 50mg daily for 7 days. 92 patients with ARDS were randomised into the placebo arm of the trial and 85 into the steroid treatment arm. The primary outcome measure was 28 day mortality, but no difference in this measure was present overall between the groups. No difference in the incidence of secondary infection was present between the study arms. If short synacthen test responders and non-responders are analysed separately, as in the parent study, improved mortality in the steroid treated arm is seen in synacthen test non-responders (75% mortality in placebo group, 33% in steroid treated group, Odds Ratio=0.35 CI= 0.15-0.82, p=0.016), but not in responding patients.[80]

1.2.4.3.2.5 Meduri 2007

This blinded placebo controlled randomised study was a follow up to the earlier 1998 study conducted by the same group. The methodology was changed by removing the controversial cross-over design of the 1998 study. The steroid dosage used was 1mg/kg loading dose, followed by 1mg/kg/day in 4 divided doses, and then weaned over 21 days.

91 patients were randomised, 63 into the treatment arm, and 28 into the control (placebo) arm. A number of cases in each arm did have protocol violations and were subsequently excluded from analysis (6 in treatment arm, 3 in control arm). Hospital mortality in the treatment group was 23.8%, and 84% in the placebo arm (relative risk=1.33 (CI=0.94 – 1.89, p=0.07). Steroid treated patients had greater incidence of lung injury score and multi-organ dysfunction score improvement, and increased ventilator free days.[84] This study was compromised in that after day 7 during the weaning phase 10 out of the 15 patients in the control arm received open label steroids, and may have derived benefit from this that is not taken into account in the in-hospital mortality.

1.2.4.3.2.6 Experience in ARDS due to H1N1 in 2009/2010

More recently the effects of exogenous steroids as a treatment for ARDS caused by the H1N1 epidemic of 2009-2010 was examined by Brun-Buisson et al in a retrospective observational study. They reported no benefit from this therapy, with increased risk of death in those treated with steroids (33.7% vs. 16.8%). Early use of steroids in this condition was more strongly associated with worse outcomes.[108] Martin-Loeche et al reported results for a similar cohort of patients; these investigators also found an increased incidence of hospital acquired pneumonia in patients receiving corticosteroids.[109]

1.3 The resolution of ARDS and the neutrophil hypothesis

1.3.1 Macrophages

Macrophages are phagocytic cells that are derived from peripheral blood mononuclear cells. They migrate into tissues either as a homeostatic mechanism to replenish the population of tissue specific macrophages, or in response to inflammation, as part of the host defence mechanism.[110] Their primary function is to remove potentially damaging extracellular material and the clearance of apoptotic cells. Macrophage recognition of pathogenic material activates an inflammatory response and the host defence response, in which these cells release pro-inflammatory cytokines. However, when performing "housekeeping" roles such as the clearance of apoptotic cells, they tend not to produce inflammatory cytokines, and in many instances produce anti-inflammatory signals. Macrophages also have a defence role, providing a continuous vigilance against threat by recognising the danger signals present in necrotic cell debris. They provide this service through a mechanism independent of the adaptive immune response, using Toll-like receptors (TLRs).[111]

Macrophages are classified into 3 subtypes, in a system derived from the classification of lymphocytes. M1 macrophages are described as "classically activated", and mature in response to the pro-inflammatory signals TNF-α and IFN-γ. They produce pro-inflammatory cytokines and have improved anti-bacterial action. M2a macrophages, also known as wound healing macrophages, have previously been termed alternatively activated macrophages. M2a macrophages are derived from resident macrophages in response to IL-4, and promote the healing of tissue injury by contributing to the extra-cellular matrix. M2b macrophages are regulatory cells. These macrophages are characterised by the production of IL-10, a potent anti-inflammatory cytokine. Despite

this nomenclature it is believed that macrophages exist in a spectrum of intermediate phenotypes, and are not limited to these strict subtypes.[111]

1.3.2 Alveolar macrophages in ARDS

Alveolar macrophages arise from both peripheral blood monocytes and proliferation of local macrophages and are responsible for the safe disposal of apoptotic cells, removal of harmful extracellular material and activation of the host defence response in the lung. As they activate the host defence response alveolar macrophages have a role in the initiation of ARDS. In early ARDS NF-kβ activation promotes the transcription of pro-inflammatory genes within alveolar macrophages.[15, 112] In an injurious ventilation model of direct ARDS macrophages are activated rapidly after the causative insult.[113] Alveolar macrophages are also activated in indirect lung injury, such as that seen in acute pancreatitis; in this instance the increase in plasma phospholipase A2 levels has been implicated due to its ability to increase the expression of inducible nitric oxide synthase in these cells.[114]

However, the main function of alveolar macrophages in ARDS is in the resolution of lung injury, with worse clinical outcomes are associated with defects in macrophage numbers and activity. It is thought that the resolution of ARDS is dependent on the action of macrophages to ingest apoptotic neutrophils, and prevent the release of their pro-inflammatory intra-cellular contents during secondary necrosis.[115] Alveolar macrophage numbers increase during the course of ARDS in patients who survive this condition, and non-survivors fail to increase their macrophage concentrations above baseline levels.[19] Even in patients with neutropaenic ARDS decreased macrophage activation is also associated with worse patient outcomes.[116]

Changes occur in the sub-populations of alveolar macrophages in ARDS. Different sub-populations of alveolar macrophages react differently to the stimuli present within the lung in ARDS, with some cells developing an inflammatory M1 phenotype, and other macrophages, primarily derived from the lung interstitium, developing an M2b, anti-inflammatory, regulatory phenotype.[117] A further sub-population of macrophages is also recruited to the lung in ARDS. Inflammation induces the differentiation of inflammatory monocytes to macrophages at the site of inflamed tissue to supplement the resident cells.[110] A study of patients with ARDS has shown that an increase in the phenotypically monocyte type alveolar macrophages is associated with a worse clinical outcome. Phenotyping in this instance was done with flow cytometric techniques using the following characteristics of "immature" macrophages: $CD14^{high}$, $27E10^{high}$, $CD11b^{high}$, $CD71^{low}$, HLA-DR^{low}, $25F9^{low}$ and $RM3/1^{low}$. The corresponding phenotype of mature alveolar macrophages is $CD14^{low}$, $27E10^{low}$, $CD11b^{low}$, $CD71^{high}$, HLA-DR^{high}, $25F9^{high}$ and $RM3/1^{high}$.[118]

1.3.3 Efferocytosis

Efferocytosis is the uptake of apoptotic cells by phagocytes, chief among which are tissue macrophages. The term is taken from the latin verb "effero" meaning "to take to the grave".[119] Apoptosis is a managed process of cell death designed to prevent harmful effects due to the release of cell contents.[120] During the process of apoptosis changes occur on the external surface of the cell membrane that make the cell recognisable as dying to phagocytotic cells such as macrophages. These cell surface changes have been termed "eat-me" signals.[121] These signals include exposed phosphatidylserine (PS) or integrin molecules recognised by the macrophage a_vb_3 integrin.[122]

The macrophage receptors for recognising eat-me signals form a complex system that can distinguish between "self", "non-self" and "altered-self" cells. Receptors for the recognition of "altered-self" on macrophages include CD36, a molecule which confers "professional" phagocytosis capacity on cells into which it is transfected,[123] the PS receptor, as well as the $a_v b_3$ integrin. Some of the eat-me signals seem subtle and context related, such as ICAM-3 and thrombospondin-1 only inducing efferocytosis in apoptotic, but not healthy cells.[124, 125]

Recognition of an apoptotic cell induces the phagosome to produce large outgrowths of the cell membrane which go on to engulf the apoptotic cell and enclose it within an "efferosome" in the cytoplasm. The apoptotic cell is then digested rapidly within this vesicle, and constituents characteristic of the apoptotic cell are indistinguishable after as little as 30 minutes.[119, 126]

Whereas the uptake of other extracellular material provides a pro-inflammatory stimulus, the uptake of apoptotic cells is part of an anti-inflammatory process.[127] Apoptotic neutrophils induce the release of the anti-inflammatory hormones IL-10 and TGF-a by macrophages, and decrease the production of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12.[120] Anti-inflammatory glucocorticoid hormones increase the rate of efferocytosis,[128] and 11β-HSD amplification of glucocorticoid effects promotes phagocytosis both in-vitro and in an animal model of abdominal sepsis.[75] Glucocorticoids also induce monocytes to exhibit increased anti-inflammatory properties such as phagocytosis as they differentiate to macrophages, confirming that the HPA system and 11β-HSD metabolism promote this anti-inflammatory process.[129] This may however be at a cost to the organism. There is some evidence that the anti-inflammatory effects of

efferocytosis decrease the anti-microbial capacity of alveolar macrophages, and can make the host more prone to secondary infection.[130]

1.3.4 The "neutrophil hypothesis"

There is evidence that a defect in the process of efferocytosis is a cause of on-going inflammation in ARDS. Neutrophils migrate in large numbers into the lungs of patients with ARDS,[19] and ultimately undergo apoptosis in the lung.[131] Their safe disposal by efferocytosis is necessary for the resolution of this condition. The continued inflammation and postponement of the anti-inflammatory phase of ARDS by the continued presence of activated neutrophils, and the release of pro-inflammatory cell contents from apoptotic neutrophils not undergoing efferocytosis, has been termed the "neutrophil hypothesis.[132]

The continued presence of activated neutrophils within the lungs of patients with ARDS is due to both delayed apoptosis and inhibited efferocytosis. The delay in apoptosis of neutrophils is part of the inflammatory response, and is mediated through inflammatory signalling and activation of the NF-κβ system.[133] Granulocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor and IL-2 concentrations are raised in the alveolar space of patients with ARDS and all act to delay neutrophil apoptosis.[134-136] Glucocorticoids levels are also raised in ARDS, and have also been shown to increase neutrophil lifespan.[137] The increased neutrophil lifespan is associated with the severity of disease, such that neutrophils from more severely-unwell patients with severe sepsis have longer delays to apoptosis and longer-lived neutrophils.[131]

In the resolution of ARDS apoptotic neutrophils are taken up by alveolar macrophages, and this task is essential to the resolution of ARDS.[138] A failure to increase the efferocytosis capacity by increasing alveolar macrophage numbers is associated with worse patient outcomes.[19] Efferocytosis is inhibited by many of the inflammatory mediators and cytokines that are present in increased concentrations in ARDS, such as LPS, TNF-α, and HMGB-1.[139-142] This process has been shown to be important in other diseases, as efferocytosis is defective in models of atherosclerosis and obesity,[143] and wound healing is delayed in diabetes due to defects in efferocytosis and the accumulation of apoptotic neutrophils.[144] Supporting evidence also comes from the study of alcoholic patients. Ethanol inhibits efferocytosis by a Rho GTPase mediated pathway: Neutrophil concentrations are higher in the lungs of alcoholic patients with ARDS, and alcoholic patients are more prone to this condition.[145]

Efferocytosis is therefore a steroid sensitive process which is under increased demand in ARDS. The metabolism of glucocorticoids by 11β-HSD in alveolar macrophages is a necessary pre-receptor amplification of the glucocorticoid signal required for adequate up-regulation of efferocytosis,[75] and defects in 11β-HSD activity are a potential cause of steroid resistance, inadequate efferocytosis and on-going inflammation in ARDS.

1.4 Biomarkers in ARDS

1.4.1 Biomarkers in ARDS

ARDS is a difficult disease to investigate. Mortality is moderately high,[4, 146]and therefore survival is the only true end-point for investigations of this condition. As patients tend to die from the causative condition rather than respiratory failure due to ARDS,[7] and there is a diverse list of potential causes of ARDS with differing associated mortalities,[3] there is large variability that requires relatively large sample sizes to account for. It has been suggested that these factors have contributed to the failure to show any pharmacological agent is of therapeutic benefit for ARDS.[96] The use of a surrogate endpoint can provide a more frequently occurring or linear scale outcome that could be used in ARDS investigation. Surrogate end-points in ARDS have traditionally been the physiological parameters which represent the measurable physiological derangements of ARDS. Chiefly this has been measures of defects in gas exchange such as the PaO_2, and the PaO_2: FiO_2 ratio, and disease severity indices such as the Murray lung injury score.[147] Other investigations have used short term measures of clinical outcome such as ventilator free days (VFDs) and extrapolated these to longer term outcomes. Unfortunately studies using such parameters have seen that even when an intervention causes an improvement in the surrogate end-point, this does not necessarily translate into meaningful mortality outcomes: An example of this is the use of prone ventilation. In this ventilatory strategy mechanically ventilated patients are nursed in the prone position, with the theoretical benefits of improving the balance between ventilation and perfusion within the lung. Although this treatment has consistently been shown to improve the measures of oxygenation, researchers have failed to demonstrate an improvement in mortality.[148-151] Other investigators have used short-term measures of

clinical outcome as surrogate end-points, such as ventilator free days (VFDs), the time within the first 30 days after onset of disease that the patient does not require ventilatory support. This measure has been statistically tested and is thought to reduce the numbers needed in future trials of ARDS treatments.[152] However, it lacks the ability to reflect pathological progression in shorter periods of time, or rates of improvement.

As yet no such biological marker (biomarker) is universally recognised in ARDS. Biomarkers are in everyday use in the management of many severe illnesses, such as troponins in the diagnosis of myocardial infarction,[153] HbA_{1c} in the monitoring of diabetes,[154] and creatinine and glomerular filtration rate in renal failure.[155] As well as being specific to the disease in question, and a sensitive marker of this condition, a potential candidate to be a useful research and clinical biomarker would ideally fulfil certain criteria: Firstly, it should have a causal role in the pathophysiology of the condition. Secondly, that the effect of any experimental or therapeutic intervention should be captured by changes in the biomarker. Thirdly, concentrations of the surrogate must reliably reflect the relevant clinical outcomes.[156] It has also been suggested that any candidate should be easily and reliably collected and measured.[157] Not only would such a marker be a valuable investigational tool, it would also be useful in the clinical management of patients with ARDS. A sufficiently sensitive marker could be used to ensure that ventilator associated damage was minimised, and guide ventilatory strategy. *See table 1.3.*

The concentrations of many molecules are altered in the plasma and BALF of patients with ARDS such as TNF-α, IL-1β, IL-6, IL-8, IL-10, Protein C, ICAM-1, and procollagen amino-terminal peptides 1 and 3; but as these molecules are markers of systemic inflammation, they lack the necessary specificity for lung injury severity.[10, 13, 101, 158] Some

more specific candidates for an ARDS biomarker have been suggested, including Clara cell secretory protein 16. Unfortunately results for this molecule have been contradictory: levels being found to be high in patients who did not survive ARDS,[159] but one group suggesting low plasma concentrations are diagnostic of ARDS,[160] while other groups suggesting high levels are diagnostic.[161] A more reliable marker is needed.

Table 1.3 Properties on an ideal biomarker[156, 157]

Properties of an ideal biomarker
Highly specific
Highly sensitive
Causative role in pathophysiology
Easily collected
Easily measured
Reflect clinical outcomes
Changes should reflect effects of intervention/therapy

1.4.2 The receptor for advanced glycation end products (RAGE) as a biomarker in ARDS

The receptor for advanced glycation end products (RAGE) has been suggested as a candidate biomarker for alveolar damage in ARDS.[157] RAGE is expressed constitutively within the lung as a cell surface receptor on type-1 alveolar cells, principally on the basal cell membrane.[162] Alveolar type 1 cells form 93% of the alveolar surface of the lung,[163] and are responsible for many aspects of host defence, as well as allowing gas exchange and ensuring the hydrostatic integrity of the alveolar membrane. RAGE has a role in the inflammatory response, and therefore has a plausible causative role in the pathophysiology of ARDS.

1.4.2.1 RAGE

RAGE is a multi-ligand receptor that is a member of the immunoglobulin super-family of proteins, coded for within the major histocompatibility class III region on the short arm of chromosome 6.[164] It is a protein of approximately 35kDa, subdivided into 3 functional elements: an extra cellular region of 332 amino acids, a trans-membrane domain of 19 amino acids, and an intra-cellular domain of 43 amino acids. The extra cellular region is composed of three domains, one "V" type and 2 "C" type immunoglobulin domains.[165] RAGE is thought to recognise families of molecules, rather than specific peptides, and as such is a key factor in the innate immune defence.[166]

It is named for the non-enzymatically produced factors for which it acts as a receptor: advanced glycation end-products (AGEs). AGEs are produced under conditions of oxidative and glycation stress,[167] conditions which are typical of those present in the inflammation associated with sepsis and ARDS. These products are detectable in high concentrations in critically ill patients using skin auto-fluorescence techniques.[168] RAGE is a multi-ligand receptor, and as well as AGEs RAGE-ligands include other pro-inflammatory molecules such as b-fibrils, TNF-α, SA100A12, and high mobility group box protein-1 (HMGB-1).[169]

As well as the full length cell surface receptor form, other forms of RAGE have been identified, most notably a secreted, soluble form of the receptor: sRAGE. This variant lacks the trans-membrane and intra-cellular domains and is actively secreted from cells. This form of RAGE has been proposed to act as a decoy receptor, neutralising RAGE ligands prior to their binding to cell surface RAGE, preventing activation of the pro-inflammatory cascade, and thus act as a negative feedback mechanism.[170] Administration of sRAGE decreases the pro-inflammatory response in models of

sepsis,[171] arthritis,[172, 173] atherosclerosis,[174] and encephalomyelitis.[175] However, the biology is complex, and differs between models. sRAGE itself appears to have pro-inflammatory effects on mouse splenocytes in vitro, acting via Mac-1 to enhance NF-kb pro-inflammatory gene expression, and acting as a chemotactic agent.[173]

Cell surface RAGE acts as an endothelial surface receptor, a counter-receptor for leucocyte integrins, and an agent instrumental in the recruitment of leucocytes to site of inflammation. The presence of the S-100 proteins, which are also RAGE ligands, augments this process.[176]

1.4.2.2 RAGE in the host defence response

Models of sepsis and critical illness have shown that RAGE has a role in the host defence response. RAGE knockout mice are protected from the lethal effects of septic shock, a process dependent on the innate immune system. This is reversed when these mice are reconstituted with RAGE.[171, 177] RAGE has also been investigated as a therapeutic target in the treatment of sepsis. In a murine model of sepsis using caecal puncture, the administration of a monoclonal anti-RAGE antibody increased survival.[177] Other investigations have shown that anti-RAGE antibodies decrease the diaphragmatic dysfunction observed in this model of sepsis.[178]

A key feature of RAGE modulated biological interactions is the up-regulation of RAGE in conditions that are rich in RAGE ligands. This mechanism is through the activation of NF-κβ and subsequent increased RAGE expression, leading to further RAGE-ligand interaction effects.[179] This is thus a positive feedback loop that will amplify the inflammatory response.[166] As NF-κβ amplification of pro-inflammatory gene expression is abrogated by glucocorticoids, this would provide a direct mechanism by which glucocorticoids could influence the expression of RAGE. Inflammatory mediators such as

reactive oxidant species also directly increase the expression of the receptor and its ligands by endothelial cells in vitro.[180]

1.4.3 RAGE as a potential biomarker

Studies by Uchida and colleagues using a rodent model of lung injury have shown that sRAGE is released in response to lung injury, and that sRAGE concentrations in both the BALF and serum varied with increased severity of the induced lung injury.[181] Similar findings were reported by Su and co-workers in a mouse model of LPS and acid induced lung injury. This group found that concentrations of RAGE in the alveolar fluid were raised in the lungs of animals with lung injury, and varied with the severity of injury. This group however only found the increase in RAGE in a direct model of lung injury, and similar experiments using indirect causes of lung injury (thiourea induced vascular injury and a model of transfusion related lung injury (TRALI)) did not show the same increases in BALF RAGE levels. Su et al also found that when treated with mesenchymal stem cells, a measure shown to decrease inflammation in animal models of ARDS, the RAGE concentrations decreased, which correlated with histological improvements.[182] Thus, although there have been few experiments in humans involving RAGE, animal models would suggest that RAGE mirrors the severity and course of ARDS, and could fulfil this criterion to be a biomarker and surrogate end-point in clinical studies. Unfortunately the support for this in human subjects is mixed. A study by Calfee and colleagues in 2008 showed that the increase in RAGE levels was only present in the patients ventilated with high tidal volumes, and not those ventilated with the low tidal volumes now in common practice.[183]

In animal studies RAGE concentrations in the alveolar lavage fluid correlates with the severity of the experimentally induced lung injury in rats.[181] In human studies also, RAGE

is found in increased concentrations in the alveolar fluid of patients with acute lung injury compared to concentrations both in the lungs of normal healthy controls, and in patients suffering from hydrostatic pulmonary oedema.[181] RAGE concentrations are linked to clinical outcomes in ARDS in patients ventilated with larger tidal volumes; with higher concentrations present in patients who did not survive.[183] RAGE ligands such as SA100A12, a calcium binding protein released from neutrophils, are also found in high concentrations in the broncho-alveolar lavage fluid of patients with acute lung injury.[169]

Although it is expressed in other cell types,[184] raised RAGE concentrations in the context of critical illness are thought to be specific to lung injury and ARDS.[181, 185] This makes RAGE a potentially more useful marker of disease due to its relative specificity. RAGE has been investigated previously as a biomarker. In 2008 Calfee reported that in the patients enrolled to the ARDSnet trial of low tidal volume ventilation,[97] RAGE concentrations were higher in patients who died compared to those who did not. These results were attributed to increased concentrations in patients ventilated with higher tidal volumes, who also had increased mortality.[183] No comparison was reported between patients with ARDS and subjects without this condition. A report in 2009 from Determann and co-workers investigated RAGE as a biomarker as part of a ventilator acquired pneumonia study involving 33 patients of which 10 developed ALI/ARDS. The results showed RAGE may have only limited utility in this setting as a diagnostic marker of ARDS, with an area under the curve statistic of only 0.61 on ROC analysis.

ARDS and ALI are not the only diseases in which RAGE may prove useful as a marker of disease severity and progression. RAGE has also been postulated as a potential biomarker in chronic obstructive pulmonary disease (COPD). In contrast to investigations of ALI and ARDS, in this chronic disease the RAGE concentrations were in fact found to

be lower in the serum of stable COPD patients than in that of healthy controls. In patients suffering an acute exacerbation of COPD, the levels were found to drop further.[186] It has also been investigated in acute exacerbations of idiopathic pulmonary fibrosis (IPF), where concentrations are approximately 50% lower than those seen in the acute inflammation seen at the onset of ALI.[187]

RAGE therefore has a potential role in the pathological process of ARDS, reflects the clinical outcomes in some patients with ARDS, is easily collected by venesection or by bronchoscopy, and is easily measured by ELISA. RAGE is therefore a promising candidate as a clinical biomarker of ARDS which could also be used as a surrogate endpoint in investigations of this condition. However further investigation is required: As yet no investigations have assessed its concentrations in the alveolar space, and RAGE has not been used to measure outcomes in a pharmacological trial.

1.5 Conclusion

Much progress has been made in unravelling the pathological processes of ARDS since it was first described in 1967. It is now established that this is an inflammatory disease, and that the massive inflammatory cell infiltration that occurs in ARDS overwhelms the lungs ability to dispose of these cells, which then perpetuate inflammation. The HPA system limits the systemic inflammation of the host defence response, and promotes the timely clearance of inflammatory cells by efferocytosis. However, ARDS is refractory to the endogenous anti-inflammatory mechanisms of the HPA system and its endogenous glucocorticoids. No pharmacological anti-inflammatory agent has yet been shown to benefit these patients, but exogenous glucocorticoids are the most promising therapeutic approach to date, and the rationale for their use is strong. However, the

biology of steroid hormones within the lung is imperfectly understood. Glucocorticoids are metabolised within the lung by the enzyme 11b-hydroxysteroid dehydrogenase, and the activity of this enzyme will influence the how cells respond to the steroid signal. An improved understanding of how glucocorticoids are metabolised within the lung, and why cells with an anti-inflammatory role are unable to respond effectively to the anti-inflammatory signal of the HPA system is required.

Improved investigational tools are required for the study of ARDS. A validated biomarker that can be used as a diagnostic inclusion criteria and surrogate outcome measure in investigations of ARDS, and in the diagnosis and ventilator management of patients with ARDS will improve our care of these critically ill people. As yet no such indicator exists, but one candidate molecule is the receptor for advanced glycation end-products.

2 Aims

This thesis is the report of a series of investigations into how glucocorticoids and glucocorticoid metabolism by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) influence alveolar macrophage efferocytosis in the acute respiratory distress syndrome (ARDS). As the investigation of ARDS is limited by the lack of an appropriate biomarker, this thesis also contains the report of an investigation into a candidate molecule. Specifically the aims of these investigations are:

1. To establish the concentrations of glucocorticoids in the alveolar space of patients with ARDS, and how they relate to lung inflammation and disease severity.
2. To determine whether factors present in the extra-cellular environment of alveolar macrophages during ARDS are responsible for any changes in 11β-HSD activity.
3. To evaluate how glucocorticoids, glucocorticoid metabolism by 11β-HSD, and the inflammatory process of ARDS influence the efferocytosis of apoptotic neutrophils by primary alveolar macrophages.
4. To measure the 11β-HSD activity of alveolar macrophages extracted from patients who have ARDS, and determine how this relates to disease severity, inflammation and steroid concentrations in the plasma and alveolar space.
5. To test whether concentrations of the receptor for advanced glycation end products (RAGE) in the plasma or alveolar space of patients with ARDS could be developed as a diagnostic marker, a marker of disease progression and resolution, or as a surrogate end-point in the investigation of ARDS

3 General methods

3.1 Patient recruitment

Patients were enrolled for the bronchoscopy studies on the intensive care units at the Heart of England NHS Foundation Trust, and the University Hospitals Birmingham in the period from January 2008 to July 2009. The Heart of England NHS Foundation Trust is a university affiliated trust that represents three hospitals in the East and North of Birmingham. There are three intensive care units in the trust totalling 22 beds. At University Hospitals Birmingham there were 4 critical care units during this period with a capacity of 52 patients. The intensive care units from which subjects were recruited admit adult patients with both medical and surgical conditions. Patients were recruited into a program of 3 complementary studies investigating ARDS: the BALTI-1 study, a bronchoscopic sub-study of the BALTI-2 trial, or into the resolution of inflammation in ARDS study. In addition to clinical samples from patients with ARDS or at risk of developing ARDS, laboratory studies used alveolar macrophages extracted from lung resection samples available through the Midlands lung tissue collaborative (MLTC). All studies had ethical approval from local research and ethics committees.

3.1.1 Diagnosis of ARDS

ARDS was diagnosed using the American European consensus conference (AECC) criteria. *See table 1.1*. Screening for eligible patients was performed daily on participating ITUs, and patients were eligible for inclusion in the studies reported in this thesis within 24 hours of the diagnosis of ARDS.

3.1.2 "At risk" patients

In order to provide a control group of patients who were ventilated on the intensive care unit with a critical illness, but who had not developed ARDS we recruited patients

with a systemic inflammatory response syndrome as defined by the ACCP/SCCM consensus conference criteria. *See table 3.1*.[188]

Table 3.1 Systemic inflammatory response syndrome diagnostic criteria

2 or more of the following criteria
• Fever of more than 38°C or less than 36°C
• Heart rate of more than 90 beats per minute
• Respiratory rate of more than 20 breaths per minute or a PaCO2 level of less than 32 mm Hg
• Abnormal white blood cell count (>12,000/μL or <4,000/μL or >10% bands)

3.1.3 The BALTI-1 study

The BALTI-1 trial (b-agonist lung injury trial 1) recruited 40 patients between 2001 and 2003 at Birmingham Heartlands hospital only. This was a double blind placebo controlled trial of intra-venous salbutamol in the treatment of ARDS with a primary endpoint of reduction in extra-vascular lung water.[95] Patients enrolled in this study underwent bronchoscopy at enrolment to the study (onset of ARDS, day 0) and after 4 days (day 4). Biological samples from this study were collected by Dr Gavin Perkins, archived and were made available for this investigation.

3.1.4 The BALTI-2 bronchoscopic sub-study

The BALTI-2 trial (β-agonist lung injury trial 2) was a double blind, placebo controlled trial of intra-venous salbutamol as a treatment for ARDS. It aimed to recruit 1334 patients with ARDS from intensive care units throughout the UK, and randomise them to receive an infusion of intravenous salbutamol or placebo for seven days. Its primary outcome was 28 day mortality, but also collected data on the secondary outcome

measures of organ failure and ventilator free days. As part of this study the protocol allowed for a bronchoscopic sub-study, in which patients who consented to inclusion underwent bronchoscopy at the same time points as the BALTI-1 study. This study was suspended on safety grounds by the trial steering committee on 26th March 2010. 326 patients had been recruited at this point, and an interim analysis was undertaken. This showed an increased mortality rate in those patients given the active drug compared to those treated with placebo.

3.1.5 The resolution of inflammation in ARDS study

This was a prospective observational study, designed as part of the BALTI series of investigations to provide a cohort of "at-risk" patients, as detailed in the at-risk section above. Inclusion criteria for this study were individuals who met criteria for the systemic inflammatory response syndrome, and were intubated on the intensive care unit. An identical protocol was used for the collection of samples and clinical data for this study as for the BALTI-1 and BALTI-2 trials, in order to make the results comparable between these investigations.

3.1.6 The Midlands lung tissue collaborative (MLTC)

The MLTC is a regional scheme involving The University of Birmingham, Glenfield Hospital Leicester and Birmingham Heartlands Hospital that allows the use of lung resection specimens obtained from patients undergoing either a lobectomy or pneumonectomy for research into lung disease. The surgery is conducted at Birmingham Heartlands Hospital Thoracic Unit as part of therapy for lung cancer. Patients recruited had provided consent for any tissue removed as a part of their surgery not required for diagnostic or clinical purposes to be used for research.

3.2 Severity scoring

After enrolment to either of the clinical studies, the severity of critical illness and any lung injury was calculated by a number of schemes, as outlined below. The purpose of these schemes is to classify disease severity to enable clearer analysis of effects of treatment, and to take into account multiple factors, impacting on survival.

3.2.1 APACHE-II

The acute physiology and chronic health evaluation (APACHE) system of scoring critically ill patients is a well established and validated system of severity assessment now in its 4th incarnation as APACHE IV. The system was originally developed in 1981 as APACHE, and updated in 1985 to APACHE II, which remains the most commonly used system. In APACHE-II 12 variables are each assigned a score from 0 to 4 depending on the most abnormal measurement in the 1st 24 hours of their ITU stay. Scores are also added to account for the variables of age, past medical history and presence of a surgical condition. This leads to a score between 0 and 71, higher scores indicating an increasing severity and risk of death. The APACHE scoring system is widely used in clinical studies as a marker of disease severity in critically ill patients. [189]

3.2.2 The SAPS-2 score

The Simplified acute physiology score version 2 (SAPS-2) is a physiological disease severity index that uses acute physiological measurements and chronic health status together with the context of the ICU admission to give a score that correlates well to mortality outcomes. It has been developed from logistic regression analysis of a large cohort of critically ill patients.[190]

3.2.3 SOFA score

The sequential organ system failure assessment (SOFA) is a severity score designed to classify the degree of organ failure a patient suffers whilst critically ill. Organ function in the respiratory, cardiovascular, hepatic, coagulation, neurological and renal systems is assigned a score between 0 and 4. Higher total scores are associated with worse outcomes.[191] *See table 3.2*.

An independent research group found excellent correlations with outcome: a minimum SOFA score greater than 9 in patients over the age of 60 had 100% specificity for death.[192] Other groups have found that this score is useful in the quantification of both organ failure on admission to the intensive care unit, and that which develops during the ICU stay, again giving good predictive values for patient outcomes.[193] Another study showed that the sequential calculation of this score gave a meaningful prediction of outcome, as not only were high initial scores prognostic of poor outcome, but a deterioration in the initial SOFA score over the first few days of ICU admission was also associated strongly with death.[194] When serial SOFA scores were combined with APACHE-2 scores the prognostic prediction is better than with either scoring system alone.[195]

Table 3.2 Components of the sequential organ failure assessment (SOFA) score

Score	**0**	**1**	**2**	**3**	**4**
P:F Ratio (kPa)	> 53.3	≤ 53.3	≤ 40	≤ 26.6	≤ 13.3
Platelets (10^9 mm^{-3})	> 150	≤ 150	≤100	≤50	≤20
Bilirubin (mmol L^{-1})	≤ 20	20-32	33-101	102-204	>204
GCS	15	13-14	10-12	6-9	<6
Creatinine (mmol L^{-1}) OR urine output (ml/day)	< 110	110-170	171-299	300-440	>440
				< 500	< 200
Hypotension (infusion rate in ml/kg/min)	None	MAP<70 mmHg	DA <5 DB -any dose	DA > 5 Adr ≤ 0.1 NA ≤ 0.1	DA > 15 Adr ≥ 0.1 NA ≥ 0.1

MAP= mean arterial pressure, DA= dopamine, DB=dobutamine, Adr= adrenaline, NA=noradrenaline

3.2.4 Murray Lung Injury Score (LIS)

The Murray lung injury score was derived as a tool to predict clinical outcomes in patients with ARDS, as part of an attempt to tighten the definition of ARDS by leading researchers into the condition.[147] The score is a calculated by the sum of individual scores for each feature (CXR appearance, hypoxia, positive end-expiratory pressure and compliance) divided by the number of features that made up the score. More than one study has failed to show that the LIS is a predictor of mortality,[196, 197] however for patients with a score greater than 2.5 it does predict those likely to follow a complicated course.[198]

Table 3.3 Components of the Murray lung injury score (LIS)

CXR Score	
0	No alveolar consolidation
1	Alveolar consolidation confined to 1 quadrant
2	Alveolar consolidation confined to 2 quadrants
3	Alveolar consolidation confined to 3 quadrants
4	Alveolar consolidation in all 4 quadrants
Hypoxia score	
0	PaO_2 / FiO_2 > 40 kPa
1	PaO_2 / FiO_2 30 – 40 kPa
2	PaO_2 / FiO_2 23.3 – 29.9 kPa
3	PaO_2 / FiO_2 13.3 – 23.2 kPa
4	PaO_2 / FiO_2 < 13.3 kPa
Positive end expiratory pressure score (when ventilated)	
0	< 5 cm H_2O
1	6-8 cm H_2O
2	9-11 cm H_2O
3	12-14 cm H_2O
4	> 15 cm H_2O
Respiratory system compliance	
0	>80 ml / cm H_2O
1	60-79 ml / cm H_2O
2	40-59 ml / cm H_2O
3	20-39 ml / cm H_2O
4	< 19 ml / cm H_2O

3.2.5 GOCA

The GOCA scoring system is specifically designed for the characterisation of disease in patients suffering from ARDS, and was advocated by the AECC. This scoring system incorporates the most important factors that influence prognosis in ARDS, and is named by the abbreviation of these components: Gas exchange, organ failure, cause and associated disease. *See table 3.4*. This system has been designed, not to predict mortality, but to standardise reporting of the spectrum of ARDS.[199] Consistent with its design it has been shown to perform less well in mortality prediction than the APACHE-2

and SAPS-2 scoring systems.[200] In this investigation it has been used to characterise the demographics of the patients.

Table 3.4 the components of the GOCA classification of ARDS

	Meaning	Scale	Definition
G	**Gas exchange**	0 1 2 3 A B C D	PaO_2/FIO_2 > 40 kPa PaO_2/FIO_2 26.8 - 40 kPa PaO_2/FIO_2 13.46 - 26.8 kPa PaO_2/FIO_2 <13.46 kPa Spontaneous breathing, no PEEP Assisted breathing, PEEP 0-5 cm H_2O Assisted breathing, PEEP 6-10 cm H_2O Assisted breathing, PEEP > 10 cm H_2O
O	**Organ failure**	1 2 3	Lung + 1 organ Lung + 2 organs Lung + 3 organs
C	**Cause**	0 1 2	Unknown Direct lung injury Indirect lung injury
A	**Associated diseases**	0 1 2	No coexisting diseases that will cause death within 5 years Coexisting disease that will cause death within 5 years Coexisting disease that will cause death within 6 months

3.3 Sample Collection

3.3.1 Bronchoscopy and collection of broncho-alveolar lavage fluid

Bronchoscopy was performed on patients using a standardised protocol. The patient was sedated and a muscle relaxant administered. The patient was then ventilated using the P-SIMV mode of ventilation on 100% inspired oxygen for at least 5 minutes prior to the procedure. An Olympus LF-TP (Olympus-Keymed, UK) tracheal intubation fiberscope was used for all procedures. This scope is 5.2mm in diameter and has a large 2.6mm suction channel. After inserting the bronchoscope through the patient's endo-tracheal

tube or tracheostomy tube (if present), the tip of the bronchoscope was wedged into a sub-segmental bronchus of the right middle lobe. Three 50ml syringes of room temperature, sterile 0.9% saline were instilled down the bronchoscope as a lavage, and the broncho-alveolar lavage fluid (BALF) aspirated back up into a 100ml collection chamber. The sample was immediately placed on ice and transported to the laboratory for processing.

Initial bronchoscopy and collection of BALF was performed at enrolment to the study. Therefore the initial bronchoscopy is on the day of diagnosis of ARDS for those patients with this condition; described as ARDS day 0. For patients with ARDS a subsequent bronchoscopy was performed after 4 days: ARDS day 4. Patients without ARDS had only one bronchoscopy performed.

3.3.2 Blood Collection

Blood was collected at the same time points as bronchoscopy. Blood was collected from pre-sited arterial or central venous lines into 7ml Lithium Heparin vacutainer® tubes (Becton Dickinson Ltd, Oxford, UK). These were then transported on ice to the laboratory for processing and analysis.

3.3.3 Lung resection specimens

Resected lung specimens collected as part of the MLTC were immediately examined in the operating theatre by a member of the surgical team and a portion of specimen without macroscopic pathology and not required for a diagnostic purpose was passed to the research team. This sample was immediately immersed in sterile 0.9% saline in a sealed container and transported on ice to the laboratory for processing. The sample

was measured and superficially washed with 0.9% saline immediately on arrival at the laboratory.

3.4 Sample processing

3.4.1 Broncho-alveolar lavage fluid processing

BALF was initially filtered through surgical gauze to remove debris and mucous and placed into a 50ml centrifuge tube. This was then spun in a pre-chilled centrifuge at 4^{o}c and 500g for 5 minutes. The supernatant was then aspirated off, divided into 1ml aliquots and immediately stored at -80^{o}c for future analysis. The cell pellet was re-suspended in 10ml RPMI culture media.

3.4.1.1 Differential cell count

A differential cell count of cells present in the BALF was performed by direct visualisation of stained cytospin slides. 50ml of the re-suspended cell pellet was placed in the cytospin apparatus and spun at 450g for 3 minutes in a Shandon Mk II cytocentrifuge (Thermo electron corporation Basingstoke, UK). The slides were air dried quickly and then stained with Diff-Quick (Baxter Incorp., UK). The differential cell count was performed by counting numbers of different cells until a total of greater than 100 cells had been seen. The percentage of each cell type seen was then calculated.

3.4.1.2 Cell viability

Cell viability was assessed using the trypan blue exclusion test: 50ml of cell solution was mixed with 50ml of filtered 0.4% trypan blue solution, and left to incubate for 5 minutes. 10ml of this mixture was then placed on a haemocytometer with an improved Neubaur

grid. Non-viable cells take up the trypan blue dye and therefore appear dark, while viable cells exclude the dye and are distinct from the non-viable cells. The total number of viable cells was calculated by multiplying the number of viable cells on the haemocytometer grid by 2 to account for trypan blue solution dilution, and then multiplying by 10^4 to give the number of cells per millilitre in the cell suspension.

3.4.2 Blood Processing

At the laboratory whole blood was placed in a centrifuge pre-chilled to 4^{o}c and spun at 500g for 10 minutes. The supernatant (plasma) was then aspirated and aliquoted into 1ml cryovials and stored immediately at -80 for future analysis.

3.4.3 Resected lung sample processing

2000ml of 0.9% saline was flushed through the sample by injection through a 14 gauge hollow needle moved through the sample from multiple sites. The washed through fluid that is produced during this process was collected, decanted into 50ml centrifuge tubes and spun in a pre-chilled centrifuge at 4^{o}c and 500g for 5 minutes. The supernatant was then discarded and the cell pellets pooled and re-suspended in 10ml of RPMI.

3.5 Separation of alveolar macrophages

Resuspended cell pellets from the resected lung sample washing and BALF were treated identically after this point. The cell solution was under-layered with 10ml of lymphoprep^{TM} (Axis-Shield, Dundee, UK) and spun in a centrifuge at ambient temperature for 20 minutes at 800g with the centrifuge brake turned off. The resultant buffy inter-phase was aspirated with a Pasteur pipette into a 15ml centrifuge tube. A cell count and viability assay was then performed on this suspension using a

haemocytometer and the trypan blue exclusion test as described above. The original cell suspension was then diluted to give a concentration of 1 million cells per ml. Cells were cultured in 24-well cell culture plates at 37^{o}c in a 5% CO_2 atmosphere.

3.5.1 Cell culture condition viability tests

To ensure the tissue culture conditions were not causing cell death an assay of cell viability was used. The commercially available Celltiter aqueous (Promega, UK) assay solution was used. This contains a tetrazolium compound, which is metabolised to a coloured formazan compound by healthy cells. A sample of each culture condition was used in the cell viability assay. 20ml Celltiter aqueous solution was added to individual cell culture wells and the cells incubated for 2 hours. After this period 100ml of the cell culture supernatant for each well was transferred to a 96 well plate, and the light absorbance measured at a wavelength of 495nm using a Synergy 2 microplate spectrophotometer (Biotek, Winooski, VT, USA). Absorbance levels for each culture condition were compared to a known viable culture, and if a condition proved to result in decreased cell viability it was excluded from analysis.

3.6 Collection of RNA

To extract RNA from alveolar macrophages in cell culture plates the commercially available RNAeasy kit (Qiagen, Hilden, Germany) was used. The cells were initially disrupted by vigorous pipetting in the solution Cell lysis buffer. This solution was transferred to a Qia-shredder column (Qiagen, Hilden, Germany) and centrifuged in a micro-centrifuge at 13000 rpm for 1 minute. The solution produced was then diluted to twice its volume with 70% ethanol. The resultant mixture was mixed by pipetting and

transferred to an RNA-easy column. This column was then spun in a micro-centrifuge at 13000 rpm for 15 seconds. The flow through solution was discarded. 700ml of the kit "RLT" buffer was added to the column and again the column was spun in a micro-centrifuge at 13000 rpm for 15 seconds. The flow-through solution was again discarded. 500ml of the kit "RPE" buffer was then added to the spin column and spun in a micro-centrifuge for 2 minutes at 13000rpm. The spin column was then placed in a new collection tube and spun for 1 minute at 13000rpm in order to dry the spin column. The column was again placed in a new RNAse free collection tube and 30ml of RNAse free water was added to the column. The column was spun in the micro-centrifuge for 2 minutes at 13000rpm and the resultant flow through; containing the RNA, was immediately sealed and placed in a freezer at -80^{o}c.

3.7 Real-time polymerase chain reaction

Real-time polymerase chain reaction, also termed quantitative PCR (qPCR) is a bio-molecular analysis technique used to determine the relative quantities of specific sequences of "target" nucleic acids present in biological samples. In PCR target sequences of deoxyribose nucleic acid (DNA) are amplified in quantity by their repeated exposure to cycles of optimum replication conditions, together with a reverse-transcriptase enzyme and DNA primers specific to the target DNA. Each cycle produces a doubling of the number of copies of the target DNA. This technique is adapted in qPCR by the addition of a DNA probe that becomes detectable under fluorescent light when bound to the target DNA. The signal generated by this probe increases in every cycle, as more copies of the target DNA are produced. The number of cycles before this signal

crosses a pre-set threshold can be used to determine the relative quantity (RQ) of target DNA in the original sample, when compared to a control.

3.7.1 qPCR methods

Synthesis of copy DNA (cDNA) from extracted RNA was performed using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA, USA) as per the manufacturers instructions. TaqMan real-time PCR was performed in collaboration with Siân Lax (The University of Birmingham, UK). TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) was used and reactions carried out on an ABI 7900 (Applied Biosystems, Carlsbad, CA, USA). Standard PCR conditions were used; 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. TaqMan β-actin primers and probe were designed using Primer Express computer software (Applied Biosystems, Carlsbad, CA, USA) and synthesised by Eurogentec (details are in table 3.5 together with primer-probe sets used). Relative quantification of target gene mRNA was calculated by directly analysing mRNA levels of the target gene to the β-actin mRNA in a duplex PCR.

Table 3.5 Primers and probes used for qPCR

Target mRNA	Forward primer	Reverse Primer	Probe	Fluoro-chrome
11β-HSD1 (HSD11B1)	Hs01547870_m1: purchased as a gene expression assay from Applied Biosystems, Carlsbad, CA, USA.			FAM
11β-HSD2 (HSD11B2)	Hs00388669_m1: purchased as a gene expression assay from Applied Biosystems, Carlsbad, CA, USA.			FAM
β-Actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT	ATCAAGATCATTGCTCCTCCTGAGCGC	Yakima yellow

Sequences are written from 5' to 3' terminal.

3.8 Efferocytosis assay

An assay of efferocytosis was used to quantify the rate at which alveolar macrophages took up apoptotic neutrophils under different experimental conditions. This technique was developed independently within the University of Birmingham lung inflammation laboratories specifically for the purpose of this investigation. The principle of the technique is that neutrophils are stained with a fluorescent dye, and then allowed to undergo apoptosis. These apoptotic cells are then introduced to macrophages that have been exposed to different experimental conditions. These macrophages engulf the apoptotic cells in the process of efferocytosis. The propensity of macrophages to engulf apoptotic neutrophils can be measured by determining the number of macrophages that have engulfed a neutrophil during a specific time period. The technique was initially developed using direct vision, with a fluorescent microscope, to ensure the concept worked. Subsequently, flow cytometry was used to distinguish macrophages that had engulfed a neutrophil, and therefore contained increased fluorescent material, from those macrophages that had failed to do so. Results of the assay were validated using the direct fluorescent light microscopy technique.

3.8.1 Preparation of neutrophils

Neutrophils were isolated from blood using discontinuous density gradients. An isotonic stock solution of percoll (Sigma-Aldrich, Poole, UK) 9:1 (volume: volume) with 1.5M sterile saline solution. This solution was then diluted further with 0.15M sterile saline solution to provide 78% and 54% percoll solutions. The discontinuous gradients were then prepared by the under-layering of 2ml of 54% percoll with 3ml of 78% percoll, in sterile 15ml centrifuge tubes.

Venous blood collected from peripheral venepuncture of healthy volunteers was collected into 7ml Lithium-heparin collection tubes. The blood was diluted to twice its original volume with 0.15M sterile saline solution. 7ml of diluted blood was layered carefully on top of the percoll gradients. The tubes were then spun in a centrifuge at 200g and 20^{o}c for 25 minutes, with no brake applied to the centrifuge. During this process blood separates into distinct phases. Uppermost being the diluted plasma, followed by an interface containing mononuclear cells. This was followed by the 54% percoll, then an interphase containing the polymorphic nuclear cells, before the 78% percoll. Bottom-most was the cell pellet containing erythrocytes. The neutrophils were aspirated from the interphase between 54% and 78% percoll using a Pasteur pipette and placed in a new sterile 15ml centrifuge tube. The cells were washed twice with sterile 0.15M saline solution, before being counted and a trypan blue exclusion test was performed as described in section 3.4.1.2. A differential cell count was also performed to ensure greater than 95% neutrophils were present in the sample. The cells were then suspended in a 1 x 10^{-6}M solution of Celltracker Green 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen, Paisley, UK) in warmed, serum free RPMI. This stain is taken up by viable cells, and converted by intra-cellular esterase enzymes to a compound that absorbs light at 492nm and emits at 517nm. They were then incubated at 37^{o}c in a 5% CO_2 atmosphere for 30 minutes to allow uptake and the intracellular modification of the fluorescent marker. After this period the cells were washed twice with warm serum free RPMI, spinning in a centrifuge at 1500g for 15 minutes, before finally re-suspending at a concentration of 2 x 10^6 cells/ml, in warm RPMI with 10% FCS. This suspension was placed in a cell culture flask in an incubator at 37^{o}c and a 5% CO_2 atmosphere for 20 hours to ensure adequate apoptosis. Apoptosis was established by

the morphological changes to the neutrophils: chromatin aggregation, and cytoplasmic vacuolation.[126]

It is known that neutrophils undergo apoptosis in a time dependent manner in vitro over 24 hours after extraction.[126] The PMN were left to undergo apoptosis for 20 hours as at this time the features of apoptosis were present in greater than 50% of cells without an increase in the number of cells taking up trypan blue and therefore no longer viable. *See figure 3.1*.

Figure 3.1Time course of viability (trypan blue exclusion test) and apoptotic features of neutrophils in vitro

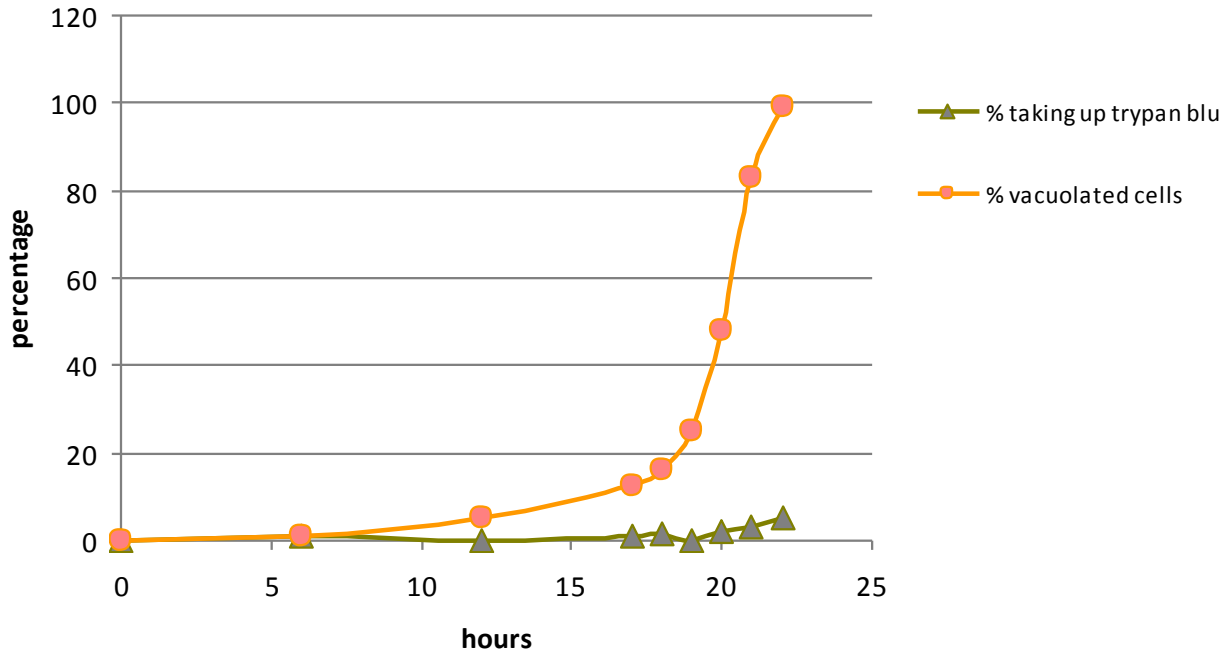
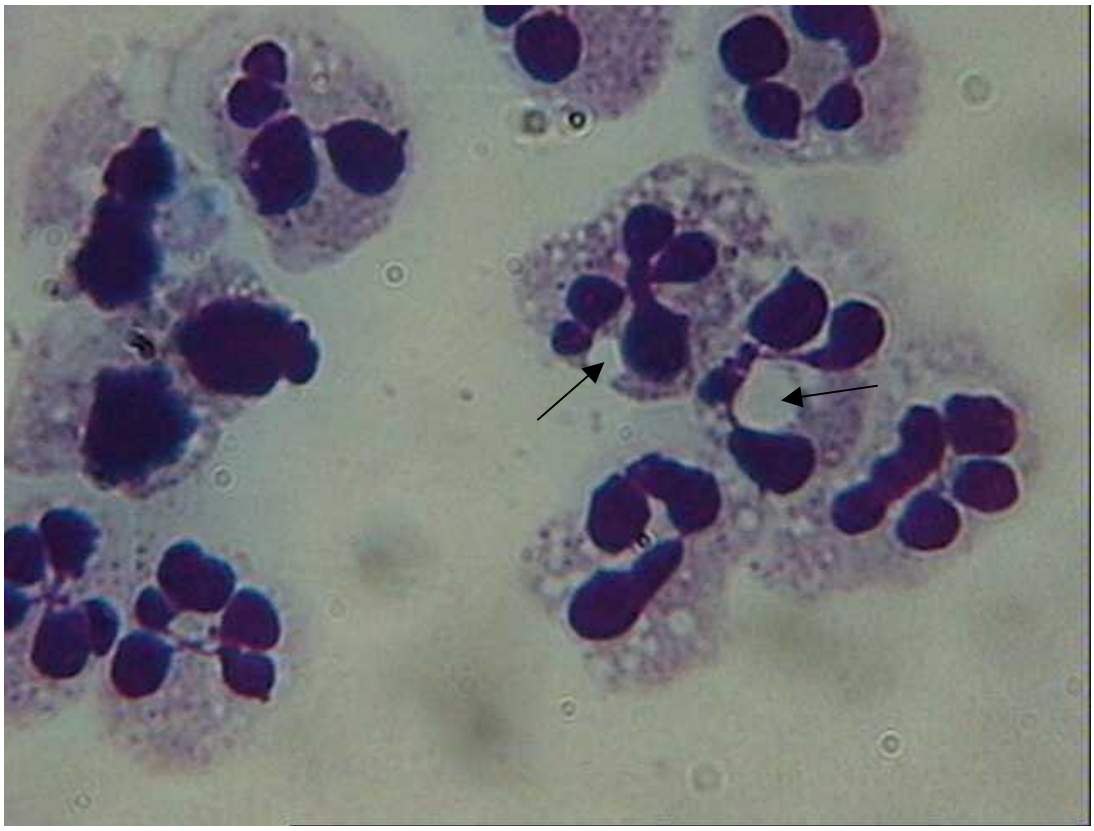


Figure 3.2 Vacuolation of apoptotic neutrophils



Arrows show large vacuoles in cytoplasm of apoptotic neutrophils

3.8.2 Flow cytometry assay of efferocytosis

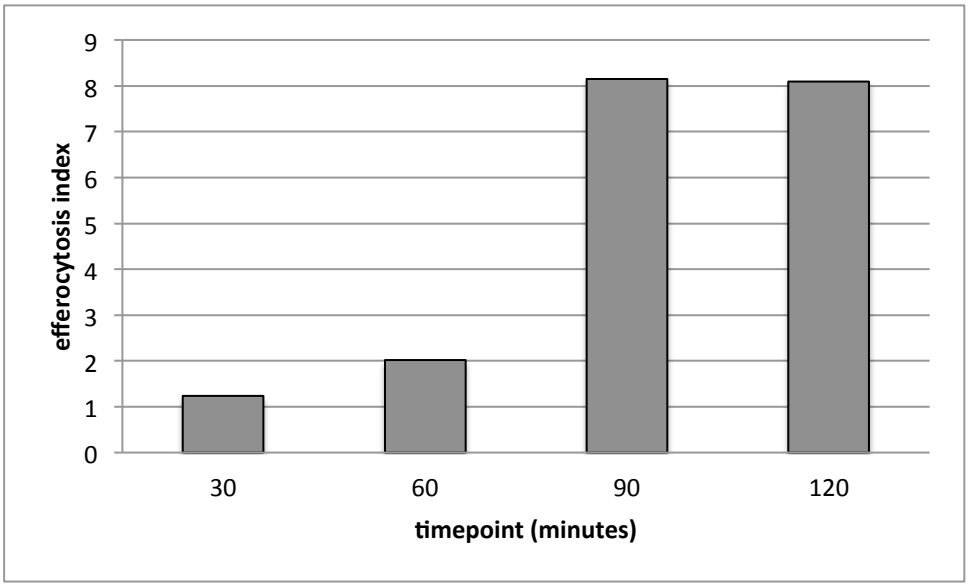
Alveolar macrophages cultured in 24 well tissue culture plates were washed with serum free medium, and the media aspirated from culture wells. The vial containing the apoptotic neutrophils was agitated to ensure the cells were in suspension, and this suspension added to the alveolar macrophages to provide a fourfold excess of neutrophils to macrophages. Blank control wells containing no macrophages, and control wells of macrophages without the addition of neutrophils were also prepared. Cells were incubated for 90 minutes in a 5% CO_2, 37^{o}C atmosphere to allow the phagocytosis of the neutrophils. This time point was found to be optimal by measuring efferocytosis at 30, 60, 90, and 120 minutes. *See figure 3.3* The media was then

aspirated, and the wells washed vigorously with room temperature PBS to remove any non-adherent and non-engulfed cells. 500ml of a 10% trypsin solution was then added to the wells, which were incubated for a further 15 minutes. The cells were then inspected to ensure that all macrophages had become loose from the well floor. If they had not, further 5-minute incubation stages were used until cells were all in suspension. The resultant cell suspension was placed in flow cytometry tubes, spun in a centrifuge for 5 minutes at 500g and 4^{o}C, and placed on ice.

Flow cytometry is a technique of cellular analysis that is used to determine the size, consistency and molecular phenotype of individual cells. Inside a flow cytometer a solution of cells is aspirated and carefully manipulated by the instrument so that individual cells are introduced in a stream to the detection apparatus. Lasers are then targeted on the cells and the distribution of the dispersion of the light provides information on the size and granularity of the cell (forward scatter and side scatter respectively). Lasers providing light of different wavelength can also be used to detect the presence of fluorescent markers on the surface or within the cell.

Pure macrophage preparation and pure neutrophil preparations were used to set gating of forward and side scatter on the flow cytometer, to ensure that only macrophages were included in analysis. The FL-1 laser was used to detect the fluorescence of the CMFDA present within macrophages that had engulfed a neutrophil. Due to the normal fluorescence of macrophages a threshold fluorescence level of detection above 2% was used. A minimum of 10000 events (cells gated as macrophages) were counted for each experimental condition. The proportion of macrophages that had fluorescence levels above the threshold, and therefore had engulfed an apoptotic neutrophil was used as an efferocytosis index (EI).

Figure 3.3 Time course of efferocytosis in un-stimulated alveolar macrophages



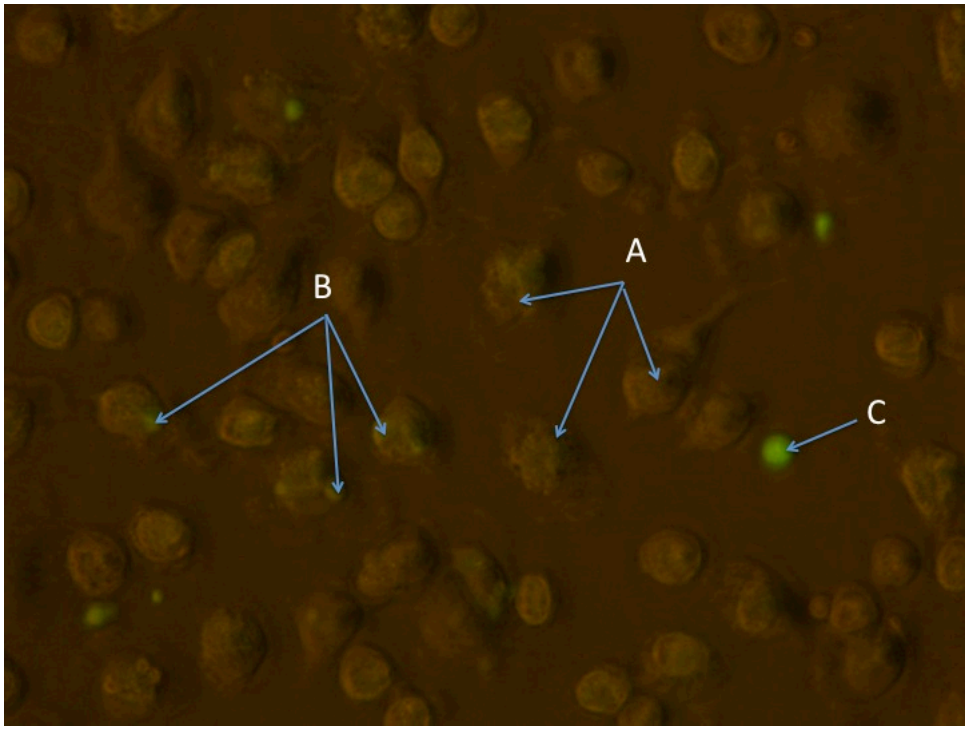
3.8.3 Validation of assay

The flow cytometric assay was validated and developed using a direct fluorescent light microscopy technique. In this method after the neutrophil solution had been removed from the macrophages and the wells washed at the end of the efferocytosis stage, 5 random fields of each cell culture well were then promptly photographed under a fluorescent microscope. *See figure 3.4* For each culture condition the total number of alveolar macrophages in the photographs was counted. The number of alveolar macrophages that had demonstrated the capacity to engulf an apoptotic neutrophil was then established by counting the number of macrophages that contained a fluorescent body. The number of macrophages that had engulfed a neutrophil, was divided by the total number of macrophages, to give the EI. Differences between the two methods were consistently less than 10%

The coefficient of variation (CV) was determined for both intra-assay and inter-assay variability. Intra-assay CV was assessed by repeating one condition four times and the EI then being divided by the standard deviation of the mean, then multiplied by 100. The

inter-assay CV was calculated by comparing control EI across eight consecutive experiments. The intra assay CV was 3.7%, however the inter-assay CV was large at 19.0%.

Figure 3.4 Sample photograph of efferocytosis assay



This photograph shows cultured alveolar macrophages (labelled ”A”) to which apoptotic neutrophils stained with CMFDA have been added. Many of these macrophages have engulfed a neutrophil, which can be seen under fluorescent light as a green body within the macrophage (labelled “B”). Also present are neutrophils that have not been engulfed (labelled C).

3.9 11β-hydroxysteroid dehydrogenase (11β-HSD) activity assay

In order to measure the alveolar macrophage conversion of cortisone to cortisol by the oxo-reduction reaction of 11β-HSD, and the conversion of cortisol to cortisone by the dehydrogenase reaction of 11β-HSD, a thin layer chromatography (TLC) assay was used.

Alveolar macrophages from lung resection or bronchoscopy samples were cultured as monolayers in 24 well plates, with 500,000 cells per well. At initiation of the assay the culture medium was aspirated and replaced with warmed serum free media containing a 10^{-7}M concentration of either cortisol or cortisone. 2ml of tritiated cortisol or cortisone was also added to each well. Cells were cultured in a 5% CO_2 atmosphere at 37^{o}c for 12-16 hours. At this point the cell culture supernatant was transferred to glass test tubes and either analysed immediately or sealed and stored at -20^{o}c for later analysis. Samples, if frozen, were thawed to room temperature prior to analysis. 5ml of dichloromethane was then added to the test tubes, which were then sealed and agitated for 5minutes to ensure adequate mixing of the aqueous and solvent layers. The tubes were then spun in a centrifuge at 1800g for 15 minutes. The top, aqueous phase was then aspirated off using glass Pasteur pipettes, and discarded. The remaining solvent was evaporated to dryness on a heating block at 50^{o}c, using an air blower. The dry samples were then re-suspended in 80ml of dichloromethane to the glass test tubes, and vortexing. TLC silica running-plates were pre-prepared by spotting mixed non-radioactive cortisol and cortisone onto the points at which the samples were to be placed, in order to ensure that the samples run out and separate adequately. Blank controls of cortisol and cortisone were also prepared. Re-suspended samples were spotted out onto the running plates, which were then placed in a TLC running tank containing the mobile phase solution. This was a ethanol:chloroform (8:92) mixture. The tank was left for 90 minutes to ensure adequate separation of cortisol and cortisone, the plates removed, and allowed to dry in a fume cupboard. The plates were read on a Bioscan TLC plate reader (Bioscan, Washington, DC, USA) scanning each line for 10 minutes. Relevant peaks were identified by checking the peaks against blank cortisol and cortisone controls.

Calculation of conversion was based on the counts present in the individual peaks of activity. The counts in the two areas that corresponded to cortisone and cortisol were summated and the conversion of steroid from one to the other calculated as the percentage of the original added steroid to its metabolite, as a percentage of the total area of the steroids. Calculation of conversion is expressed in pmol/million cells/hour to allow direct comparison of steroid conversion.

3.9.1 Validation of the assay

The coefficient of variation (CV) was determined for both intra-assay and inter-assay variability. Intra-assay CV was assessed by the 11β-HSD activity in one sample being measured four times on a single plate and divided by the standard deviation of the mean, and multiplied by 100. The inter-assay CV was calculated from control conditions across eight experiments. The intra-assay CV was 3.5%, and the inter-assay CV was 12.6%.

3.10 Protein concentration measurement

Protein concentration of BALF and plasma was measured using the Pierce® BCA Protein Assay kit. (Pierce biotechnology, Illinois, USA). This method is based on the ability of bicinchoninic acid (BCA) to detect the reduction of Cu^{2+} to Cu^{+} in an alkaline environment containing sodium potassium tartrate (the biuret reaction). Cu^{2+} ions produce a violet colour in the presence of peptide bonds in this reaction. The intensity of colour produced is proportional to the concentration of peptide present according to the Beer-Lambert law.

A standard curve was produced for each assay using seven serial dilutions of known concentrations of bovine serum albumin (BSA), and a blank absorbance using distilled water. The individual samples were diluted to an appropriate degree and then 25ml diluted sample added to a 96 well plate with 200ml of the working solution provided in the commercially available kit. The plate was covered and incubated for 30 minutes at 37^{o}c, and subsequently the absorbance at 570nm was calculated using a Synergy HT microplate spectrophotometer (Biotek, Winooski Vermont). Mean absorbance of the blank wells was subtracted from the absorbance of standards and samples, and regression analysis of the absorbance curve constructed from standards performed. The result equation was then used to calculate protein concentration of each sample.

3.10.1 Validation of the assay

The coefficient of variation (CV) was determined for both intra-assay and inter-assay variability. Intra-assay CV was assessed by the protein concentration in one sample being measured four times on a single plate and divided by the standard deviation of the mean, and then multiplied by 100. The inter-assay CV was measured in a single sample across four consecutive plates. The intra assay CV was 1.7%, and the inter assay CV was 4.0%.

3.11 Urea assay

Urea concentrations were measured in BALF and plasma using a commercially available kit. (Quantichrom urea assay, BioAssay systems, Hayward, CA USA). This technique uses the improved Jung method, in which urea reacts specifically with the chromogenic reagent to form a coloured compound.[201] The intensity of colour is proportional to the

concentration of the chromatic compound, and therefore to the concentration of urea, according to the Beer-Lambert law.

Analysis of BALF samples was performed by placing 50ml of each of the samples together with 200ml of the working solution from the commercially available kit in a well of a 96-well plate. A blank well containing 50ml distilled water and 200ml of reaction solution was used for calibration. Duplicates were used for each sample and blank. The plate was then incubated at room temperature, protected from light for 50 minutes. After this period the plate was read using a Synergy-2 microplate spectrophotometer (Biotek, Winooski Vermont) at an absorbance of 520nm. The concentration of urea in each sample was then calculated using the following formula:

$$\text{Concentration} = \frac{\text{OD(sample)-OD(blank)}}{\text{OD(standard)-OD(blank)}} \times n$$

Where n=concentration of standard (5ng/ml for BALF, 50ng/ml for plasma).

Concentrations were then converted into mmol/l.

3.12 Enzyme linked immunosorbant assays (ELISAs)

A sandwich type enzyme linked immunosorbant assay (ELISA) technique is a method of calculating the concentration of a specific substance in a given sample. The principle of this technique is that the wells of a polystyrene microplate are coated with a “capture” antibody specific to the substance under investigation. A sample containing the investigation substance is then added to the wells, allowed to bind to the capture antibody and then any unbound substance washed off. An enzyme bound detection antibody is then added which binds to the sample-capture antibody complex. After an

incubation period unbound detection antibody is washed off. A substrate solution containing hydrogen peroxide and tetramethylbenzidine is then added. This solution is converted to a blue colour by the enzyme bound to the detection antibody. The reaction is stopped by the addition of a solution of sulphuric acid, which turns the solution yellow. The intensity of this colour is proportional to the concentration of the investigation substance in the sample.[202]

3.12.1 HMGB-1 ELISA

High mobility group box 1 (HMGB-1) concentrations were measured in plasma and BALF using a commercially available ELISA kit (Shino-Test Corporation, Kanagawa, Japan). The kit contained a 96-well assay plate that is pre-coated with an anti-HMGB-1 capture antibody. To this plate were added 100ml of assay diluent and 10ml of sample and standards. The standard range of concentrations was created by serial dilution to of an 80ng/ml concentration of HMGB-1 to produce a range of 80 to 2.5ng/ml, with each concentration half that of its predecessor. These were also added to the assay plate. A blank sample was also measured, using sample diluent from the ELISA kit. Duplicates of each sample and standard were measured. The addition of reagents and samples was completed within 15 minutes, after which the assay plate was covered with an adhesive strip, and incubated overnight at 37^{o}c. The assay plate was washed 5 times on an automated plate washer, each time using 400ml of the assay kit wash buffer. 100ml of the assay kit anti-HMGB-1 detection-antibody solution was then added to each well. The assay plate was re-covered and incubated at room temperature for two hours before being washed 5 times with 400ml of the assay kit wash buffer as before. 200ml of a hydrogen peroxide and tetramethylbenzidine mixture was then added to each well, the plate covered again, protected from light and incubated for 30 minutes at room

temperature. 100ml of the assay kit stop-solution was then added to each well and the plate shaken briefly. The optical density of each well was measured at 450nm using a Synergy-2 microplate spectrophotometer (Biotek, Winooski, Vermont, USA). Mean absorbance of the blank wells was subtracted from the absorbance of standards and samples, and regression analysis of the standard absorbance curve performed. The result equation was used to calculate the HMGB-1 concentration of each sample.

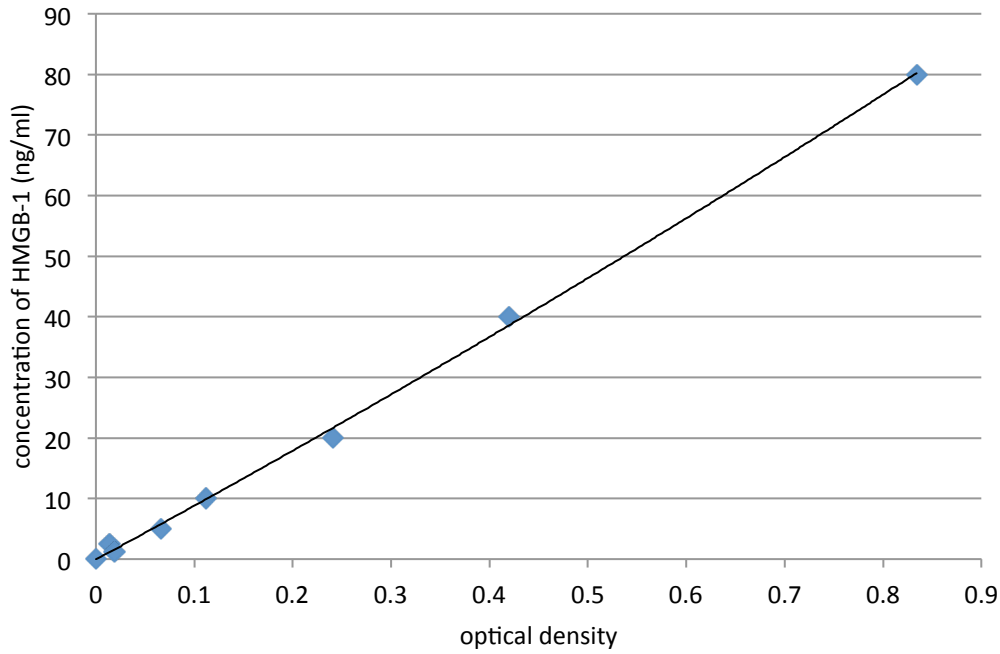
3.12.1.1 Validation of the assay

The lower limit of detection was determined by adding two standard deviations of the mean optical density of ten zero standard replicates and calculated the corresponding HMGB-1 concentration. Intra-assay CV was assessed by the HMGB-1 concentration in one sample being measured four times on a single plate, divided by the standard deviation, and then multiplied by 100. The inter-assay CV was measured in a single sample across three consecutive plates. The percentage recovery was measured by adding a known quantity of HMGB-1 to a sample and calculating the resulting concentration. This was then compared to the predicted value to obtain the proportion recovered. The lower limit of detection, Intra-assay and inter-assay CV, and the percentage recovery are shown in table 3.6 with an example standard curve in figure 3.4

Table 3.6 HMGB-1 ELISA validation statistics

Lower limit of detection (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)	Recovery (%)
1.0ng/ml	6.2% (n=4)	9.0% (n=3)	98.57%

Figure 3.5 Sample standard curve for HMGB-1 ELISA



3.12.2 RAGE ELISA

The receptor for advanced glycation end products (RAGE) concentrations in plasma and BALF were measured using a commercially available ELISA kit (R&D systems, Minneapolis, Minnesota, USA). This kit contains a 96-well assay plate that is pre-coated with an anti-RAGE capture antibody. To this plate were added 100ml of the assay diluent and 50ml of standards and sample that had been diluted to an appropriate degree for the range of the assay. The standard range of concentrations was created by serial dilution of a 5000pg/ml concentration of HMGB-1 to produce a range of 5000 to 78pg/ml, with each concentration half that of its predecessor. These concentrations were also added to the assay plate. A blank sample was measured, using sample diluent from the ELISA kit. Duplicates of each sample and standard were measured. The addition of reagents and samples was completed within 15 minutes, after which the assay plate was covered with an adhesive strip and incubated for 2 hours at room temperature. The

assay plate was then washed 4 times on an automated plate washer, each time using 400ml of the assay kit wash buffer, ensuring complete removal of liquid after each wash. 100ml of the assay kit anti-RAGE detection-antibody solution was then added to each well. The assay plate was re-covered and incubated at room temperature for two hours before being washed 4 times with 400ml of the assay kit wash buffer as before. 200ml of a substrate mixture of hydrogen peroxide and tetramethylbenzidine was then added to each well, the plate covered again, protected from light and incubated for 30 minutes at room temperature. 50ml of the assay kit stop-solution was then added to each well and the plate shaken briefly. The optical density of each well was measured at 450nm using a Synergy-2 microplate spectrophotometer (Biotek, Winooski, Vermont, USA). Corrections were made at 540nm absorbance. Mean absorbance of the blank wells was subtracted from the absorbance of standards and samples, and regression analysis of the standard absorbance curve performed. The result equation was used to calculate the RAGE concentration of each sample.

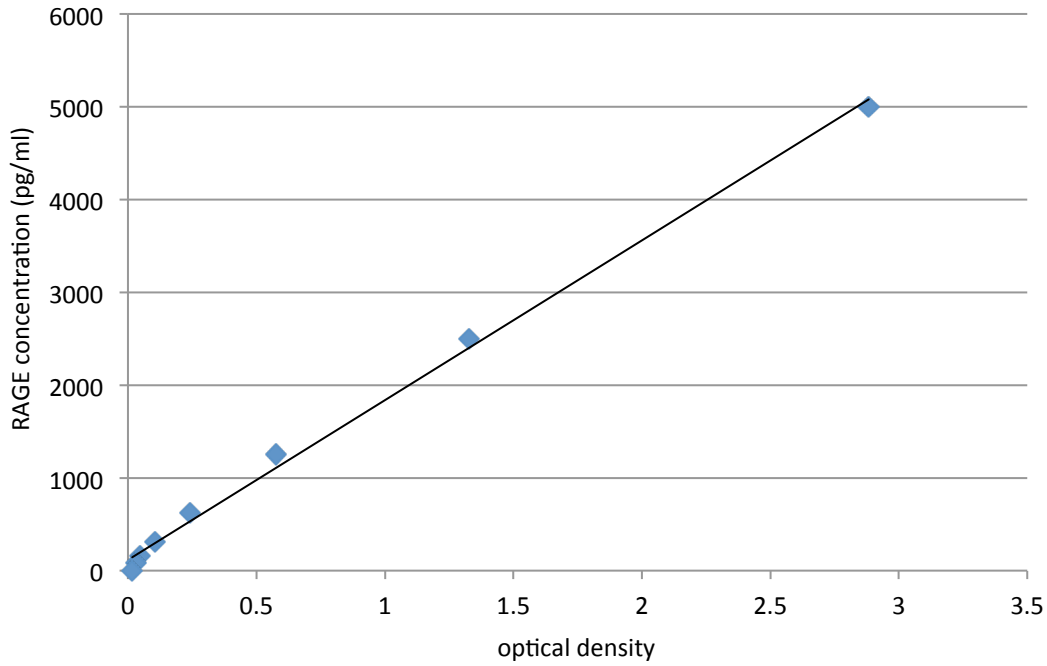
3.12.2.1 Validation of the assay

The lower limit of detection, inter-assay CV, intra-assay CV and percentage recovery were calculated as described for HMGB-1 above. The results of these calculations are shown in table 3.7 and a sample standard curve in figure 3.5

Table 3.7 RAGE ELISA validation statistics

Lower limit of detection (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)	Recovery (%)
95pg/ml	4.1% (n=4)	10.6% (n=3)	96.25%

Figure 3.6 Sample standard curve for RAGE ELISA



3.13 Liquid chromatography/mass spectrometry

Liquid chromatography/mass spectrometry (LC/MS) is an analytical chemistry technique that consists of two parts, the liquid chromatography system and a mass analyser (triple quadrupole mass spectrometer). The LC system separates a mixture of analytes based on their polarity using two liquids (such as methanol and water). Specific compounds will elute from the column at specific times which equate to a specific concentration of organic solvent. The elute from the column is then sampled into the mass spectrometer due to differences in pressure, temperature and voltage; this produces an ionised (charged) compound. The mass analyser then detects these compounds based on their mass-to-charge (m/z) ratio. To increase sensitivity to a specific compound that compound can be fragmented and specific daughter ions produced using MS/MS mode.

Two daughter ions are generally required for accurate quantitation of a compound; these MS/MS mass transitions are usually specific to the compound of interest. By comparison to the retention time and mass transitions of a reference compound it is possible to identify and quantify compounds within a mixture using LC/MS/MS.

Mass spectrometry quantification of steroids in BALF and plasma was performed by Dr. Angela Taylor at the University of Birmingham, UK.

3.13.1 Sample preparation

Steroids were extracted from BALF samples via a solid phase extraction method using a C18 SPE cartridge. The cartridge was washed with 1mL methanol followed by 1ml water. 5ml of the BALF was then applied to the cartridge after the addition of 20ml of internal standard ([9,11,12,12-2D_4]-cortisol (cortisol-D_4 1000ng/mL solution)). The column was subsequently washed with 1mL of water before the steroids were eluted with 2ml of methanol. The methanol elute was then evaporated to dryness under nitrogen at 55°C, and reconstituted in 100µL of a 50:50 methanol/water solution and transferred to a low volume LC/MS vial for analysis.

Steroids were extracted from plasma samples via a liquid-liquid extraction method. Plasma sample were prepared by adding 20µL of cortisol-D_4 (1000ng/mL solution) as an internal standard to 200ml of plasma. This was then heated at 55^{o}c for 5 minutes, vortexed and then 1ml of Methyl-tert-butyl-ether (MTBE) added. The solution was then mixed and centrifuged at 1200rpm for 5 minutes, due to differences in polarity the steroids move into the organic phase. The MTBE (organic) layer was then removed and evaporated to dryness. The samples were then reconstituted in 100µL of 50:50 methanol/water solution and transferred to a low volume LC/MS vial for analysis.

3.13.2 Mass spectrometry parameters

All samples and calibrators were prepared in 50/50 methanol/water (LC/MS grade), 20µL of each sample was analysed. A Waters Xevo mass spectrometer with Acquity uPLC system was used fitted with a HSS T3, 1.8µm, 1.2x50mm column. The column temperature was maintained at 60°C throughout the experiments. The following optimised settings were used: an electrospray source in positive ionisation mode, capillary voltage 3.6kV, cone voltage 24-26V, collision energy 16-30eV (depending on the mass transition), a source temperature of 150°C, and a desolvation temperature of 600°C.

A gradient system of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) was applied. The starting gradient was 45% B, increasing to 47% over 1 minute, 57% B at 2 minutes, 70% B at 2.5 minutes, B was increased to 98% at 4 minutes. This concentration was then held for 1 minute to wash the column. Following this the column was equilibrated for 2 minutes at 45% B as the starting gradient.

Each steroid was quantified by comparison to a calibration series with respect to the internal standard cortisol-D_4. The assay was linear over a range of 0.05 to 100ng/ml for cortisol (R^2=0.997) and 1 to 100ng/ml for cortisone (R^2=0.999). Intra-assay reproducibility was <10%. Quantitation was completed using TargetLynx software version 4.1 (MassLynx 4.1). The mass transitions for each steroid are described in table 3.8.

Table 3.8 Specific mass transitions for each steroid in LC/MS

Steroid	Quantifier ion (m/z)	Qualifier ion (m/z)	Cone voltage (V)	Collision energy (eV)
Cortisol	363.3 > 121.1	363.3 > 97.1	26, 26	23, 30
Cortisone	361.2 > 163.1	361.2 > 105.1	28, 28	28, 46
Cortisol-d4 (Internal standard)	367.2 > 121.1	367.2 > 327	24, 24	28, 16

3.14 Data handling and analysis

3.14.1 Data handling

Data were collected prospectively on specifically designed proformas incorporated into case report forms (CRFs). Data was collected from the daily ICU bedside charts and the patient's medical notes. These CRFs were stored securely in locked rooms in security pass protected rooms in the MIDRU building at Birmingham Heartlands Hospital. Once collected, data was transferred to electronic format for analysis, ensuring patient anonymity. The results of laboratory analysis of data was stored in an anonymised form electronically, together with the appropriate methodology. Electronic data was stored securely in password protected encrypted format. Data integrity was validated by the random selection of 1 in 10 of raw data entry cells and comparing these with the appropriate source data.

3.14.2 Data analysis

Statistical analysis was done using the commercially available software packages SPSS 17.0 and Sample Power 3 (both IBM, Somers, New York, USA), and Sigmaplot 11.0 (Systat Software, San Jose, California, USA).

Data was assessed for normality using the Kolmogorov-Smirnov test. Normally distributed data was analysed using two-tailed t-tests to compare means, and analysis of variance (ANOVA) test for multiple means. Non-parametric data has been analysed using Mann Whitney tests, and ANOVA on ranks for comparison of multiple means.

The test for statistical significance was set at a probability of $p=0.05$. Changes to the level of statistical significance to account for multiple analyses have not been made in the course of this study. Such corrections (for example the Bonferonni correction) are intended for use where a number of statistical comparisons are made with multiple null-hypotheses using the same data, in order to avoid type 2 error.[203] Much debate and controversy on this subject can be found in the literature.[204-206]

4 Glucocorticoid concentrations and the cortisol: cortisone ratio in the alveolar space and plasma of patients with ARDS

4.1 Abstract

Introduction: Acute respiratory distress syndrome (ARDS) is an inflammatory disease of the lung seen in critically ill patients. The glucocorticoid hormone cortisol is an endogenous anti-inflammatory hormone whose concentration is raised in the plasma of patients with ARDS and critical illness. Cortisol and cortisone are inter-converted in peripheral tissues by 11β-hydroxysteroid dehydrogenase (11β-HSD). Increased expression of the type 2 isoenzyme, which deactivates cortisol, has previously been seen in the lungs of patients who have died with ARDS. Alveolar concentrations of glucocorticoids and the influence of 11β-HSD activity on these levels have not previously been examined.

Methods: Invasively ventilated adult patients with ARDS and patients with a systemic inflammatory response, who were considered at risk of ARDS, were included. Bronchoscopy was performed and blood collected at recruitment to the study and repeated at day 4 in the patients with ARDS. Cortisol and cortisone concentrations were measured by mass spectrometry in plasma and broncho-alveolar lavage fluid (BALF) and the cortisol: cortisone ratio calculated as an indicator of the activity of 11β-HSD in plasma and the alveolar space. Results from BALF were corrected by urea dilution method to estimate epithelial lining fluid (ELF) concentrations. Associations of these measures with clinical and laboratory markers of disease severity were then tested.

Results: Samples were available from 68 patients with ARDS and 5 at-risk controls. The cortisol concentration in the ELF was higher at day 0 of ARDS compared to that in the at-risk group (median= 110.64 vs. 49.98pg/ml, n=50, p=0.027). At onset of ARDS a positive relationship existed between severity indices and concentration of cortisol in the ELF and the SOFA score (n=45, ρ=0.362, p=0.015), the SAPS-2 score (n=45, ρ=0.381,

p=0.010), and the APACHE-2 score (n=45, ρ=0.393, p=0.008). Negative associations of protein permeability with ELF cortisol (ρ=-0.320, n=44, p=0.034), ELF cortisone (ρ=-0.709, n=19, p=0.001) and plasma cortisol (ρ=-0.329, n=51, p=0.018) were present at day 0 of ARDS. Negative associations of BALF neutrophil concentration with both ELF cortisol concentration (ρ=-0.351, n=41, p=0.024) and plasma cortisol concentration (n=48, ρ=-0.297, p=0.041) were detected. Increased cortisol: cortisone ratios were detected in the alveolar space of patients at both day 0 (median= 4.16 vs. 0.72, p=0.012) and day 4 of ARDS (median= 4.60 vs. 0.72, p=0.019) compared to at-risk control subjects, suggesting increased conversion of cortisone to cortisol. Positive associations between the alveolar cortisol: cortisone ratio and disease severity indices were seen.

Conclusion: Cortisol concentrations are higher in the alveolar space of patients at onset of ARDS and are a marker of global disease severity. Negative associations of cortisol with cellular inflammation and protein permeability suggest cortisol is responsible for decreased cellular inflammation and improved membrane integrity. No evidence for increased 11β-HSD deactivation of cortisol was found.

4.2 Introduction

The acute respiratory distress syndrome (ARDS) is a severe inflammatory condition of the lung seen in critically ill patients and characterised by dysregulated inflammatory mechanisms.[8] Glucocorticoid steroids are important endogenous anti-inflammatory agents produced in response to inflammation by the adrenal gland after activation of the hypothalamic-pituitary-adrenal (HPA) system. These steroids bind to the intra-cellular glucocorticoid receptor which then migrates to the nucleus and promotes the

transcription of anti-inflammatory genes and suppresses the transcription of pro-inflammatory genes. Glucocorticoids thus have a key role in promoting the resolution of acute inflammation.[30] The biology of the HPA axis in critical illness is complex, and can become dysregulated; potentially prolonging illness. Causes of dysregulated HPA function include tissue resistance to glucocorticoid and altered glucocorticoid tissue distribution. [29] Although total concentrations of glucocorticoids are increased in the plasma of critically ill patients,[67, 207-209] very little has been published on the concentration or metabolism of these steroids in the alveolar space of patients with ARDS.

11β-hydroxysteroid dehydrogenase (11β-HSD) inter-converts cortisone and cortisol. The type 1 isoenzyme (11β-HSD1) in-vivo primarily activates cortisone to cortisol, whilst the type 2 isoenzyme (11β-HSD2) inactivates cortisol to cortisone. Increases in type 1 isoenzyme expression and activity have been described in many different tissues in response to inflammatory stimuli.[51, 54, 62, 63, 65, 210] This increase in the production of active cortisol causes a local tissue amplification of glucocorticoid effects,[45] that will promote the resolution of inflammation.[75] Suzuki et al have reported post mortem studies of patients dying with ARDS showing increased expression of 11β-HSD2 in their lungs,[69] and the administration of carbenoxolone in an animal model of ARDS to block 11β-HSD activity was found to promote the effects of endogenous glucocorticoids.[72] This has led to the speculation that decreased bioavailability of active glucocorticoids within the lungs of patients with ARDS due to inactivation of cortisol by 11β-HSD2 was responsible for steroid resistance and prolonged inflammation.[69, 72] The ratio of cortisol to cortisone present in plasma and tissues is thought to represent global and local 11β-HSD activity

respectively. The plasma cortisol: cortisone ratio in patients with critical illness is increased.[67]

Glucocorticoids are transported in the blood bound primarily to corticotrophin binding globulin and albumin. In critical illness concentrations of both these proteins drop, and the plasma concentration of free cortisol rises.[211, 212] Furthermore, damage to the alveolar-capillary membrane in ARDS leads to an increased permeability to these proteins,[213, 214] and the intra-alveolar presence of these proteins may therefore increase further the pulmonary concentration of glucocorticoids in ARDS.

The aim of this study was to determine the concentrations of the glucocorticoids cortisone and cortisol within the alveolar space and plasma of patients with ARDS, and to examine their relationship with markers of alveolar protein permeability, cellular inflammation, disease severity and mortality. We also aimed to test the hypothesis that in ARDS increases in the expression and activity of 11β-HSD2 within the lung would alter the local cortisol: cortisone ratio, and to test the relationship of this measure to disease severity and clinical outcomes.[67]

4.3 Methods

The methods used in this chapter are in line with those set out in chapter 3 and are summarised below:

4.3.1 Design of the investigation

This investigation was designed as an observational study of the concentration of cortisol and cortisone, and the ratio between these two steroids within the plasma and alveolar space of patients with ARDS. We also planned to examine the relationships

between these steroid parameters and markers of cellular inflammation (neutrophil count), alveolar membrane dysfunction (protein permeability index) as well as clinical severity indices (the Murray lung injury score,[147] the SOFA score,[191] the SAPS-2 score[190] and the APACHE-2 score[189]) and mortality. ARDS was diagnosed by the European-American consensus conference (EACC) criteria.[3] Patients with ARDS not being treated with corticosteroids were recruited as part of either the BALTI-1 study[95] or the bronchoscopic sub-study of the BALTI-2 trial. Patients defined as being at risk of ARDS were those meeting criteria for the systemic inflammatory response syndrome (SIRS),[215] but not meeting the EACC criteria for ARDS, and were enrolled in either the resolution of inflammation in ARDS investigation, or the BALTI-1 investigation.

4.3.2 Sample processing and laboratory technique

For patients with ARDS, bronchoscopy and Broncho-alveolar lavage fluid (BALF) collection was performed on the day of diagnosis of ARDS (day 0) and repeated after 4 days (day 4). For normal subjects and patients at risk of ARDS, BALF collection was performed at enrolment to the study. Plasma was extracted from blood collected at the same time points.

Samples were immediately frozen at -80 degrees centigrade for subsequent analysis. Cortisol and cortisone concentrations were measured using mass spectrometry. The cortisol: cortisone ratio was then calculated from these results. Protein concentrations were measured using a commercially available assay (Biorad protein assay, Biorad laboratories, Hercules, California, USA). The passage of protein through the alveolar membrane was then estimated by calculating the protein permeability index using the formula below:[214]

Protein permeability index= (BALF protein (mg/l)/plasma protein (mg/l))*100

Epithelial lining fluid (ELF) concentrations were estimated using the urea dilution method: Urea concentration was determined in BALF and plasma using a commercially available kit (Quantichrom urea assay, bioassay systems, Hayward, California, USA). Concentrations of glucocorticoids in ELF were then estimated by the following equation:[216]

ELF conc. = BALF conc. x (plasma urea conc.) / (BALF urea conc.)

This estimate cannot be used for the cortisol to cortisone ratio, as it returns the BALF ratio only; the dilution factor being both a numerator and a denominator in the calculation of the ELF cortisol: cortisone ratio.

4.3.3 Statistical analysis

Statistical analysis was done using the statistical software SPSS (IBM, Somers, New York, USA). Normality was tested using the Kolmogorov-Smirnov test. Normally distributed data were further analysed using the parametric techniques t-tests and ANOVA. If data were not normally distributed analysis was done using the non-parametric techniques, Mann Whitney and ANOVA by ranks (Kruskal Wallis). Linear associations were tested using Pearson's correlation coefficient for parametric data, and Spearman's correlation coefficient for non-parametric data. All significance tests are done using an α-value of 0.05, using two-tailed tests, unless stated otherwise within the text.

4.4 Results

4.4.1 Patient characteristics

40 adult patients with ARDS were recruited to the BALTI-1 investigation and had BALF collected at baseline. Cortisone concentrations are not available for BALF or ELF for subjects recruited to the BALTI-1 investigation as the relevant technique was unavailable at the time of this investigation. A further 24 patients were recruited to the BALTI-2 study, of which 20 patients had BALF collected at day 0 and 21 had BALF collected at day 4 of ARDS. 5 patients who were at risk of ARDS but who had not developed this condition were recruited to the ARDS resolution study. Plasma was collected from all patients undergoing bronchoscopy. Biological samples were available for a total of 68 subjects.

Of the 68 patients, 41 (57.4%) were male. The mean age of subjects was 62.5 (SD=16.00). The causes of lung injury are documented in table 4.1.

Table 4.1 Predisposing factors for lung injury in the ARDS and at-risk groups

Predisposing condition	**ARDS patients**	**"At risk" patients**
Pneumonia	22 (34.9%)	1 (20%)
Sepsis	29 (46.0%)	3 (60%)
Aortic aneurysm repair	4 (6.3%)	-
Chest trauma	3 (4.7%)	1 (20%)
Pancreatitis	1 (1.6%)	-
Transfusion related lung injury	1 (1.6%)	-
other	3 (4.7%)	-

4.4.2 Glucocorticoid concentrations between day 0 and day 4 of ARDS and patients at risk of ARDS

As the day 0 and day 4 samples were repeated measures from the same subjects, and the at-risk group was not, separate analysis with appropriate statistical repeated measures was undertaken to determine whether differences were present: *See tables 4.2, 4.3 and 4.4*.

No difference in the plasma or ELF concentrations of cortisol was detectable between subjects at day 0 or day 4 of ARDS. Similarly, no difference in the cortisone concentrations was detected between these time-points in either plasma or ELF. The cortisol concentration was higher in the plasma at day 0 of ARDS compared to patients at risk of ARDS (median=551.15 vs. 370.17 pg/ml, U(61)=59.5, p=0.031), and was also higher in the plasma at day 4 of ARDS compared to the at risk group (median= 536.36 vs. 370.17pg/ml, U(50)=45.0, p=0.027). *See figure 4.1*.

The cortisol concentration in the ELF was also higher at day 0 of ARDS compared to that measured in the at-risk group (median= 110.64 vs. 49.98pg/ml, n=50, U=81.0, p=0.027). *See figure 4.2*. No differences in cortisone concentrations were detected between the at-risk group and subjects at either day 0 or day 4 of ARDS in either ELF or plasma

Table 4.2 Differences between glucocorticoid concentrations between day 0 and day 4 of ARDS

	Median cortisol concentration (pg/ml)		Wilcoxon signed rank test	
	Day 0 (n, range)	**Day 4 (n, range)**	**Z**	**P value**
plasma	551.15 (56, 84.25-2716.00)	536.36 (45, 4.63-2343.00)	-0.121	0.904
ELF	110.64 (45, 25.33-1831.24)	188.55 (37, 9.97-3153.02)	-0.566	0.572
	Median cortisone concentration (pg/ml)			
	Day 0 (n, range)	**Day 4 (n, range)**		
plasma	63.20 (56, 20.83-250.00)	47.26 (44, 19.10-179.00)	-1.055	0.291
ELF	26.86 (20, 9.84-167.62)	55.53 (21, 9.22-236.35)	-1.590	0.112

(*indicates results that have met pre-defined criteria for statistical significance (a=0.05))

Table 4.3 Differences between glucocorticoid concentrations between day 0 of ARDS and at risk group

	Median cortisol concentration (pg/ml)		Mann- Whitney test	
	Day 0 (n, range)	**At risk (n, range)**	**U**	**P value**
plasma	551.15 (56, 84.25-2716.00)	370.17 (5, 145.03-379.32)	59.5	0.031*
ELF	110.64 (45, 25.33-1831.24)	49.98 (5, 11.00-445.63)	81.0	0.027*
	Median cortisone concentration (pg/ml)			
	Day 0 (n, range)	**At risk (n, range)**		
plasma	63.20 (56, 20.83-250.00)	38.89 (5, 30.56-58.33)	67.0	0.055
ELF	26.86 (20, 9.84-167.62)	96.39 (5, 15.49-391.26)	28.0	0.148

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Table 4.4 Differences between glucocorticoid concentrations between day 4 of ARDS and at risk group

	Median cortisol concentration (pg/ml)		Mann- Whitney test	
	Day 4 (n, range)	**At risk (n, range)**	**U**	**P value**
plasma	536.36 (45, 4.63-2343.00)	370.17 (5, 145.03-379.32)	45.0	0.027*
ELF	188.55 (37, 9.97-3153.02)	49.98 (5, 11.00-445.63)	68.0	0.362
	Median cortisone concentration (pg/ml)			
	Day 4 (n, range)	**At risk (n, range)**		
plasma	47.26 (44, 19.10-179.00)	38.89 (5, 30.56-58.33)	86.5	0.449
ELF	55.53 (21, 9.22-236.35)	96.39 (5, 15.49-391.26)	37.0	0.340

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 4.1 Differences in plasma cortisol concentration between patients at onset of ARDS, at day 4 of ARDS and in patients at risk of ARDS

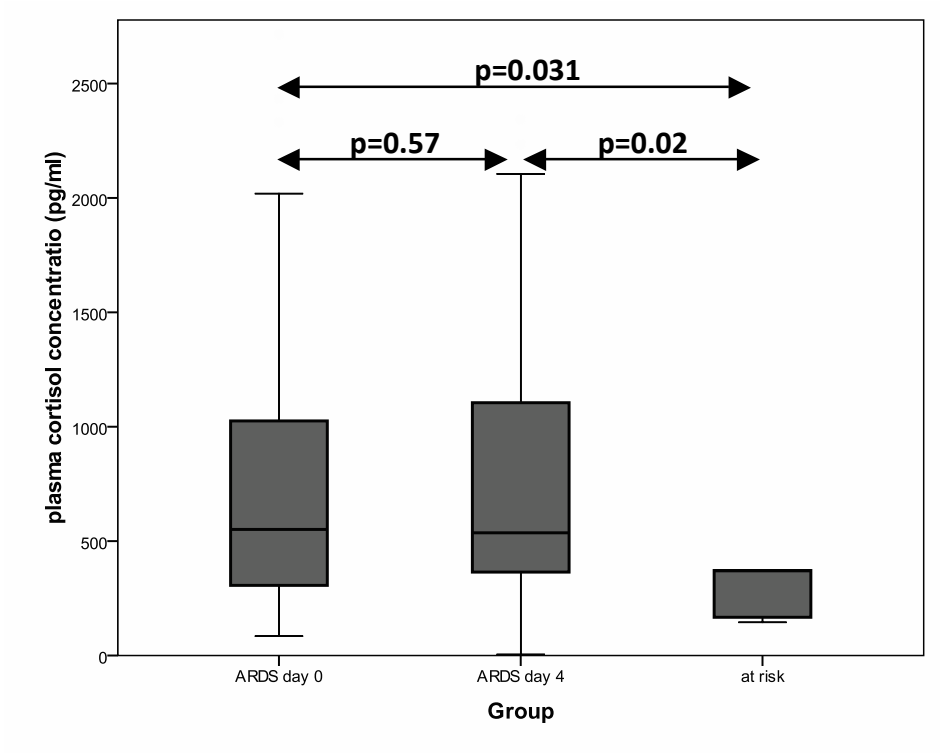
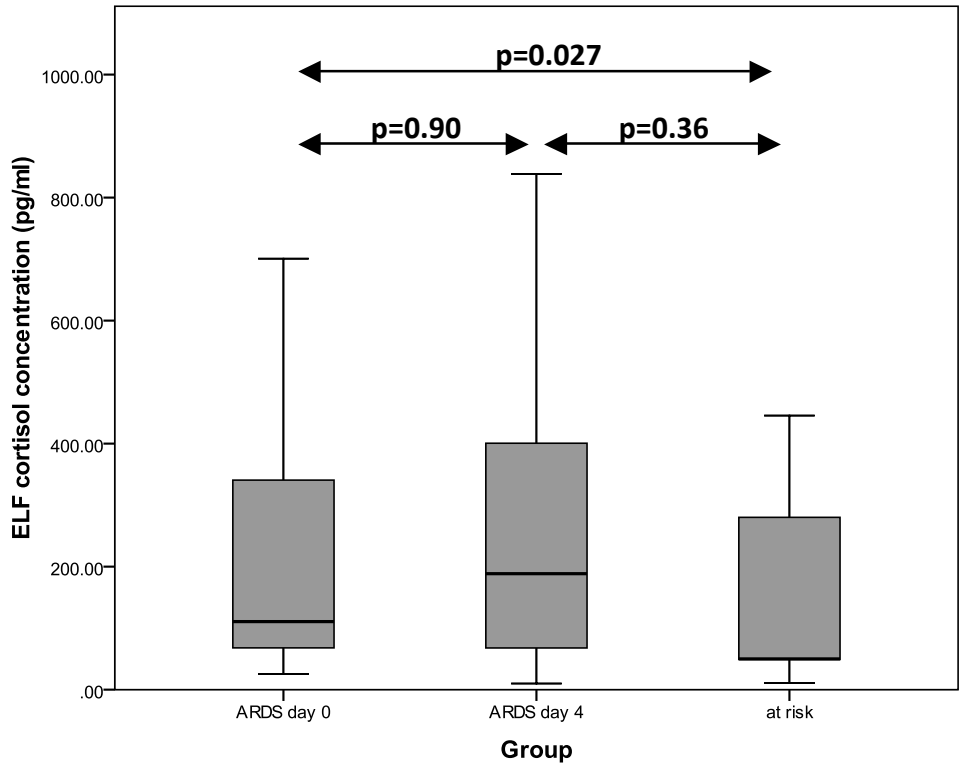


Figure 4.2 Differences in ELF cortisol concentration between patients at onset of ARDS, at day 4 of ARDS and in patients at risk of ARDS



4.4.3 Glucocorticoid concentration and severity scores

Relationships were tested between glucocorticoid concentrations and disease severity indices at day 0 and day 4 of ARDS. *See table 4.5 and 4.6*.

4.4.3.1 Glucocorticoid concentration and severity scores at day 0 of ARDS

At onset of ARDS the plasma cortisol had a positive relationship with the SOFA score (n=56, ρ=0.495, p<0.001) only. Plasma cortisone concentrations had a negative linear relationship between the SAPS-2 score (n=56, ρ=-0.307, p=0.021). No other correlations between plasma glucocorticoid concentrations and disease severity indices could be shown. *See figure 4.3*.

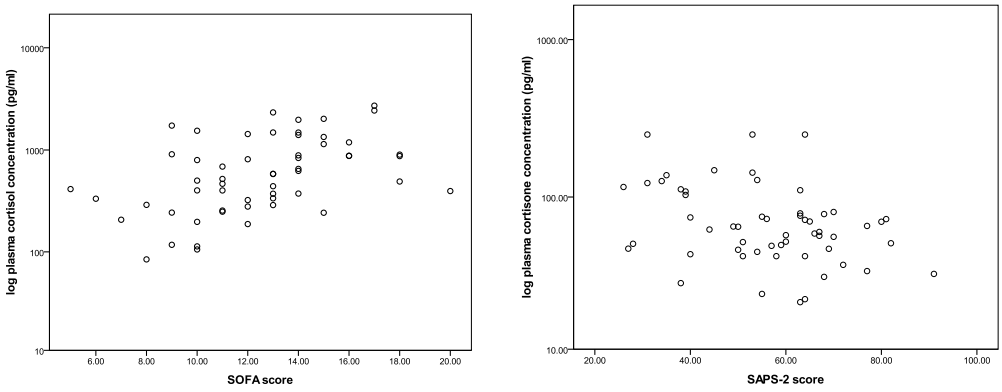
In the alveolar space a positive relationship was present between the ELF cortisol concentration and the SOFA score (n=45, ρ=0.362, p=0.015), the SAPS-2 score (n=45, r=0.381, p=0.010), and the APACHE-2 score (n=45, ρ=0.393, p=0.008). The ELF cortisone concentrations also had a relationship with SOFA scores (n=20, ρ=0.490, p=0.028), and SAPS-2 scores (n=20, ρ=0.458, p=0.042). *See Figure 4.4*.

Table 4.5 Table of correlations of day 0 glucocorticoid concentrations with severity indices. Cells show correlation coefficient (Spearman's "ρ"), significance value, and "n" number.

			LIS	SOFA	SAPS-2	APACHE-2
Cortisol	**plasma**	ρ =	-0.032	0.495*	0.158	0.252
		p =	0.817	<0.001	0.245	0.061
		n =	56	56	56	56
	ELF	ρ =	-0.185	0.362*	0.381*	0.393*
		p =	0.223	0.015	0.010	0.008
		n =	45	45	45	45
Cortisone	**plasma**	ρ =	0.096	-0.122	-0.307*	-0.181
		p =	0.481	0.372	0.021	0.183
		n =	56	56	56	56
	ELF	ρ =	-0.130	0.490*	0.458*	0.327
		p =	0.584	0.028	0.042	0.159
		n =	20	20	20	20

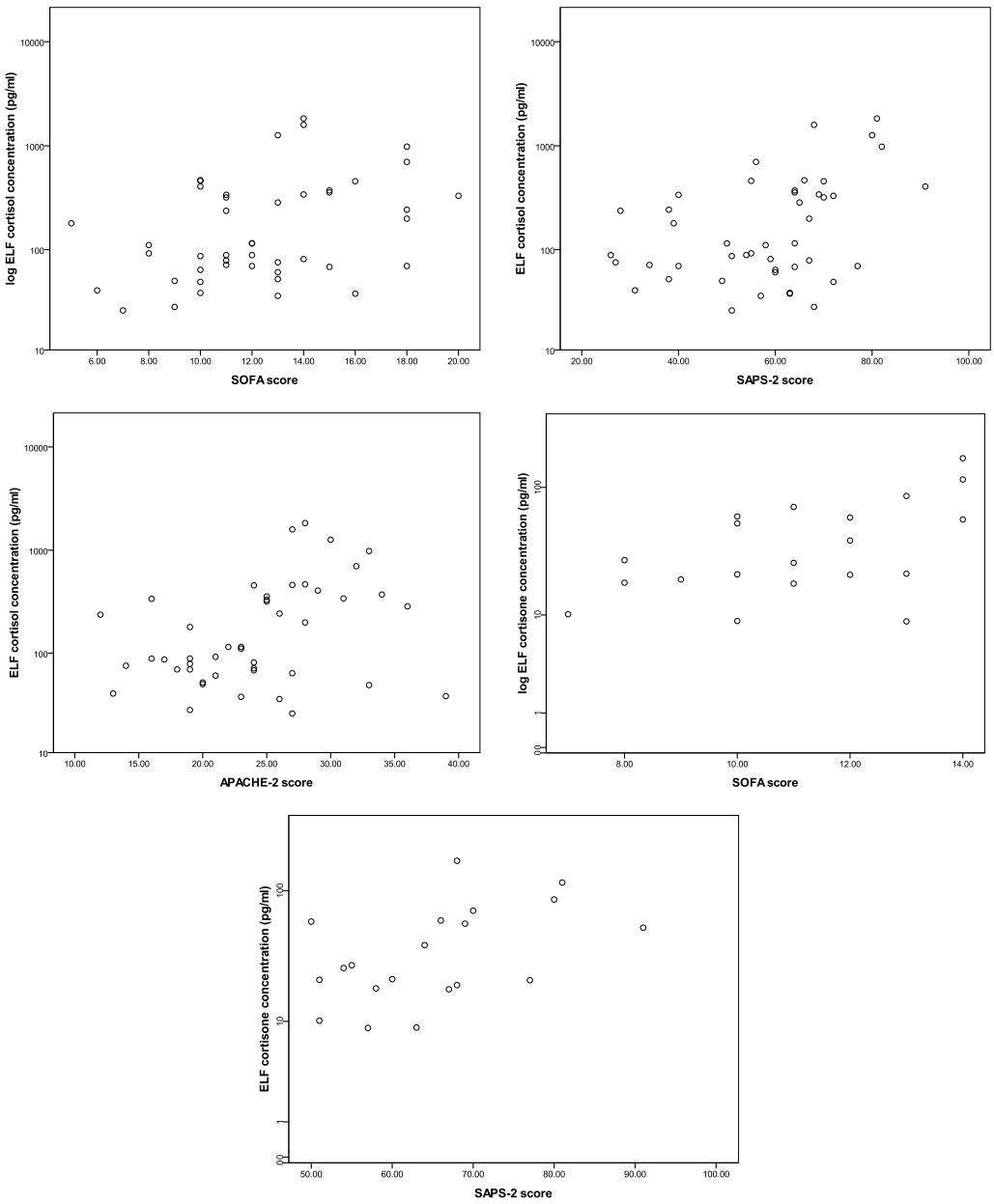
(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 4.3 Relationships between glucocorticoid concentrations in plasma and disease severity indices at onset of ARDS



At onset of ARDS a positive relationship is present between (a) SOFA score and plasma cortisol concentration (n=56, ρ=0.495, p<0.001), but (b) there is a negative relationship between the SAPS-2 score and the plasma cortisone concentration (n=56, ρ=-0.307, p=0.021).

Figure 4.4 Relationships between glucocorticoid concentration in the alveolar space and disease severity indices



Positive relationships exist between (a) the SOFA score and ELF cortisol concentration (n=45, ρ=0.362, p=0.015), (b) the SAPS-2 score and ELF cortisol concentration (n=45, ρ=0.381, p=0.010), and (c) the APACHE-2 score and ELF cortisol concentration (n=45, ρ=0.393, p=0.008). Positive relationships also exist between the ELF cortisone concentration and (d) the SOFA score (n=20, ρ=0.490, p=0.028), and (e) the SAPS-2 score (n=20, ρ=0.458, p=0.042).

4.4.3.2 Glucocorticoid concentration and severity scores at day 4 of ARDS

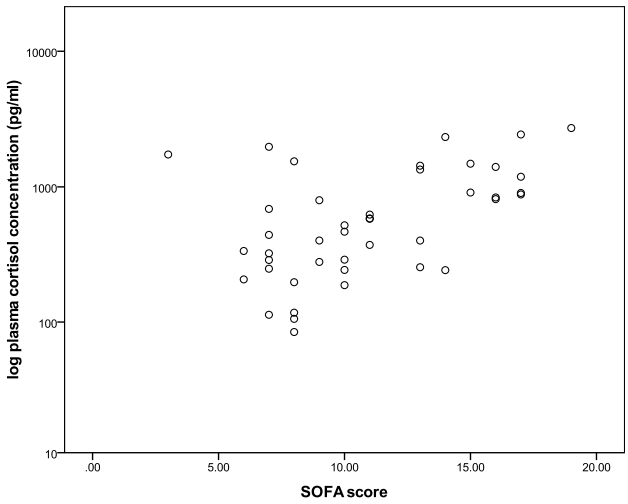
A positive relationship was present between the SOFA score at day 4 of ARDS and the simultaneously measured plasma cortisol concentration (n=44, ρ=0.497, p=0.001). *See Figure 5.9.* No other relationships between glucocorticoid concentrations in plasma or the alveolar space were demonstrated. *See table 4.6*.

Table 4.6 Table of correlations of day 4 glucocorticoid concentrations with severity indices. Cells show correlation coefficient (Spearman's "ρ"), significance value, and "n" number.

			LIS	SOFA	SAPS-2
Cortisol	plasma	r	-0.029	0.497*	0.296
		p =	0.854	0.001	0.181
		n =	43	44	22
	ELF	r	-0.039	0.305	0.315
		p =	0.825	0.074	0.164
		n =	35	35	21
Cortisone	plasma	r	0.153	0.211	0.143
		p =	0.332	0.175	0.525
		n =	42	43	22
	ELF	r	-0.206	0.150	0.136
		p =	0.371	0.515	0.558
		n =	21	21	21

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 4.5 Relationship between SOFA score and plasma cortisol concentration at day 4 of ARDS (n=44, ρ=0.497, p=0.001)



4.4.4 Glucocorticoid concentration and changes in severity scores

Differences in glucocorticoid concentrations were examined between subjects showing an improvement in disease severity indices. *See tables 4.7 and 4.8*.

At onset of ARDS higher plasma cortisol concentrations were present in patients who failed to go on to show an improvement in their SOFA score compared to those who did improve (Median=871.40 vs. 401.93pg/ml, U(56)=237.0, p=0.011). *See figure 4.6*. Plasma cortisone concentrations were also higher in this group (median=76.84 vs. 53.63 pg/ml, U(54)=235.0, p=0.010). *See Figure 4.7*.

Table 4.7 Differences in glucocorticoid concentrations at day 0 of ARDS between groups as defined by improvements in severity scores

		Any improvement in LIS		Any improvement in SOFA score	
		Yes	**No**	**Yes**	**No**
Plasma cortisol concentration (pg/ml)	Mdn	656.08	496.5	401.93	871.40
	range	105.60-2716.00	84.25-2019.00	105.6-1975.14	84.25-2716.00
	n	22	34	27	29
	U	364.0		237.0*	
	P value	0.867		0.011	
ELF cortisol concentration (pg/ml)	Mdn	199.16	90.28	88.57	145.16
	range	25.33-1590.74	27.46-1831.24	25.33-1831.24	37.16-1590.74
	n	17	28	24	21
	U	212.0		224.0	
	P value	0.543		0.553	
Plasma cortisone concentration (pg/ml)	Mdn	64.50	61.90	53.63	76.84
	range	20.83-250.00	23.61-250.00	20.83-250.00	23.61-250.00
	n	21	35	28	28
	U	342.0		235.0*	
	P value	0.666		0.010	
ELF cortisone concentration (pg/ml)	Mdn	56.74	26.20	23.86	56.75
	range	8.91-166.62	8.84-114.86	8.84-114.86	18.27-166.62
	n	9	11	16	4
	U	40.0		20.0	
	P value	0.503		0.290	

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05)

Table 4.8 Differences in glucocorticoid concentrations at day 4 of ARDS between groups as defined by improvements in severity scores

	Mann Whitney	Any improvement in LIS		Any improvement in SOFA score	
		Yes	**No**	**Yes**	**No**
Plasma cortisol concentration (pg/ml)	Mdn	724.87	444.64	464.96	991.79
	range	99.45-2105.00	4.63-2343.00	99.45-2234.81	4.63-2343.00
	n	23	22	31	14
	U	214.0		152.0	
	P value	0.376		0.111	
ELF cortisol concentration (pg/ml)	Mdn	200.63	168.0	147.56	269.93
	range	9.97-1593.07	28.56-3153.02	9.97-3153.02	31.87-1593.07
	n	20	20	27	10
	U	165.0		105.0	
	P value	0.869		0.305	
Plasma cortisone concentration (pg/ml)	Mdn	44.44	48.61	44.44	54.40
	range	19.10-170.50	24.10-179.00	23.20-179.00	19.10-176.20
	n	23	21	31	13
	U	191.0		165.5	
	P value	0.235		0.354	
ELF cortisone concentration (pg/ml)	Mdn	66.98	29.13	53.43	84.64
	range	9.22-236.65	19.53-86.35	9.22-236.35	22.20-108.33
	n	10	11	18	3
	U	35.0		19.0	
	P value	0.173		0.421	

Figure 4.6 Differences in plasma cortisol concentration at onset of ARDS in patients with and without an improvement in SOFA score between onset and day 4 of ARDS (Median=871.40 vs. 401.93pg/ml, U(56)=237.0, p=0.011)

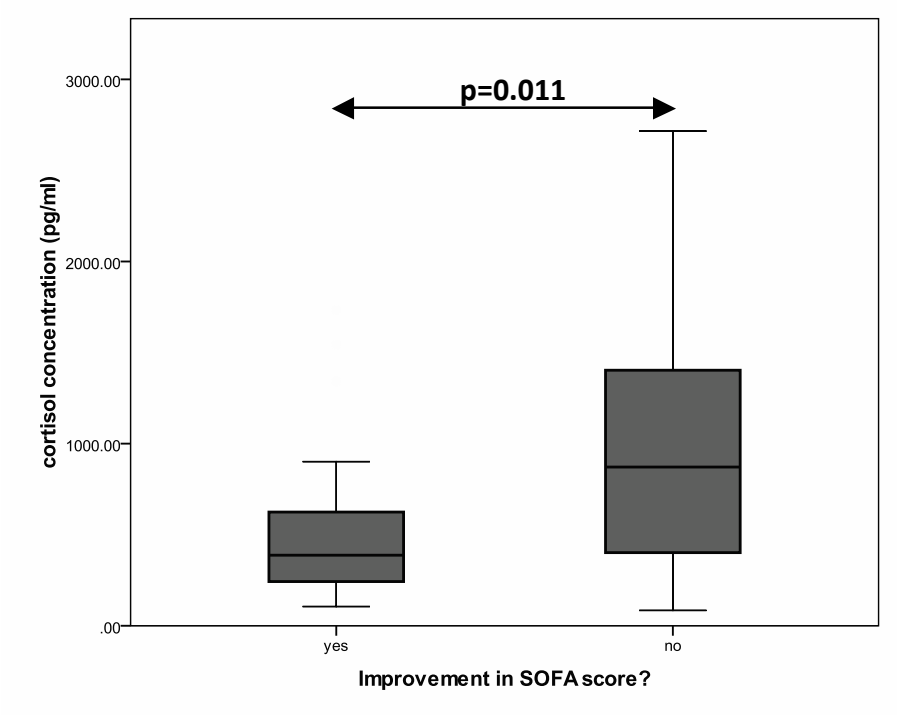
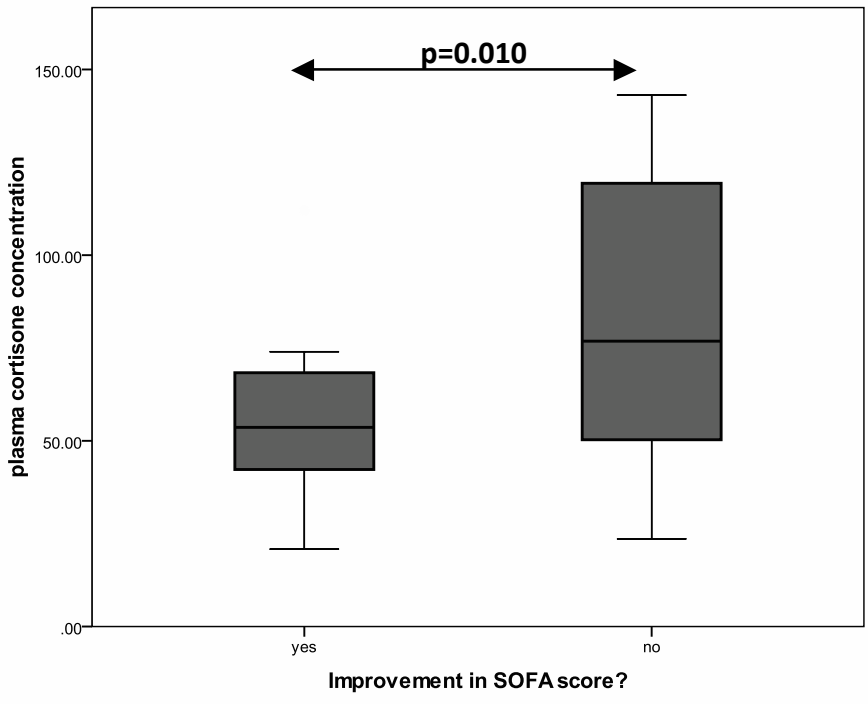


Figure 4.7 Differences in plasma cortisone concentration at onset of ARDS in patients with and without an improvement in SOFA score between onset and day 4 of ARDS (median=76.84 vs. 53.63 pg/ml, U(54)=235.0, p=0.010)



4.4.5 Glucocorticoid concentration and mortality

At day 0 of ARDS the plasma cortisol concentration was lower in patients who survived ITU compared to those patients that succumbed (median=687.85 vs. 323.20 pg/ml, U(55)=241.0, p=0.021). However differences in plasma cortisol concentrations were not present between patients for 28 day, or hospital survival. ELF cortisol concentration were also higher in patients failing to survive ITU (median= 264.15 vs. 75.29 pg/ml, U(45)=147.0, p=0.017), and failing to survive 28 days (median= 237.23 vs. 72.31pg/ml, U(45)=162.0, p=0.044), but no difference was present between for hospital survival. *See tables 4.9 and 4.10.*

Table 4.9 Glucocorticoid concentrations at day 0 of ARDS and mortality

	Mann Whitney	ICU survival		28 day survival		Hospital survival	
		Alive (n)	**Dead (n)**	**Alive (n)**	**Dead (n)**	**Alive (n)**	**Dead (n)**
Plasma cortisol (pg/ml)	Mdn conc.	323.20	687.85	442.0	624.31	336.30	624.31
	N	23	33	21	35	17	39
	range	84.25-2716.00	113.26-2434.00	105.60-2716.00	84.25-2434.00	84.25-2716.00	113.26-2434.00
	U	241.0*		329.0		269.0	
	P value	0.021		0.515		0.265	
ELF cortisol (pg/ml)	Mdn conc.	75.29	264.15	72.31	237.23	72.31	179.68
	N	20	25	19	26	15	30
	range	25.33-1263.43	35.36-1831.24	25.33-1590.74	27.46-1831.24	25.33-1263.43	27.46-1831.24
	U	147.0*		162.0*		159.0	
	P value	0.017		0.044		0.083	
Plasma cortisone (pg/ml)	Mdn conc.	54.05	70.96	62.11	63.59	60.72	63.59
	N	24	32	22	34	18	38
	range	21.80-250.00	20.83-250.00	20.83-250.00	23.61-250.00	21.80-250.00	20.83-250.00
	U	306.0		361.0		336.0	
	P value	0.196		0.827		0.916	
ELF cortisone (pg/ml)	Mdn conc.	21.52	52.94	21.52	39.11	24.52	32.66
	N	11	9	9	11	6	14
	range	10.14-85.97	8.84-166.62	8.91-166.62	8.84-114.86	10.14-85.97	8.84-166.62
	U	40.0		43.0		42.0	
	P value	0.503		0.656		1.0	

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Table 4.10 Glucocorticoid concentrations ratio at day 4 of ARDS and mortality

	Mann Whitney	ICU survival		28 day survival		Hospital survival	
		Alive (n)	**Dead (n)**	**Alive (n)**	**Dead (n)**	**Alive (n)**	**Dead (n)**
Plasma cortisol (pg/ml)	Mdn conc.	587.10	530.48	548.21	536.36	597.78	524.60
	N	25	20	22	23	18	27
	range	99.45-2105.00	4.63-2343.00	99.45-2105.00	4.63-2343.00	99.45-2105.00	4.63-2343.00
	U	248.0		248.0		223.0	
	P value	0.964		0.910		0.643	
ELF cortisol (pg/ml)	Mdn conc.	96.85	298.23	68.71	259.19	55.60	243.30
	N	22	18	18	19	15	25
	range	9.97-838.67	27.90-3153.02	9.97-1593.07	67.78-3153.02	9.97-838.67	27.90-3153.02
	U	92.0*		74.0		67.0*	
	P value	0.017		0.003		0.003	
Plasma cortisone (pg/ml)	Mdn conc.	44.44	51.39	44.44	51.19	43.17	49.81
	N	25	19	22	22	18	26
	range	19.10-176.20	34.72-179.00	19.10-176.20	34.72-179.00	19.10-176.20	34.72-179.00
	U	154.0*		177.5		170.0	
	P value	0.048		0.130		0.126	
ELF cortisone (pg/ml)	Mdn conc.	55.95	50.82	55.95	53.43	55.95	53.43
	N	11	10	9	12	5	16
	range	20.34-236.35	9.22-108.33	9.22-108.33	20.34-236.35	25.80-87.24	9.22-236.35
	U	46.0		51.0		34.0	
	P value	0.557		0.862		0.660	

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

4.4.6 Glucocorticoid concentrations and cellular inflammation

At day 0 of ARDS a negative relationship is present between the concentration of neutrophils in BALF and both plasma cortisol (n=48, ρ=-0.297, p=0.041) and cortisone (n=48, ρ=-0.298, p=0.040). *See figure 4.8*. Negative linear relationships are also present at day 0 of ARDS between BALF neutrophil concentration and both ELF cortisol (n=41 ρ=-0.351, p=0.024) and cortisone concentrations (n=19, ρ=-0.511, p=0.026). *See figure 4.9*.

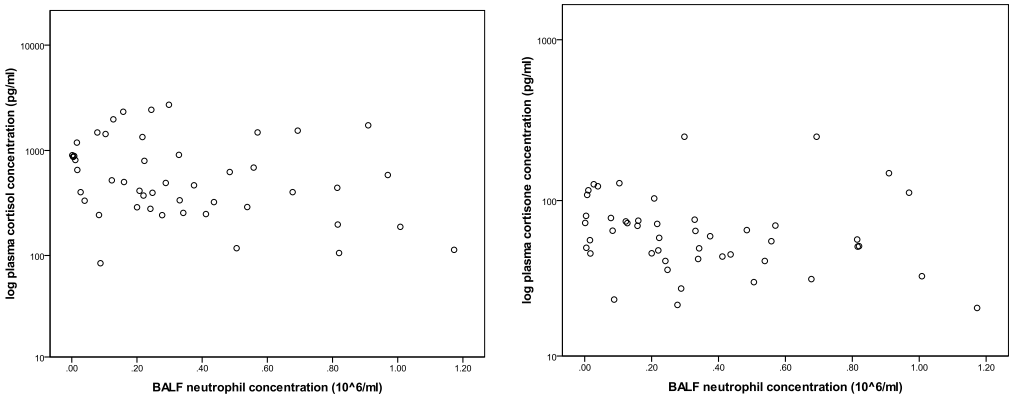
At day 4 of ARDS no relationships were found between neutrophil count and any of the steroid concentrations measured. *See table 4.11*.

Table 4.11 Correlations between concentrations of glucocorticoids and the neutrophil concentration in BALF from patients at day 0 and day 4 of ARDS

BALF neutrophil concentration time point:			Day 0	Day 4
Cortisol	plasma	Spearman's "ρ"	-0.297*	-0.123
		p =	0.041	0.437
		n =	48	42
	BALF	Spearman's "ρ"	0.128	-0.026
		p =	0.425	0.875
		n =	41	38
	ELF	Spearman's "ρ"	-0.351*	-0.154
		p =	0.024	0.376
		n =	41	35
Cortisone	plasma	Spearman's "ρ"	-0.298*	-0.236
		p =	0.040	0.142
		n =	48	40
	BALF	Spearman's "ρ"	-0.075	-0.144
		p =	0.759	0.534
		n =	19	21
	ELF	Spearman's "ρ"	-0.511*	-0.366
		p =	0.026	0.103
		n =	19	21

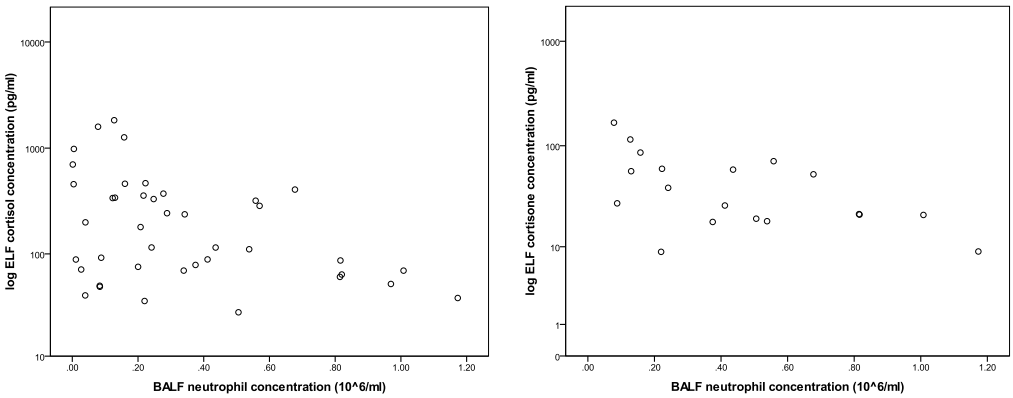
(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 4.8 Relationships between glucocorticoid concentration in plasma and the BALF neutrophil concentration at onset of ARDS



At onset of ARDS a negative relationship exists between the neutrophil concentration in the BALF of patients with ARDS and (a) the plasma cortisol concentration (n=48, ρ=-0.297, p=0.041) and (b) the plasma cortisone concentration (n=48, ρ=-0.298, n=48, p=0.040).

Figure 4.9 between glucocorticoid concentrations in the alveolar space and the BALF neutrophil concentration at onset of ARDS.



Negative linear relationships between BALF neutrophil concentration at day 0 of ARDS and (a) ELF cortisol concentration (ρ=-0.351, n=41, p=0.024) and (b) ELF cortisone concentration (ρ=-0.511, n=19, p=0.026).

4.4.7 Glucocorticoid concentrations and protein permeability

Correlations were calculated between glucocorticoid concentrations and the protein permeability index to determine whether increased protein concentration in the alveolar space could be responsible for increased alveolar glucocorticoid concentrations. *See table 4.12*. At day 0 of ARDS the protein permeability index had a negative relationship with plasma cortisol (ρ=-0.329, n=51, p=0.018). *See figure 4.9*. Negative relationships were also present between the protein permeability index and ELF concentrations of cortisol (r=-0.320, n=44, p=0.034) and cortisone (ρ=-0.709, n=19, p=0.001) at day 0 of ARDS and the ELF cortisone concentration at day 4 of ARDS (ρ=-0.672, n=18, p=0.002). *See figures 4.10 and 4.11*.

Table 4.12 Relationships between the alveolar membrane protein permeability index and glucocorticoid concentrations.

Protein permeability time point:			Day 0	Day 4
Cortisol	plasma	Spearman's "ρ"	-0.329*	0.025
		p =	0.018	0.883
		n =	51	38
	ELF	Spearman's "r"	-0.320*	-0.229
		p =	0.034	0.208
		n =	44	32
Cortisone	plasma	Spearman's "ρ"	-0.091	-0.057
		p =	0.523	0.740
		n =	51	36
	ELF	Spearman's "ρ"	-0.709*	-0.672*
		p =	0.001	0.002
		n =	19	18

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 4.10 Relationship between plasma cortisol concentration and protein permeability index at day 0 of ARDS (ρ=-0.329, n=51, p=0.018)

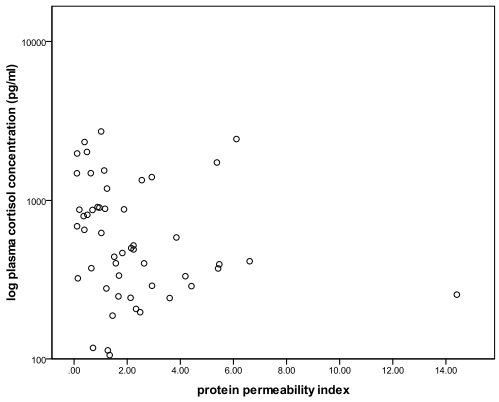
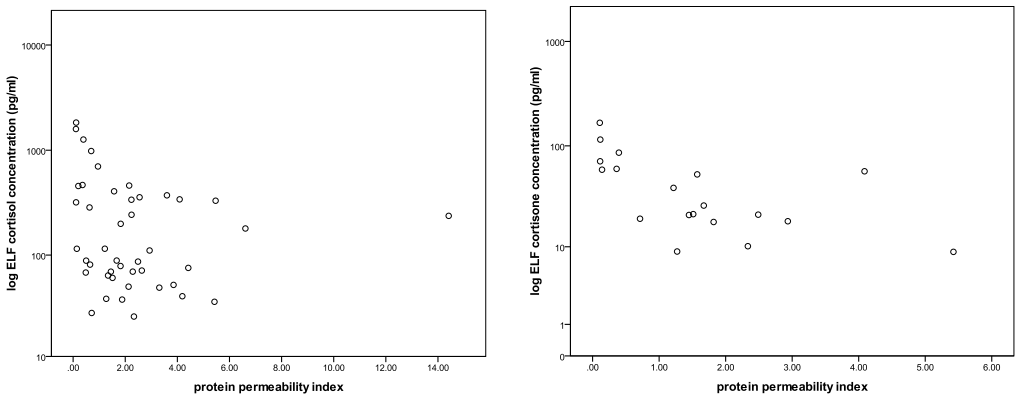
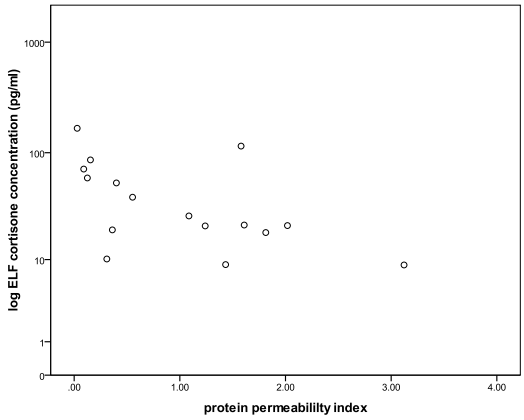


Figure 4.11 Relationships between alveolar space glucocorticoid concentrations and the protein permeability index at day 0 of ARDS



At day 0 of ARDS a negative relationship is present between the protein permeability index and (a) the ELF cortisol concentration (ρ=-0.320, n=44, p=0.034), and (b) the ELF cortisone concentration (ρ=-0.709, n=19, p=0.001).

Figure 4.12 Relationships between alveolar space glucocorticoid concentrations and the protein permeability index at day 4 of ARDS



At day 4 of ARDS a negative association is present between the protein permeability index and ELF cortisone concentration (ρ=-0.672, n=18, p=0.002).

4.4.8 The cortisol: cortisone ratio

To test the hypothesis that 11β-HSD activity within the lung was altered in ARDS this parameter was calculated from the steroid concentrations.

4.4.8.1 The cortisol: cortisone ratio between plasma and BALF

Plasma cortisol: cortisone ratios are higher than BALF cortisol: cortisone ratios both at day 0 (median= 10.29 vs. 5.50, t(18)=4.56, p<0.001) and day 4 (median= 12.20 vs. 6.55, t(20)=6.47, p<0.001) of ARDS, suggesting that the pulmonary inactivation of steroids is greater than that in the body as a whole. *See figures 4.13 and 4.14*. This investigation could not determine whether this was greater than that which would be seen in normal subjects. However, the BALF cortisol: cortisone ratio was higher at day 0 of ARDS compared to the at-risk group (median=4.16 vs. 0.72, U(25)=14.0, p=0.012), and also higher at day 4 of ARDS compared to the at-risk group (median=4.60 vs. 0.72, U(26)=17.0, p=0.019). *See figure 4.15*.

Figure 4.13 Differences between plasma and BALF cortisol: cortisone ratio at day 0 of ARDS

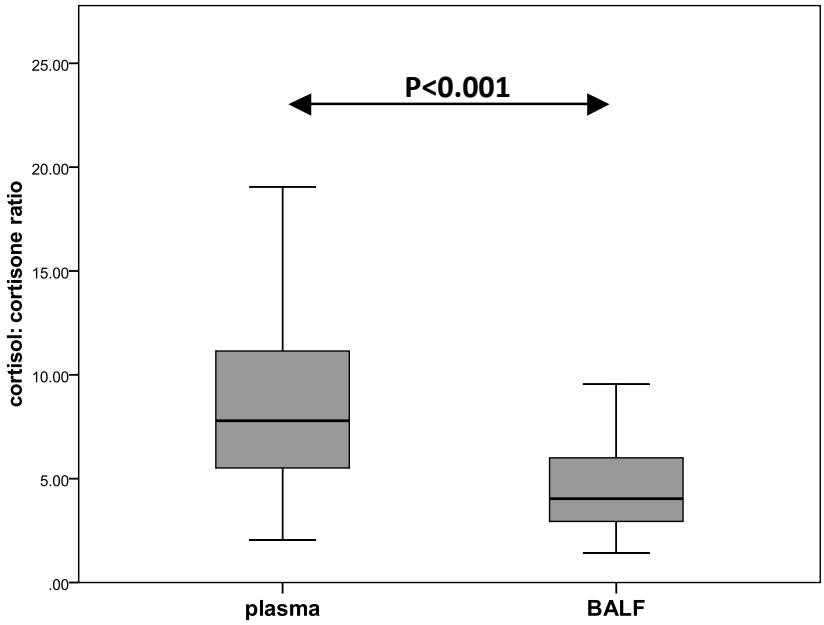


Figure 4.14 Differences between plasma and BALF cortisol: cortisone ratio at day 0 of ARDS

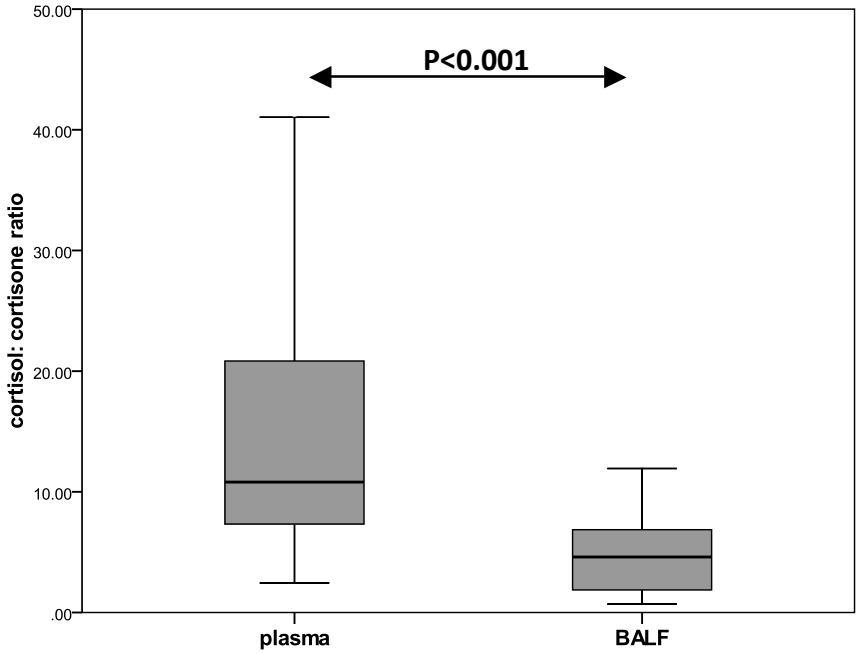
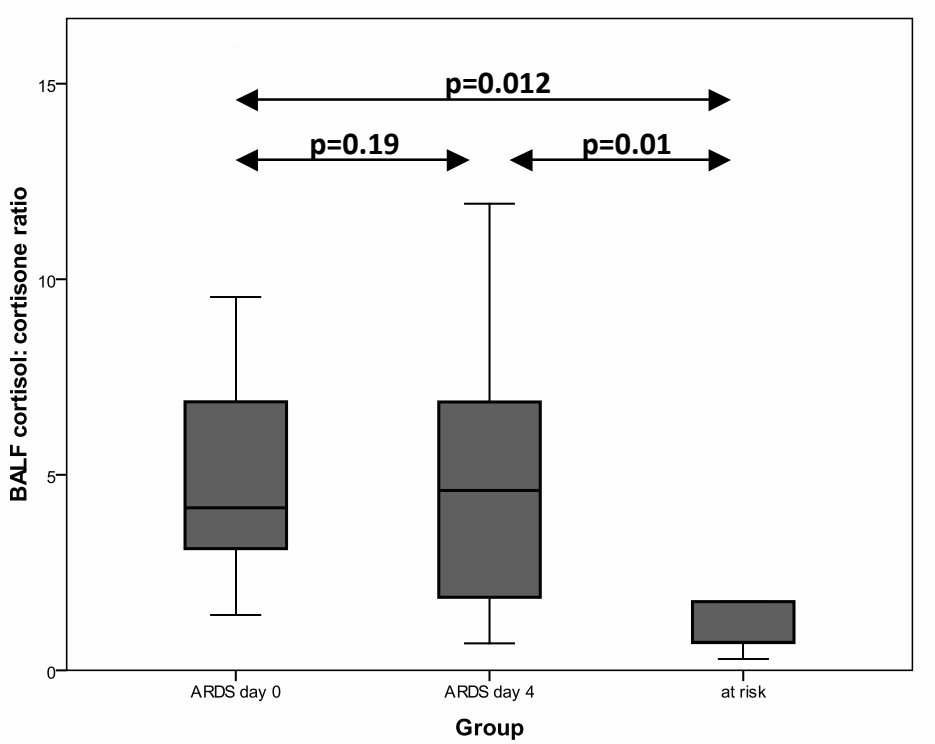


Figure 4.15 BALF cortisol: cortisone ratio between patients at onset of ARDS, at day 4 of ARDS and in patients at risk of ARDS



4.4.8.2 Cortisol: cortisone ratio and severity scores at day 0 of ARDS

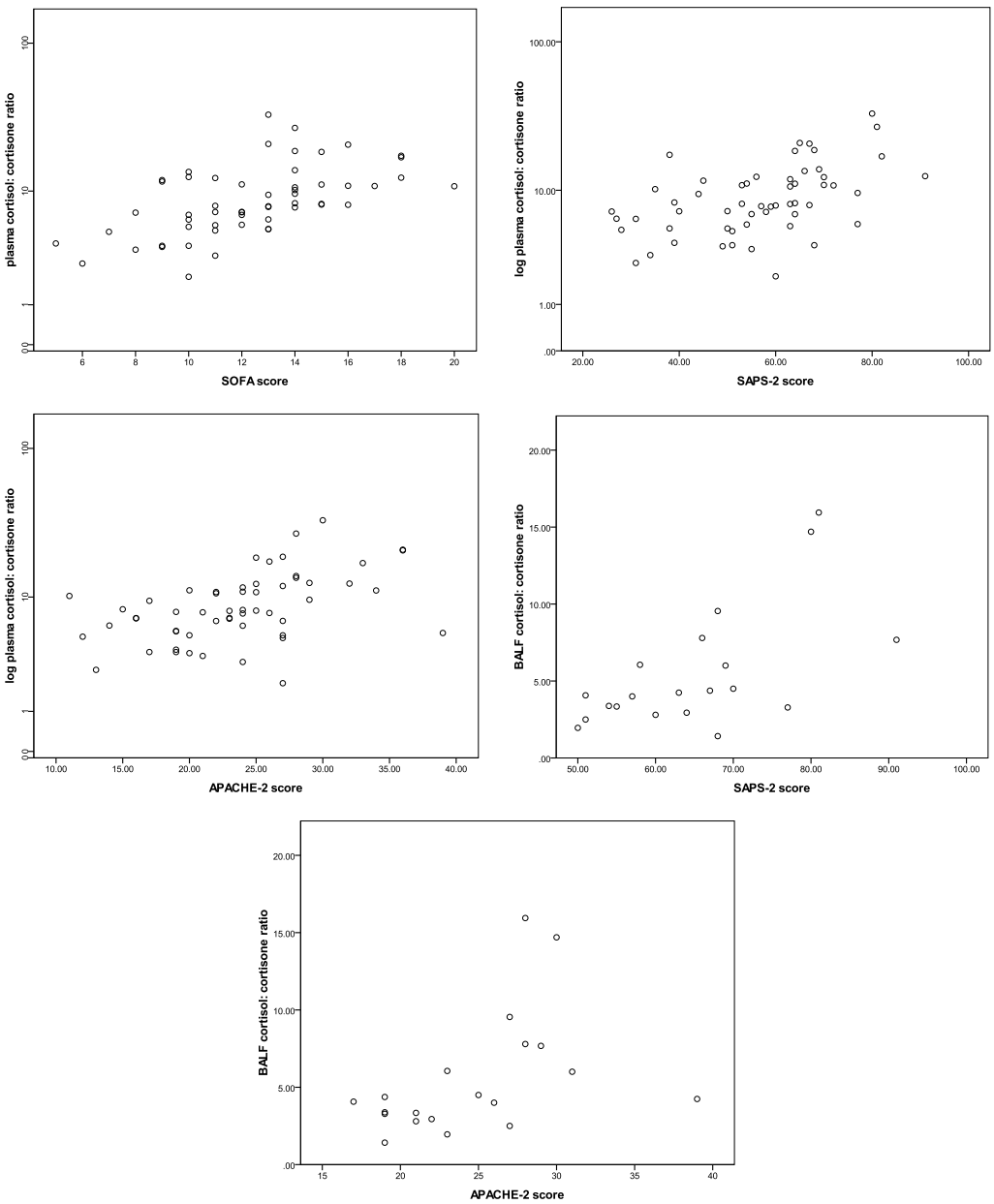
At onset of ARDS the cortisol: cortisone ratio in plasma had a positive relationship with the SAPS-2 score (n=55, ρ=0.536, p<0.001) and the SOFA score (n=55, ρ=0.597, p<0.001). Similar results were seen with the APACHE-2 score, which had a positive relationship with the cortisol: cortisone ratio in plasma (n=55, ρ=0.536, p<0.001).

The cortisol: cortisone ratio in BALF also had a positive relationship with the SAPS-2 score (n=20, ρ=0.581, p=0.007) at day 0 of ARDS. The APACHE-2 score also had a moderate positive relationship with the cortisol: cortisone ratio in BALF (n=20, ρ=0.575, p=0.008). *See figure 4.16*.

4.4.8.3 The cortisol: cortisone ratio and severity scores at day 4 of ARDS

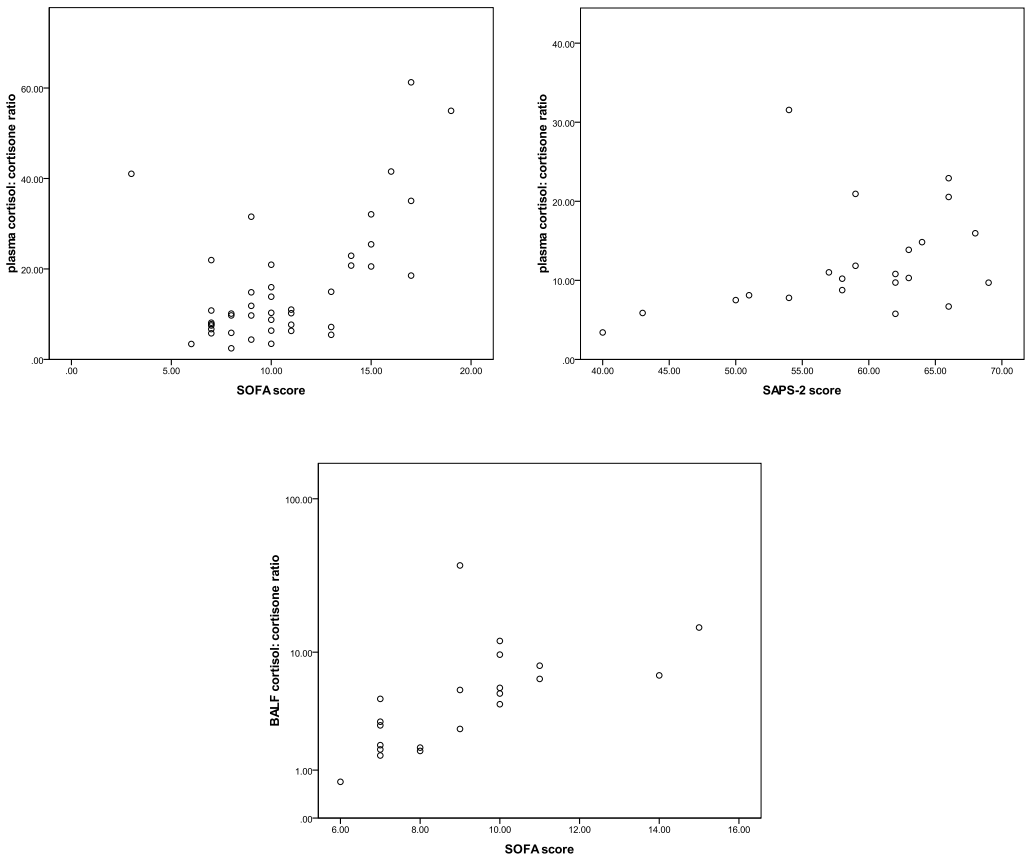
The plasma cortisol: cortisone ratio had a positive relationship with The SOFA score (n=42, ρ=0.493, p=0.001), and the SAPS-2 score (n=21, ρ=0.432, p<0.001) at day 4 of ARDS. The BALF cortisol: cortisone ratio also had a positive linear relationship with the SOFA score at day 4 of ARDS (n=21, ρ=0.432, p<0.001). *See figure 4.17.*

Figure 4.16 Relationships between the cortisol: cortisone ratio in the plasma and alveolar space and disease severity indices at onset of ARDS



At onset of ARDS a positive relationship exists between the plasma cortisol: cortisone ratio and (a) the SOFA score (n=55, ρ=0.597, p<0.001), (b) the SAPS-2 score (n=55, ρ=0.536, p<0.001), and (c) the APACHE-2 score (n=55, ρ=0.536, p<0.001), Similar positive relationships are present between the cortisol: cortisone ratio in BALF and (d) the SAPS-2 score (n=20, ρ=0.581, p=0.007) and (e) the APACHE-2 score (n=20, ρ=0.575, p=0.008).

Figure 4.17 Relationships between the cortisol: cortisone ratio in plasma and alveolar space and disease severity indices at day 4 of ARDS



At day 4 of ARDS a positive relationship was present between (a) SOFA score and plasma cortisol: cortisone ratio (n=42, ρ=0.493, p=0.001), and (b) the SAPS-2 score and plasma cortisol: cortisone ratio (n=22, ρ=0.432, p=0.045), Relationship between A positive relationship also exists between the SAPS-2 score and BALF cortisol: cortisone ratio at this time-point (n=21, ρ=0.432, p<0.001).

4.4.8.4 The cortisol: cortisone ratio and mortality

The cortisol: cortisone ratio was compared between patients who died and those who survived their hospital stay, their ICU stay and 28 days. Plasma cortisol: cortisone ratios were higher in those patients who died in ITU compared to those who survived (median ratio= 9.48 vs. 6.19, U(45)=238.0, p=0.027), as was the BALF cortisol: cortisone ratio (median ratio= 4.50 vs.3.34, U(20)=23.0, p=0.046). No other difference in this ratio between mortality groups was detectable. *See tables 4.18 and 4.19.*

Figure 4.18 Differences in cortisol: cortisone concentration at day 0 of ARDS between subjects who died and those who survived

	Mann Whitney	ICU survival		28 day survival		Hospital survival	
		Alive (n)	Dead (n)	Alive (n)	Dead (n)	Alive (n)	Dead (n)
Plasma cortisol: cortisone ratio	Median	6.19	9.48	7.76	8.78	6.19	8.12
	N	23	32	21	34	17	38
	range	2.04-33.57	3.18-27.35	2.04-33.57	3.18-27.35	2.04-33.57	3.18-27.35
	U	238.0*		296.0		243.0	
	P value	0.027		0.291		0.145	
BALF cortisol: cortisone ratio	Median	3.34	4.50	4.24	4.00	3.71	4.31
	N	11	9	9	11	6	14
	range	1.42-14.70	2.94-15.94	2.50-14.70	1.42-15.94	2.50-14.70	1.42-15.94
	U	23.0*		45.0		36.0	
	P value	0.046		0.766		0.659	

Figure 4.19 Differences in cortisol: cortisone concentration at day 4 of ARDS between subjects who died and those who survived

	Mann Whitney test	ICU survival		28 day survival		Hospital survival	
		Alive (n)	Dead (n)	Alive (n)	Dead (n)	Alive (n)	Dead (n)
Plasma cortisol: cortisone ratio	Median	11.84	10.51	10.99	10.81	13.90	10.30
	N	25	19	22	22	18	25
	range	3.41-61.26	2.45-41.54	3.41-61.26	2.45-41.54	3.41-61.26	2.45-41.54
	U	214.0		221.0		214.0	
	P value	0.787		0.808		0.787	
BALF cortisol: cortisone ratio	Median	2.81	4.98	3.03	5.21	1.87 (5)	4.82 (16)
	N	11	10	9	12	5	16
	range	1.21-11.93	1.47-37.52	1.21-14.71	1.47-37.52	1.21-9.61	1.47-37.52
	U	40.0		46.0		29.0	
	P value	0.314		0.602		0.398	

4.5 Discussion

Although glucocorticoid concentrations in plasma are known to be markers of disease severity in critical illness,[67, 207-209] no information has previously been available on glucocorticoid concentrations in the alveolar space of patients with ARDS, or how these concentrations relate to disease severity. Our results show that cortisol concentrations in the alveolar space of patients with ARDS are higher at onset of ARDS than those in critically ill patients who are at risk of developing ARDS. This investigation was unable to detect a difference between cortisol levels in the alveolar space at day 4 of ARDS and the at-risk group. In concordance with the findings of other studies,[67, 207-209] the plasma glucocorticoid concentrations were also raised at both day 0 and day 4 of ARDS.

The increase in glucocorticoid levels in the alveolar space of patients with ARDS is also supported by positive relationships between ELF cortisol and cortisone concentrations and indices of global disease severity measured at both day 0 and day 4 of ARDS. However, our results provide only limited support for plasma concentration of glucocorticoids as a marker of disease severity in ARDS. Although plasma cortisol and cortisone concentrations are higher in patients whose SOFA score failed to improve, a positive association between cortisol and the SOFA score was only present at day 0 of ARDS. This is at odds with the negative relationship detected between the cortisone concentration in the plasma and a second severity index, the SAPS-2 score. Nevertheless, our findings are that the positive associations of global disease severity with cortisol concentration in the plasma, which have previously been described by other investigators, [67, 207-209] are mirrored in the alveolus. We were unable to detect any link between alveolar glucocorticoid concentration and the Murray lung injury score (LIS), a severity scoring index which has been advocated as a tool specifically for the

assessment of ARDS severity.[147] The lack of a link between glucocorticoids and the LIS, while a link was found between measures of more general critical illness and glucocorticoids was found would suggest that the general host response to illness determines glucocorticoid concentrations within the lung.

The BALF: plasma protein ratio is an established estimate of the permeability of the alveolar capillary barrier to large molecules, and can be seen as a marker of alveolar injury.[217] Positive associations with other markers of disease severity in ARDS might therefore be expected. However, we demonstrated uniformly negative relationships between glucocorticoid concentrations in both plasma and the alveolar space and the protein permeability index. This finding suggests that increased steroid concentrations within the lung are associated with decreased or resolving lung injury. This link between glucocorticoids and decreased lung inflammation is corroborated by the negative associations between glucocorticoids and neutrophil concentration in BALF. This negative relationship of steroids with neutrophil concentration is likely to be part of the inflammatory resolution process. Glucocorticoids increase the disposal of apoptotic neutrophils by macrophages in the alveolar space. They also decrease the production of chemo-attractant mediators such as IL-8.[218, 219] Therefore patients with increasing concentrations of steroids in their lungs will have decreased recruitment and increased disposal of neutrophils. This relationship is complicated by the cleavage of cortisol-binding globulin by neutrophil elastase. This process releases free cortisol into the tissue which acts as a negative feedback of inflammation.[220] The negative association of steroid and neutrophil concentration therefore provides circumstantial evidential support for increased protein in the alveolar space not being responsible for increased alveolar concentration of glucocorticoids.

The increased cortisol concentrations detected in the plasma and ELF of patients who died in ICU and the higher cortisol concentrations in patients whose SOFA scores failed to improve reflects the severity of the causative disease. Although we have shown that cortisol is linked to decreased alveolar damage and lower neutrophil counts, these measures are lung specific and do not represent global severity of illness. These findings suggest that cortisol within the lung is at least partially fulfilling its anti-inflammatory role and any glucocorticoid receptor mediated corticosteroid resistance is therefore far from complete.

The cortisol: cortisone ratio is thought to be a measure of the net activity of the enzyme 11β-hydroxysteroid dehydrogenase, which inter-converts cortisone and cortisol.[48] 11β-HSD amplifies the local effect of glucocorticoids by increasing the available active intracellular steroid concentration, a process that is important to the resolution of inflammation.[75] This investigation measured the cortisol: cortisone ratio to try to detect an increase in the deactivation of cortisol to cortisone by increased expression and activity of the 11β-HSD type 2 iso-enzyme. Cortisol: cortisone ratios were higher in the plasma of patients with ARDS compared to that seen in BALF at day 0 and day 4 of ARDS, suggesting net 11β-HSD inactivation of cortisol within the lung. However increased ratios were present in the alveolar space of patients with ARDS at both day 0 and day 4 when compared to at-risk subjects, which would suggest either increased 11β-HSD type 1 production of cortisol from cortisone, or decreased 11β-HSD type 2 conversion of cortisol to cortisone. Positive relationships between the cortisol: cortisone ratio and disease severity indices at both day 0 and day 4 of ARDS were also demonstrated. Previous investigations have shown that systemic activity of steroids by 11β-HSD type 1 is increased in patients with sepsis.[67] Models of inflammation in other tissues have also

shown increased expression and activity of 11β-HSD type 1 is induced by TNF-α and other pro-inflammatory cytokines,[54, 64, 65, 210] the same mediators that are culpable for inflammation in ARDS. Our findings in this study cannot determine whether an alteration in either 11β-HSD isoenzyme expression occurs in the lungs of patients with ARDS, but we have detected no evidence for an increased 11β-HSD type 2 inactivation of cortisol to cortisone in ARDS as suggested by the immunohistochemistry study by Suzuki et al. that. Our results suggest that within the lungs of patients with ARDS an increase in the activity of 11β-HSD type 1 is more likely. However, no investigations of the effect of inflammatory mediators on 11β-HSD activity involving primary lung cells have as yet been published.

In conclusion, this investigation has demonstrated that cortisol concentrations in the alveolar space of patients with ARDS are increased at onset of disease, and are a marker of generalised disease severity. However they have a negative association with both alveolar permeability and cellular inflammation within the lung. We have found no evidence for increased intra-pulmonary 11β-HSD type 2 activity influencing alveolar steroid concentrations in ARDS, but some evidence that 11β-HSD type 1 activity may be increased.

5 Alveolar macrophage 11β-hydroxysteroid dehydrogenase activity

5.1 Abstract

Introduction: Investigations in the preceding chapter have shown increased alveolar concentration of cortisol in patients with ARDS compared to critically unwell patients without ARDS. Increased cortisol: cortisone ratios were also seen in the alveolar space of patients with ARDS. These findings may be explained by altered intra-pulmonary activity of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). This enzyme has two iso-enzymes: type 1 that bi-directionally metabolises the oxo-reduction of cortisone to cortisol, and type 2, that can only metabolise cortisol to cortisone. Alveolar macrophages (AM) are important cells in the resolution of severe inflammation in the lung such as that seen in ARDS. These cells take part in a number of steroid sensitive processes that are essential for the resolution of this inflammation. We conducted a series of experiments using primary alveolar macrophages to determine how humoral mediators of lung injury influence their 11β-HSD activity.

Methods: Primary alveolar macrophages from healthy areas of lung were taken from lung resection specimens. These cells were then cultured for 24 hours in stimulation conditions and the activity 11β-HSD activity of the AM subsequently measured using tritiated thin layer chromatography.

Results: TNF-α induced a dose dependent increase in 11β-HSD oxo-reductase activity (p=0.026), but not dehydrogenase activity. IL-1β also increased the oxo-reductase activity (p=0.009). LPS caused a down-regulation of 11β-HSD oxo-reductase activity (p=0.003). Salbutamol had no effect on the enzyme activity in un-stimulated cells, but caused an abrogation of the increase in oxo-reductase activity caused by TNF-α stimulation (p=0.015). Stimulation of AM with BALF from patients with ARDS caused an up-regulation in AM 11β-HSD oxo-reductase activity (p=0.010)

Conclusions: Primary alveolar macrophages have increased 11β-HSD oxo-reductase activity in response to inflammatory mediators known to be culpable in ARDS. This altered activity will increase the bioavailability of active cortisol and is consistent with the findings of increased cortisol concentrations and increased cortisol: cortisone ratios in the alveolar space of patients with ARDS.

5.2 Introduction

The acute respiratory distress syndrome (ARDS) is a severe inflammatory condition of the lung seen in critically unwell patients. Although ARDS can be caused by many different precipitants, infective causes are the most common; manifested as either pneumonia or systemic sepsis.[146] The inflammation of ARDS is mediated by the increased concentrations of pro-inflammatory cytokines that are found within the lungs of patients with this condition.[23, 146] Such inflammatory mediators include TNF-α and IL-1β, and persistently high concentrations of these cytokines are associated with poor outcomes for these patients.[10, 221] Lipopolysaccharide (LPS) is a component of the bacterial cell wall that stimulates an inflammatory response by activating Toll like receptors.[222] This molecule has been used as an initiating agent of ARDS in both in-vitro and in-vivo models of sepsis induced ARDS. More recently HMGB-1 has also been shown to be an important pro-inflammatory mediator of late inflammation in sepsis and ARDS.[223-225]

Glucocorticoids are endogenous anti-inflammatory steroid hormones produced by activation of the hypothalamic-pituitary-adrenal (HPA) system. The production of these hormones is increased in critical illness and ARDS in order to balance and limit the

inflammatory response. Glucocorticoids act by binding to the intracellular glucocorticoid receptor, which then moves to the nucleus and binds to promoter regions of DNA. This causes a decrease in the production of pro-inflammatory mediators and enhances the production of anti-inflammatory cytokines.[29] Glucocorticoids decrease the recruitment of inflammatory cells, and promote the phagocytosis of spent neutrophils.[75, 128, 219, 226, 227] They have been seen to promote the resolution of inflammation in many models of acute and chronic disease.[30]

The conversion of cortisone to cortisol in peripheral tissues is catalysed by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). The activity of this enzyme, as well as the circulating hormone concentrations determines active hormone concentrations in the tissues.[45] In inflamed tissues this enzymes expression and activity are up-regulated. This provides an enhancement of the action of glucocorticoids in target cells by increasing the production of active cortisol.[54, 63-65, 210] There exist 2 iso-enzymes of this molecule: 11β-HSD type 1 which catalyses the conversion of cortisone to cortisol in-vivo by an oxo-reduction reaction, but can also catalyse the reverse reaction; and 11β-HSD type 2 which catalyses the conversion of cortisol to cortisone in a dehydrogenation reaction, it is unable to act in the reverse direction.[48]

In the preceding chapter we have demonstrated that cortisol concentrations are increased in the alveolar space of patients with ARDS and that there is an increase in the cortisol: cortisone ratio within the lung. Findings that would be consistent with an increased 11β-HSD type 1 activity; as seen in other cell types in response to inflammatory mediators. However, immunohistochemistry studies of patients who have died with ARDS show increased expression of 11β-HSD type 2 in the lungs of patients with ARDS, localised to the alveolar wall and cells of the monocyte lineage within the

alveolar lumen.[69] Furthermore, blockage of this enzymes activity caused an increase in steroid effects.[72]

We therefore conducted a series of experiments using primary alveolar macrophages to determine whether the key inflammatory mediators implicated in ARDS cause an alteration in the 11β-HSD activity of these cells, and whether oxo-reductase or dehydrogenase activity predominates.

5.3 Methods

The methodology used in the experiments reported in this chapter is described in detail in chapter 3, and is summarised below:

5.3.1 Sample collection and processing

Patients undergoing lung resection as part of lung cancer surgery were recruited as part of the Midlands lung tissue collaborative scheme at Birmingham Heartlands Hospital. After resection a portion of healthy lung was cut from the resected lung tissue and taken on ice to the laboratory for further processing. The lung sample was then washed through with 0.9% saline solution. The wash off was collected and spun at 500G for 5 minutes. The resultant cell pellet was re-suspended in 20ml of 0.9% saline solution and under-layered with lymphoprep™ (Axis-Shield, UK). This was then spun in a centrifuge for 30 minutes at 800G. Mononuclear cells were aspirated from the resultant interphase, and alveolar macrophages counted and assessed for viability. AM were placed in 24 well cell culture plates at a concentration of 500,000 cells/well.

5.3.2 Cell culture conditions

The alveolar macrophages were incubated at 37^{o}C in a 5% CO_2 atmosphere overnight, and then washed to remove non-adherent cells. Experimental conditions were set up

using differing concentrations of LPS (R&D Systems, Minneapolis, USA), as a direct cause of ARDS and the inflammatory response, TNF-α (R&D Systems, Minneapolis, USA), and IL-1β (R&D Systems, Minneapolis, USA), as early mediators of inflammation in ARDS, and HMGB-1(R&D Systems, Minneapolis, USA), a late mediator of inflammation. Cells were also incubated with broncho-alveolar lavage fluid (BALF) taken from patients to simulate in vivo conditions during ARDS. The BALF was collected from patients enrolled in a bronchoscopic sub-study of the BALTI-2 investigation and the resolution of inflammation in ARDS investigation. ARDS was diagnosed by AECC criteria.[3] Cells were incubated in experimental conditions for 24 hours. As our study was intended to provide biological mechanisms behind changes seen in the BALTI-2 sub-study, we also used differing concentrations of salbutamol in culture media. Viability of cells cultured in all conditions were verified using celltiter aqueous (Promega, UK), and only conditions where no difference in viability between stimulation and control were included in analysis.

5.3.3 11β-HSD activity assay

A tritiated thin layer chromatography (TLC) technique was used to measure both the oxo-reduction of cortisone to cortisol and the dehydrogenation of cortisone to cortisol. AM were incubated in a 10^{-7} molar concentration of cortisol or cortisone in RPMI together with tritiated cortisol or cortisone for 18 hours. Glucocorticoids were extracted using dichloromethane and separated by TLC. TLC plates were then read using a Bioscan plate reader (Bioscan, Washington, USA). Conversion of cortisol and cortisone was then calculated. Results are recorded as pmol/million cells/hour. All experiments were carried out in duplicate, and average value of duplicated tests analysed.

5.3.4 Statistical analysis

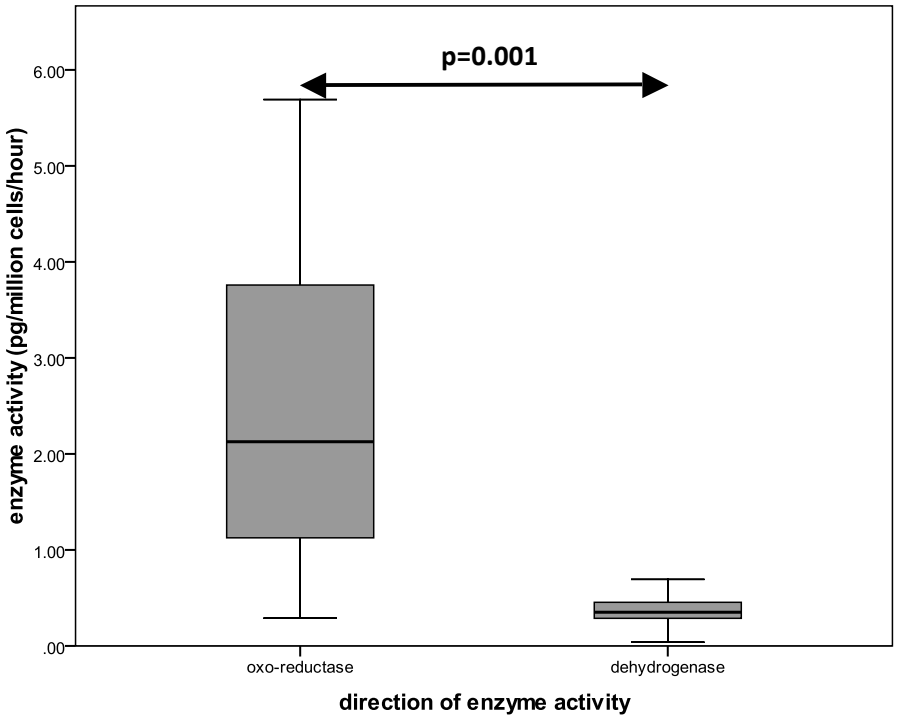
Statistical analysis was done using SPSS statistical software (IBM, Somers, New York, USA). Due to the sample sizes used in these experiments non-parametric statistical tests were used throughout. The Mann Whitney test was used for testing for differences between two unrelated groups the Wilcoxon signed rank test for differences between related groups. The ANOVA by ranks (Kruskal Wallis) test was used for examining differences between multiple groups of unrelated samples, and the Friedman tests for multiple groups of related samples. Linear associations were tested using Spearman's correlation coefficient.

5.4 Results

5.4.1 11β-HSD oxo-reductase activity in primary alveolar macrophages has a large variability between individuals.

11β-HSD oxo-reductase activity and dehydrogenase activity was compared in un-stimulated cells. Although dehydrogenase activity was normally distributed (K-S test, $D(25)=0.114$, $p=0.20$), the oxo-reductase activity for our cells was not (K-S test, $D(29)=0.178$, $p=0.019$). Non-parametric tests were used to evaluate differences between these groups. 11β-HSD oxo-reductase was considerably greater than the dehydrogenase activity measured in the same cells (median activity= 2.127 vs. 0.353 pg/million cells/hour, $U(55)=45.0$, $p<0.001$). *See figure 5.1*. Considerable variability was observed within the results of the oxo-reductase activity and the dehydrogenase activity.

Figure 5.1 Comparison between AM 11β-HSD oxo-reductase and deydrogenase activity



5.4.2 TNF-α increases 11β-HSD oxo-reductase activity in primary alveolar macrophages

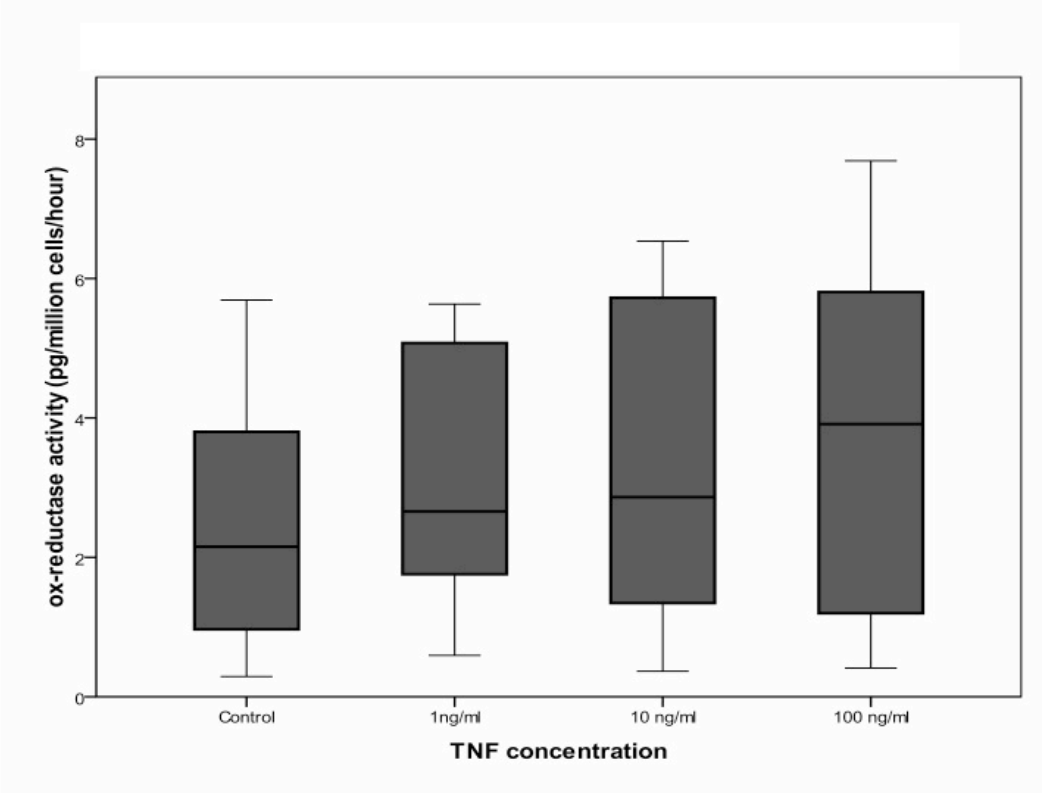
Incubation of primary alveolar macrophages with increasing concentrations of the pro-inflammatory cytokine TNF-α caused these cells to increase their conversion of cortisone to cortisol with a clear dose response (Friedman's test, $\chi^2(3)=9.30$, p=0.026). *See figure 5.2.* Post hoc analysis was therefore performed to test differences between groups. No corrections were made for multiple analyses. Differences were present between all of the conditions tested, except for between the oxo-reductase activity in the cells incubated in 1ng/ml TNF-α and 100ng/ml TNF-α (Wilcoxon signed rank test, Z=-1.461, p=0.144). *See table 5.1*.

No difference between the control cell culture condition and the different TNF-α concentrations was detected overall in their 11β-HSD dehydrogenase activity (Friedman's test, $\chi^2(3)=1.00$, p=0.801).

Table 5.1 Between Groups analysis of TNF-α influence on AM 11β-HSD oxo-reductase activity

Comparison between experimental conditions (median oxo-reductase activity, pg/million cells/hour)		**Wilcoxon Z statistic**	**P value**
Control (2.13)	TNF-α 1ng/ml (2.66)	-3.845	<0.001
Control (2.13)	TNF-α 10 ng/ml (2.86)	-3.516	<0.001
Control (2.13)	TNF-α 100 ng/ml (3.91)	-2.023	0.043
TNF-α 1ng/ml (2.66)	TNF-α 10 ng/ml (2.86)	-2.669	0.008
TNF-α 1ng/ml (2.66)	TNF-α 100 ng/ml (3.91)	-1.461	0.144
TNF-α 10 ng/ml (2.86)	TNF-α 100 ng/ml (3.91)	-2.023	0.043

Figure 5.2 Dose response of TNF-α on AM 11β-HSD oxo-reductase activity (Friedman's test, n=5, $\chi^2(3)=9.30$, p=0.026).



5.4.3 IL-1β increases 11β-HSD oxo-reductase activity in primary alveolar macrophages

Similarly to the findings for TNF-α, oxo-reductase activity of the primary AM cells was increased by IL-1β, another of the inflammatory cytokines implicated in ARDS. Increasing concentrations of IL-1β in the culture medium causing increased conversion of cortisone to cortisol (Friedman's test, $\chi^2(2)=9.45$, p=0.009). *See figure 5.3*. Differences between groups were assessed, again without correction for multiple analyses. Increased 11β-HSD oxo-reductase activity was found in cells treated with 1ng/ml of IL-1β compared to control (Wilcoxon signed rank test, Z=-2.490, p=0.013), and 10ng/ml compared to control (Wilcoxon signed rank test, Z=-2.934, p=0.003). However no difference in oxo-reductase activity was present between cells treated with 1ng/ml and 10ng/ml of IL-

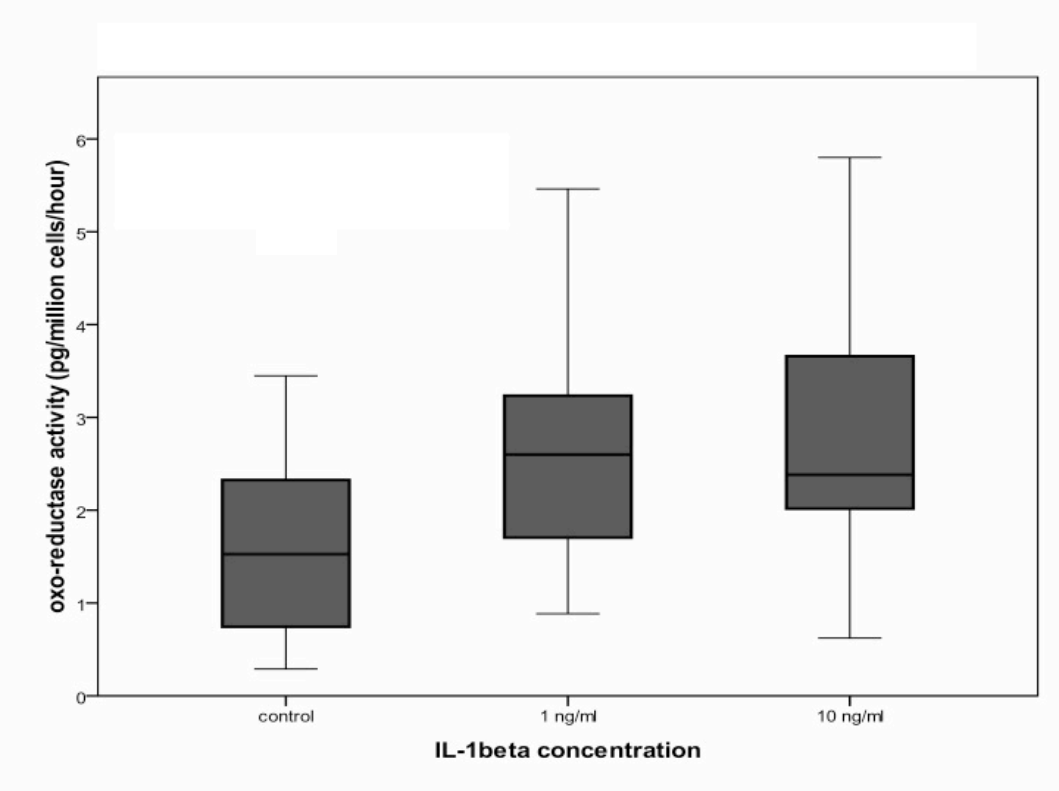
1β. *See table 5.2*. Given these between groups effects, a clear dose response for IL-1β on AM 11b-HSD oxo-reductase activity cannot be assumed.

Again similarly to the TNF-α cell culture experiments, no difference in the 11β-HSD dehydrogenase activity was demonstrable between cells cultured in different concentrations of IL-1β (Friedman's test, $\chi^2(2)=4.533$, p=0.339).

Table 5.2 Comparisons of 11β-HSD oxo-reductase activity induced by IL-1β

Comparison between experimental conditions (median oxo-reductase activity, pg/ml/hour)		**Wilcoxon Z statistic**	**P value**
Control (2.13)	IL-1β 1ng/ml (2.60)	-2.490	0.013
Control (2.13)	IL-1β 10 ng/ml (2.38)	-2.934	0.003
IL-1β 1ng/ml (2.60)	IL-1β 10 ng/ml (2.38)	-0.889	0.374

Figure 5.3 Influence of IL-1β on AM 11β-HSD oxo-reductase activity (Friedman test, n= 5, $\chi^2(2)=9.45$, p=0.009)



5.4.4 Lipopolysaccharide decreases 11β-HSD oxo-reductase activity in primary alveolar macrophages

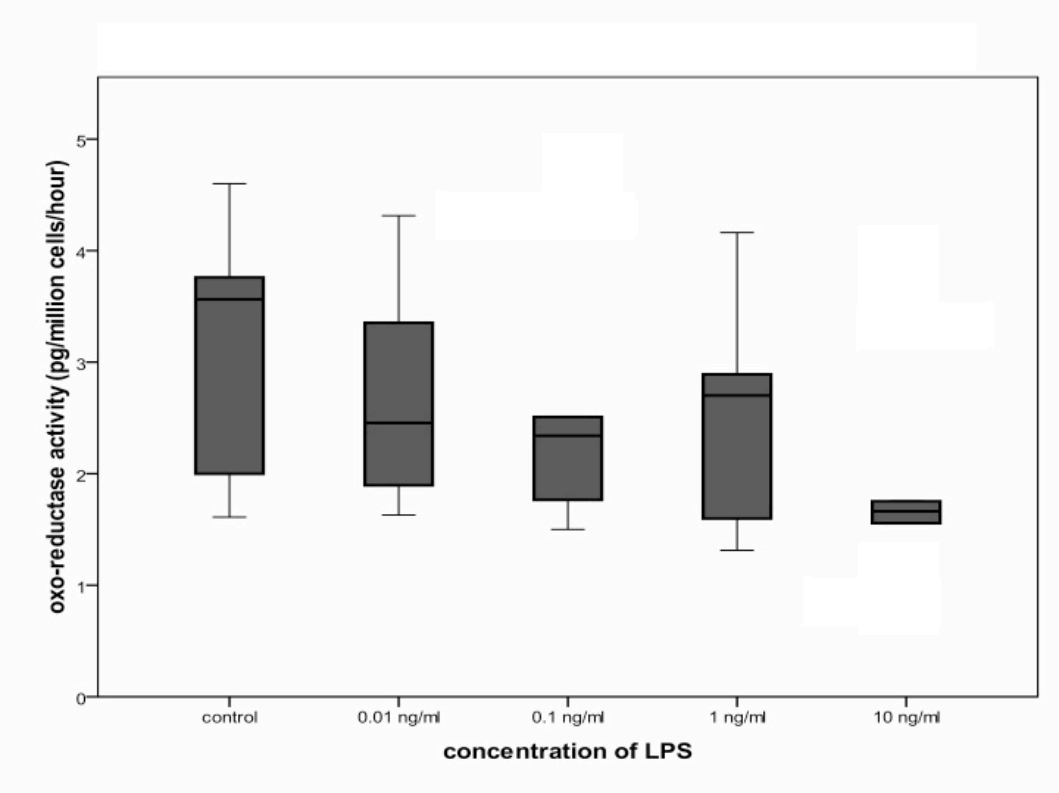
Unlike the effect of both IL-1β and TNF-α, Lipopolysaccharide (LPS) caused a decrease in the oxo-reductase activity of primary alveolar macrophages (Friedman's test, n=5, $\chi^2(3)=16.16$, p=0.003). *See figure 5.4*. As an overall difference between groups was found, we assessed individual differences between groups. *See table 5.4*. No difference was demonstrated between the control group and the group incubated in 0.01ng/ml LPS (Wilcoxon test Z(5)= -1.753, p=0.08), but as we were able to demonstrate differences between many of the other groups a dose response of LPS on AM 11β-HSD oxo-reductase activity can be seen.

We were again unable to demonstrate differences in the 11β-HSD dehydrogenase activity between these cell culture conditions (Friedman's test, $\chi^2(3)=1.288$, p=0.863).

Table 5.3 Comparisons of 11β-HSD oxo-reductase activity induced by differing concentrations of LPS

Comparison between experimental conditions (median oxo-reductase activity, pg/ml/hour)		**Wilcoxon Z statistic**	**P value**
Control (2.13)	LPS 0.01ng/ml (2.45)	-1.753	0.08
Control (2.13)	LPS 0.1ng/ml (2.34)	-2.023	0.043*
Control (2.13)	LPS 1ng/ml (2.70)	-2.023	0.043*
Control (2.13)	LPS 10ng/ml (1.66)	-2.023	0.043*
LPS 0.01ng/ml (2.45)	LPS 0.1ng/ml (2.34)	-1.483	0.138
LPS 0.01ng/ml (2.45)	LPS 1ng/ml (3.91)	-0.944	0.345
LPS 0.01ng/ml (2.45)	LPS 10ng/ml (1.66)	-2.023	0.043*
LPS 0.1ng/ml (2.34)	LPS 1ng/ml (2.70)	-0.405	0.686
LPS 0.1ng/ml (2.34)	LPS 10ng/ml (1.66)	-2.023	0.043*
LPS 1ng/ml (2.70)	LPS 10ng/ml (1.66)	-2.023	0.043*

Figure 5.4 Influence of LPS on AM 11β-HSD oxo-reductase activity (Friedman test, n= 5, $\chi^2(2)=9.45$, p=0.009)



5.4.5 HMGB-1 has no clear effect on 11β-HSD activity

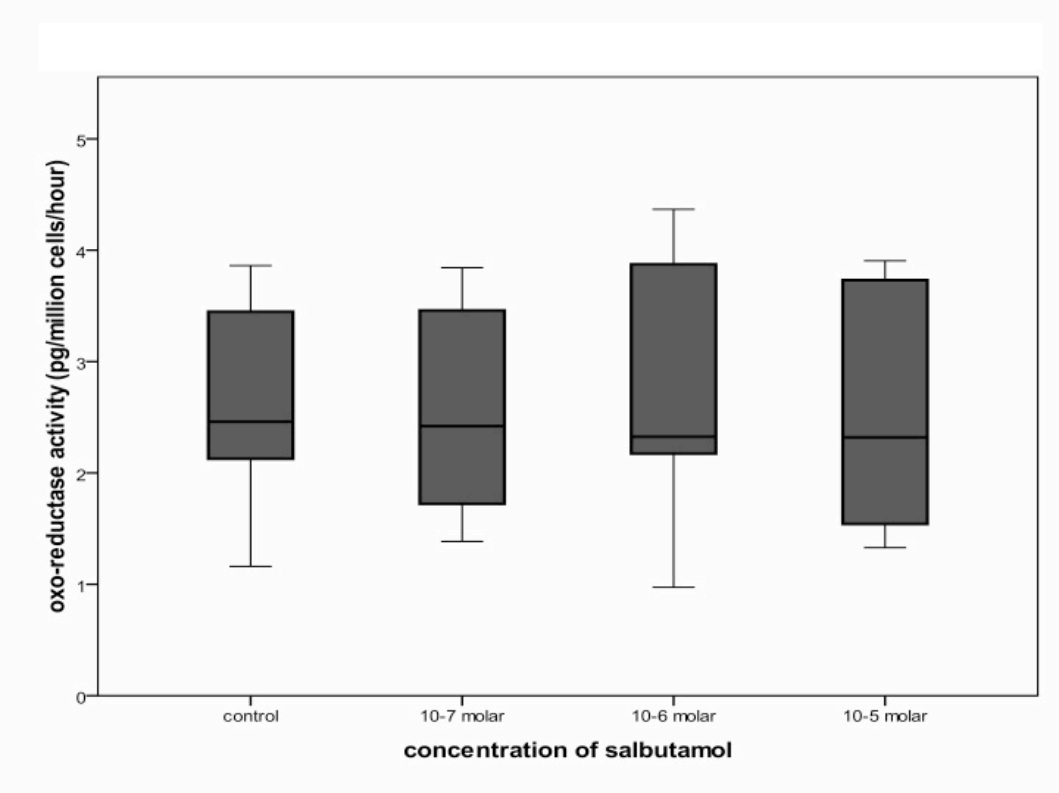
Incubation of our primary AM in differing concentrations of HMGB-1 failed to induce any clear difference in the 11β-HSD oxo-reductase activity of these cells (Friedman test $\chi^2(3)=2.70$, p=0.440). No demonstrable difference in the 11β-HSD dehydrogenase activity was induced by HMGB-1 using the dose range investigated either (Friedman test $\chi^2(3)=1.20$, p=0.753). No between groups effects were calculated.

5.4.6 Salbutamol has no detectable effect on 11β-HSD oxo-reductase activity in un-stimulated primary alveolar macrophages

The effect of salbutamol on the 11β-HSD activity was investigated by incubating primary alveolar macrophages, which had been stimulated in no other way, with differing concentrations of this drug prior to the enzyme activity assay. A 10^{-6} molar concentration of salbutamol was used as a midpoint of our concentration range as this is considered to be a significant therapeutic concentration present within the alveolar space.[228]

No discernable effect of this drug was demonstrable overall in the oxo-reductase (Friedman's test, $\chi^2(4)=2.70$, $p=0.440$), or the dehydrogenase (Friedman's test, $\chi^2(4)=1.80$, $p=0.615$. *See figure 5.5.* No difference was therefore present between any of the specific concentrations used in these dose response experiments. Data was available from a larger number of AM sources for the difference between a salbutamol 10^{-6} molar concentration and control, and despite this larger sample, no difference in the 11β-HSD oxo-reductase activity (Wilcoxon signed rank test: $Z(19)=-0.037$, $p=0.970$) or the dehydrogenase activity (Wilcoxon signed rank test: $Z(18)=-0.369$, $p=0.711$) was demonstrated.

Figure 5.5 Influence of salbutamol on AM 11β-HSD oxo-reductase activity (Friedman's test, n=5, $\chi^2(4)=2.70$, p=0.440)



5.4.7 Effect of salbutamol on the 11β-HSD activity induced by pro-inflammatory mediators

As salbutamol has been shown to have inflammatory action, we tested whether the co-incubation of this drug with our stimulated AMs would cause a decrease in the changes in 11b-HSD activity observed. As no difference in the 11β-HSD dehydrogenase activity had been detected in the experiments using pro-inflammatory mediators, this was not investigated in this series of experiments.

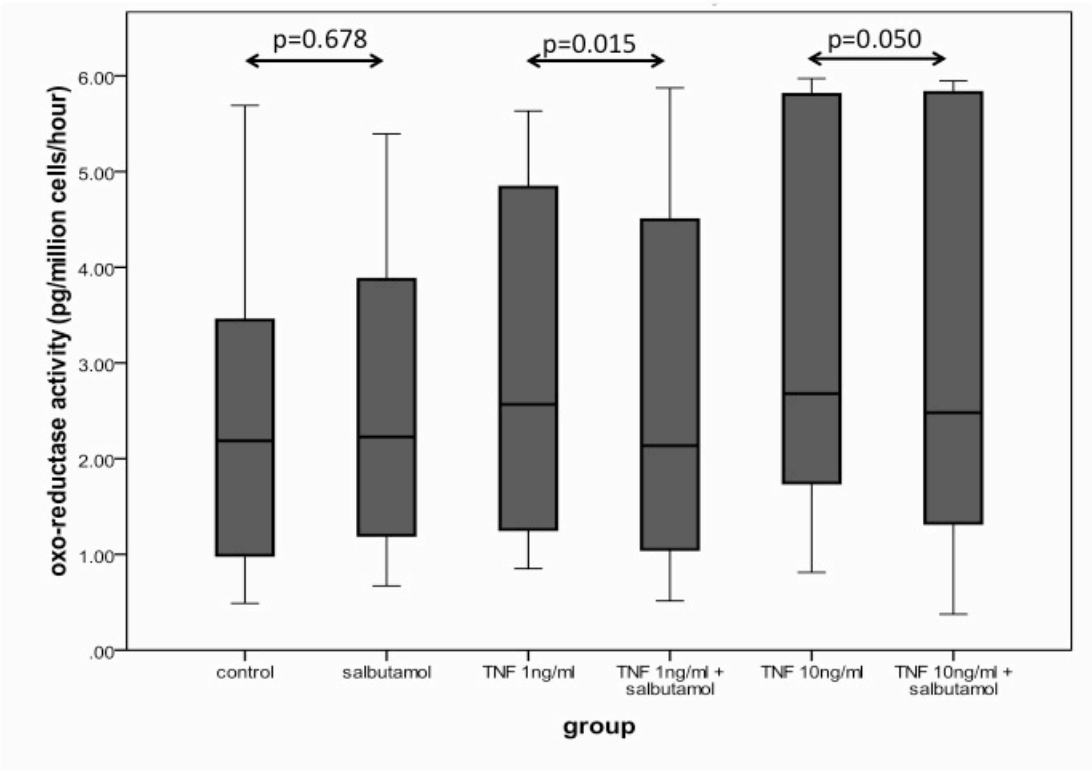
5.4.7.1 Salbutamol abrogates the TNF-α induced up-regulation of 11β-HSD in primary alveolar macrophages through a mechanism mediated by the β-adrenoceptor.

Comparisons were made between AMs incubated in TNF-α at concentrations of both 1ng/ml and 10ng/ml, and AMs in the same concentrations of TNF-α incubated together with a 10^{-6} molar concentration of salbutamol. Although, as before, no difference was present between un-stimulated AM and those cultured in salbutamol, Salbutamol decreased the 11β-HSD oxo-reductase activity caused by 1ng/ml TNF-α (Wilcoxon signed rank test: Z(17)= -2.438, p=0.015) and 10ng/ml TNF-α (Wilcoxon signed rank test: Z(12)=-1.961, p=0.050). *See figure 5.6*. We then tested the hypothesis that the effect of salbutamol on these AM was mediated through the β-adrenoceptor on the cell surface, and that blockade of this receptor would cause an up-regulation of oxo-reductase activity. We therefore conducted a series of experiments using the β-adrenoceptor antagonist propanolol. Propanolol caused a diminution of the effect of salbutamol on the TNF-α 1ng/ml stimulated AM (median oxo-reductase activity= 3.41 vs. 3.73 pg/million cells/hour, Wilcoxon signed rank test: Z=-1.826, single tailed significance: p=0.034).

5.4.7.2 Salbutamol does not influence the up-regulation of 11β-HSD oxoreductase activity induced by IL-1β

As for the experiments with TNF-α, we co-incubated cells in a 10^{-6} concentration of salbutamol and concentration of IL-1β of 1ng/ml and 10ng/ml. No differences were detectable between the salbutamol and IL-1β treated cells, and the cells treated with IL-1β alone for either 1ng/ml IL-1β (Wilcoxon signed rank test, Z(5)=-1.511, p=0.131), or 10ng/ml (Wilcoxon signed rank test, Z(5)=-0.296, p=0.767).

Figure 5.6 The effect of co-stimulation of AM with TNF-α and salbutamol on 11β-HSD oxo-reductase activity

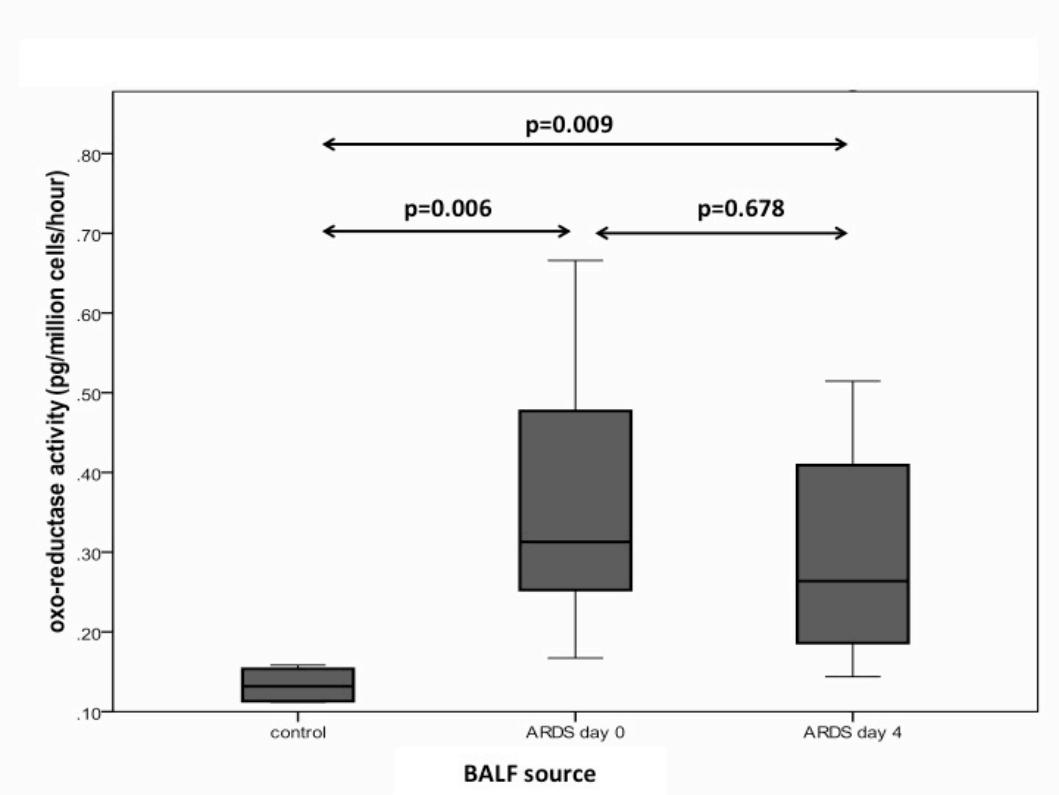


5.4.8 BALF from patients with ARDS increases alveolar macrophage 11β-HSD oxo-reductase activity

To determine whether factors present in the extra cellular milieu of the alveolar macrophages patients with ARDS was responsible for differences in 11β-HSD activity observed, we used BALF taken from patients with ARDS to model as closely as possible the extracellular environment *in vivo* that these cells are exposed to. AMs taken from lung resection specimens were therefore incubated with a mixture of RPMI /10% FCS and BALF from patients with ARDS, and normal controls.

Cells were incubated with either BALF from patients at day 0 of ARDS, day 4 of ARDS, or normal controls. An overall difference in the conversion of cortisone to cortisol was present between these groups (Kruskal Wallis test n=24, H(3)=8.86, p=0.010). *See Figure 5.7*. When performing sub-group analyses, the 11β-HSD oxo-reductase activity of the AMs was increased by incubation with BALF of patients at onset of ARDS (median= 0.313 vs. 0.127 pg/million cells/hour, U(16)=0.0, p=0.006), and day 4 of ARDS (median= 0.127 vs. 0.264 pg/million cells/hour, U(16)=1.0, p=0.009), when compared to that of normal controls. No difference was seen between the up-regulation in the oxo-reductase activity between the BALF taken at onset of ARDS, and day 4 of the condition (median= 0.313 vs. 0.264 pg/million cells/hour, U(16)=44.0, p=0.678).

Figure 5.7 Influence of incubation of AM with BALF from patients at day 0 (n=8) and day 4 (n=8) of ARDS and normal controls (n=8) on 11β-HSD oxo-reductase activity



5.5 Discussion

The preceding chapter in this thesis demonstrated that cortisol concentrations were increased in the lungs of patients with ARDS. It also showed that the cortisol: cortisone ratio was increased in the alveolar space of patients with ARDS, suggesting increased local activity of 11β-HSD type 1 oxo-reduction of cortisone to cortisol. In this chapter we have demonstrated that the inflammatory mediators TNF-α and IL-1β, which have both previously been demonstrated to be key mediators in ARDS,[10, 221] cause an up-regulation of 11β-HSD oxo-reductase activity in primary human alveolar macrophages.

The effect of the pro-inflammatory cytokines TNF-α and IL-1β on 11β-HSD activity has been observed previously in other cell types. Cooper et al. showed in osteoblasts that not only did these cytokines increase the 11β-HSD type 1 conversion of cortisone to cortisol, a suppression of the deactivation of cortisol to cortisone by 11β-HSD type 2 was also observed.[64] Similar responses to these cytokines have been seen in many other cell types including adipocytes, hepatocytes,[62] smooth muscle,[65] and amnion and skeletal muscle fibroblasts. [54, 63]

That LPS caused a decrease in the 11β-HSD oxo-reductase activity is unexpected given the finding that the other inflammatory molecules caused an up-regulation of this activity. We are unable to find any other reports of a similar response to LPS stimulation. Indeed, other investigators have demonstrated that human macrophage 11β-HSD type 1 mRNA and activity are up-regulated by LPS.[210] Why we should have observed a different result is not clear, especially as the action of LPS on macrophages is to cause them to produce TNF-α, which we have shown to up-regulate rather than down-regulate oxo-reductase activity. An altered response to LPS in these macrophages is a possibility, but further investigation is needed before such a finding should be accepted.

HMGB-1 is released from activated macrophages in response to pro inflammatory mediators such as TNF-α, IL-1β or LPS, and also causes the release of TNF-α and IL-1β from macrophages.[223] It is thought to be a late mediator of sepsis and inflammation.[224] We were not able to show any alteration in 11β-HSD activity induced by stimulation of the alveolar macrophages with this mediator. The absence of an increase in activity in response to HMGB-1, together with the declining increase in activity induced by BALF from day 4 of ARDS suggest therefore that the induction of 11β-HSD oxo-reductase

activity may be a phenomenon situated early, rather than late, in the signalling pathway of ARDS.

We were also unable to demonstrate a consistent effect of salbutamol on otherwise un-stimulated alveolar macrophages. However, when co-stimulated with the pro-inflammatory cytokine TNF-α, salbutamol caused a down-regulation of the induced increase in oxo-reductase activity, through the activation of the β-adrenoceptor. Why salbutamol should only cause such a response in co-stimulated cells is not clear: It is possible that the salbutamol induced changes are only detectable with greater activity, but also that the mechanism by which salbutamol acts is through a pathway activated only by the cytokines in question. In contrast to our findings Friedberg et al. demonstrated that although the 11β-HSD type 1 activity in adipocytes was increased by pro-inflammatory cytokines, it was also increased by salbutamol.[56] That we showed an opposite effect is likely to represent phenotypic differences between the cells studied. A tissue specific response of 11β-HSD to similar stimulations has been demonstrated by Tomlinson et al. between adipocytes and hepatocytes.[62]

The experiments incubating alveolar macrophages with BALF from patients with ARDS were designed to approximate the conditions that alveolar macrophages might be exposed to in-vivo, and in so doing examine the mechanisms by which the observed enzyme activity develops. These experiments have many factors that require a caution to be used in their interpretation. The culture conditions consist of BALF mixed in a 1:1 ratio with the normal culture medium, leading to a less physiological environment for these cells. Despite these caveats the increase in the oxo-reductase activity of alveolar macrophages caused by incubation with ARDS BALF is nevertheless convincing. These

results are in keeping with our findings that pro-inflammatory cytokines up-regulate the 11β-HSD oxo-reductase activity.

Macrophage 11β-HSD type 1 activity is important in the host response to injury and the resolution of inflammation. Macrophages are responsible for the safe disposal of spent inflammatory cells that, and 11β-HSD activity promotes this process in animal models by increasing available intracellular cortisol.[75] Failure of uptake of apoptotic cells leads these dying cells to undergo secondary necrosis and release further pro-inflammatory mediators,[229] leading to further tissue damage and the perpetuation of the inflammatory process.[230] Any failure of these mechanisms, and an insufficient up-regulation of 11β-HSD oxo-reductase activity in alveolar macrophages would therefore be of functional importance in the resolution of inflammation in ARDS. If LPS were to be present in sufficient concentrations to suppress 11β-HSD oxo-reductase activity, this could lead to prolonged disease in ARDS.

One further consideration of the interpretation of these experiments is that in this only one cell type has been studied. Alveolar macrophages are essential effector cells in the resolution of ARDS, and 11β-HSD activity within these cells will influence their function. However, the total lung cortisol: cortisone ratio cannot be solely attributed to these cells. The response of other cell types within the lung to inflammatory stimuli should be further investigated. The histopathology study performed by Suzuki et al located an increase in 11β-HSD type 2 in the alveolar cell wall, as well as in CD68 positive cells in the alveolar lumen.[69] Our results show that at least in these CD68 positive resident alveolar macrophages, inflammatory stimuli do not increase net local cortisol production. We cannot as yet inform on the activity present in other lung cells.

In conclusion, these experiments have shown that the 11β-HSD activity oxo-reductase activity of primary human alveolar macrophages is increased by pro-inflammatory cytokines, and in a BALF model of ARDS. LPS However decreases this activity. These findings provide a cellular mechanism for the increased cortisol: cortisone ratio present in the lungs of patients with ARDS that was identified in the previous chapter. This increase in 11β-HSD oxo-reduction of cortisone to cortisol amplifies the anti-inflammatory signal by increasing available cortisol.

6 Determinants of efferocytosis in ARDS

6.1 Abstract

Introduction: Efferocytosis is the process by which apoptotic cells are taken up by phagosomes such as the alveolar macrophage. It is an essential process to prevent apoptotic cells undergoing secondary necrosis and releasing inflammatory mediators. Increased numbers of apoptotic neutrophils are found in the lungs of patients with the inflammatory condition ARDS, and represent a particular challenge for this system of apoptotic cell disposal. Previous investigations have shown that glucocorticoids increase the rate of efferocytosis in monocyte-derived macrophages. We used primary alveolar macrophages to investigate how efferocytosis is influenced by glucocorticoids and salbutamol, which augments glucocorticoid action. Investigations in this thesis have demonstrated that glucocorticoid metabolism by 11β-hydroxysteroid dehydrogenase in these cells is altered by inflammatory mediators. Therefore we investigated whether this enzymes function and the inflammatory mediators that coordinate inflammation in ARDS cause a change in the rate of efferocytosis.

Methods: Primary alveolar macrophages were collected from lung resection specimens and incubated in conditions to model the inflammation of ARDS and test the influence of glucocorticoids and salbutamol. Neutrophils from healthy volunteers that had been stained and induced to undergo apoptosis were then added in a fourfold excess to the macrophages. The proportion of macrophages that had engulfed a neutrophil was then measured and expressed as the efferocytosis index (EI)

Results: *Results*: Cortisol increases efferocytosis compared to control (median EI=9.65 vs. 2.26%, n=6, p=0.005). Cortisone also increased efferocytosis (median EI=9.60 vs. 2.26%, n=6, p=0.028), but this was negated by blockade of enzymatic conversion of cortisone to cortisol (median EI=9.60 vs. 7.30, n=6, p=0.043). Salbutamol increases the

rate of efferocytosis in a dose dependent manner (n=9, p=0.005), this effect is abrogated by the b-blocker propranolol, suggesting that this effect is through the β-adrenoceptor (median EI= 8.25 vs. 6.95%, n=6, p=0.028). Efferocytosis was increased by TNF-α, but decreased by HMGB-1 (n=9, p=0.017). Efferocytosis was decreased by macrophage incubation with BALF from day 0 of ARDS (median EI= 8.05 vs. 11.32%, n=8, p=0.036), but increased by incubation with BALF from patients at day 4 of ARDS (median EI=15.51 vs. 11.32%, n=8 , p=0.012). The extent of change in EI had a negative linear relationship with HMGB-1 concentration in BALF used to stimulate the macrophages (n=18, ρ=-0.581, p=0.011).

Conclusion: Alveolar macrophage efferocytosis of apoptotic neutrophils is augmented by β-adrenoceptor agonists and the glucocorticoid axis; systems that have been postulated as treatments for patients with ARDS. HMGB-1 and TNF-α influence ARDS in opposite directions; which may be responsible for changes in efferocytosis at different times in the course of ARDS.

6.2 Introduction

The resolution of acute inflammation relies upon the recognition of apoptotic inflammatory cells, followed by their uptake and safe disposal by phagosomes, in a process termed efferocytosis.[126, 138, 231] Failure of this process leads to apoptotic cells undergoing secondary necrosis, and in so doing release pro-inflammatory cell contents into the extra-cellular environment, causing further tissue damage.[232, 233] Efferocytosis is itself an anti-inflammatory process, by both preventing further inflammation of secondary necrosis, decreasing macrophage release of pro-inflammatory mediators[234] and increasing the release of anti-inflammatory cytokines from the phagosome.[233]

Neutrophils exposed to conditions of ARDS live longer than un-stimulated cells;[235] however increased quantities of necrotic cells are seen within the lungs in animal models of ARDS.[236] This suggests that the demand for the process of efferocytosis exceeds the capacity of lung phagosomes to remove apoptotic cells. This failure of efferocytosis will therefore result in a perpetuation of inflammation of ARDS.

Glucocorticoids influence efferocytosis in a number of different ways: they contribute to the longevity of neutrophils,[137] but the process of efferocytosis is also promoted by glucocorticoids.[128, 227] In tissue the active glucocorticoid cortisol is produced from the inactive molecule cortisone by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1.[76, 126] This enzymes expression and activity are increased in various cells in response to inflammatory stimuli. Indeed our own experiments in the preceding chapter have shown that the oxo-reduction of cortisone to cortisol is increased in primary alveolar macrophages by inflammatory mediators, and by broncho-alveolar lavage fluid (BALF) taken from patients with ARDS. Peripheral blood monocytes only express the 11β-HSD type 1 iso-enzyme as they mature to macrophages, simultaneously developing the capacity to take up other cells by phagocytosis.[126] 11β-HSD has previously been linked to the up-regulation of efferocytosis required for the resolution of acute inflammation.[75] β-adrenoceptor agonists are known to augment the activity of glucocorticoids in other models of disease,[237] as well as having anti-inflammatory effects themselves.[238] Little information is available on the effect of β-adrenoceptor agonists on efferocytosis.

In this investigation we tested the influence of various factors on the process of efferocytosis of apoptotic neutrophils by alveolar macrophages. We conducted experiments using lipopolysaccharide (LPS), an initiator of inflammation,[222] TNF-α, an

inflammatory cytokine known to be a mediator of ARDS,[10, 221] and HMGB-1, a late mediator of ARDS,[223-225] to determine their effect on efferocytosis, and how different processes might predominate during the course of this condition. We also used BALF taken from patients at onset of ARDS and after 4 days of ARDS to model this condition at these time-points. As efferocytosis is known to be increased by glucocorticoids we also investigated the effect these steroids, and 11β-HSD activity have on the efferocytosis of apoptotic neutrophils by alveolar macrophages. Further we tested whether the β-adrenoceptor agonist salbutamol had an effect on efferocytosis.

6.3 Methods

This investigation is a laboratory study of the effects of cell stimulation on alveolar macrophage efferocytosis of apoptotic neutrophils. Experimental methods are described in detail in Chapter 3, and summarised below:

6.3.1 Sample collection, processing and cell culture

Alveolar macrophages (AM) were extracted from lung resection specimens collected as part of the midlands lung tissue collaboration, using density gradient techniques. They were incubated at 37^{o}c in a 5% CO_2 atmosphere overnight and then washed, prior to setting up stimulation conditions. They were incubated in these conditions for 24 hours and subsequently used in the efferocytosis assay as described below. BALF was collected at bronchoscopy from patients with ARDS, as diagnosed by AECC criteria.[3] These patients were recruited as part of a bronchoscopic sub-study of the BALTI-2 investigation of salbutamol treatment for ARDS. BALF was filtered and frozen at -80^{o}c for subsequent analysis. HMGB-1 concentration in BALF was measured using a commercially available ELISA kit (Shino-Test Corporation, Tokyo, Japan).

6.3.2 Efferocytosis assay

Polymorphic neutrophils from healthy volunteers were extracted from peripheral venous blood using density gradient media, and incubated for 30 minutes with Celltracker green^{TM} (Invitrogen life sciences, Paisley, UK) to render them visible under fluorescent microscopy, and detectable using a FL1 channel of a flow cytometer. The neutrophils were then left in culture at 37^{o}c in a 5% CO_2 atmosphere for 20 hours to undergo apoptosis.[126] The culture medium was then removed from the macrophages, and replaced with the suspension of apoptotic neutrophils, in a fourfold excess of neutrophils to macrophages. Cells were incubated in these conditions for 90 minutes, prior to aspiration of non-engulfed neutrophils. Culture wells were then vigorously washed with PBS to remove all non-adherent neutrophils. The number of macrophages that had engulfed a neutrophil and the total number of macrophages was calculated using direct microscopy and flow cytometry techniques. An efferocytosis index (EI) was then calculated as follows:

$$\text{Efferocytosis index (EI)} = \frac{\text{macrophages that have engulfed a neutrophil}}{\text{Total number macrophages}} \times 100$$

6.3.3 Statistical analysis

Statistical analysis was done using the statistical software SPSS (IBM, Somers, New York, USA). Data was analysed using the non-parametric techniques, Mann Whitney and ANOVA by ranks. Linear associations were tested using Spearman's correlation coefficient for non-parametric data.

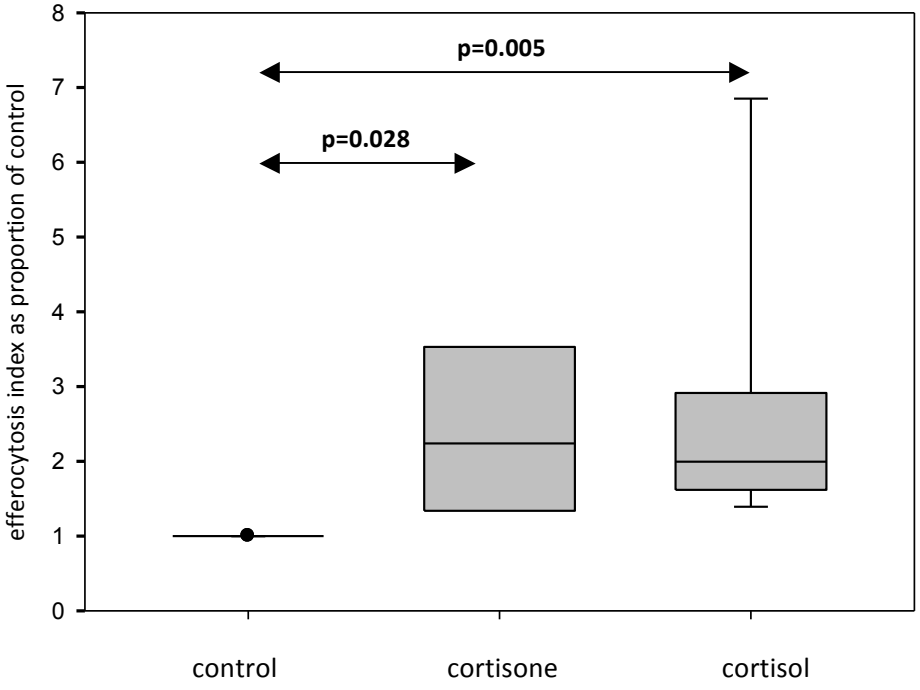
6.4 Results

6.4.1 Glucocorticoids up-regulate efferocytosis

When stimulated with the glucocorticoids cortisol or cortisone an increase in the rate of uptake of apoptotic neutrophils was found. A physiological 10^{-7}molar concentration of cortisone increased median EI from 2.26 to 9.60 (Z(6)=-2.201, p=0.028), and the same concentration of cortisol increased EI from 2.26 to 9.65 (Z(6)=-2.807, p=0.005). *See figure 6.1.* To assess this further dose response experiments were conducted to determine the half maximal effective concentration (EC_{50}) of cortisol and cortisone. The 4-parameter logistic non-linear regression analysis was used to calculate this value. The EC_{50} for cortisol was calculated using a concentration range between 10^{-9} molar and 10^{-4} molar concentrations of cortisol. *See figure 6.2*. The EC_{50} was found to be a $10^{-8.38}$ molar concentration. A similar experiment was performed for cortisone, using the same dose gradient. *See figure 6.3*. The EC_{50} for cortisone was a $10^{-8.68}$ molar concentration.

To assess the influence of glucocorticoid metabolism by alveolar macrophages on efferocytosis the 11β-HSD inhibitor Glycyrrhetinic acid (GE) was used to block the inter-conversion of cortisol to cortisone within AM. No difference was seen between un-stimulated cells and those incubated in a 10^{-7} molar concentration of GE (median EI= 4.90 vs. 5.5, Z(6)= -1.682, p=0.093). GE caused a down-regulation of efferocytosis in cortisone treated cells (median EI=9.60 vs. 7.30, Z(6)=-2.023, p=0.043), but no clear effect in cortisol treated cells (median EI= 13.4 vs. 9.65, Z(6)=-1.363, p=0.173). These results are strongly suggestive that cortisol is required for the up-regulation of efferocytosis by glucocorticoids, and that activity of the enzyme 11β-HSD is required to convert cortisone to cortisol in order for cortisone stimulation to increase efferocytosis. *See figure 6.4.*

Figure 6.1 Effect of stimulation of alveolar macrophages with cortisol or cortisone on efferocytosis of apoptotic neutrophils



Results are expressed as proportion of control

Figure 6.2 The effect of increasing concentrations of cortisol on efferocytosis of apoptotic neutrophils by alveolar macrophages (EC_{50}= $10^{-8.38}$ M).

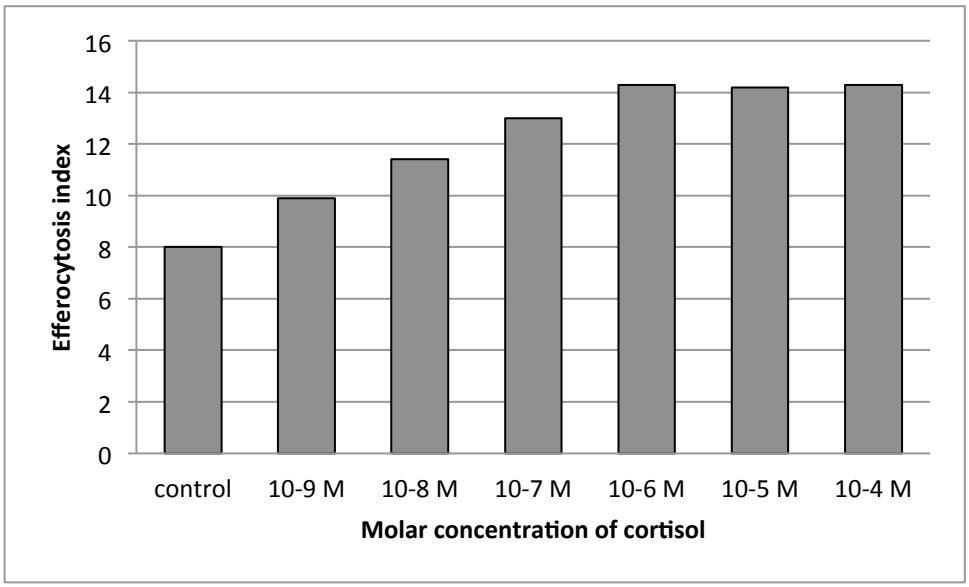


Figure 6.3 The effect of increasing concentrations of cortisone on efferocytosis of apoptotic neutrophils by alveolar macrophages (EC_{50}= $10^{-8.68}$ M).

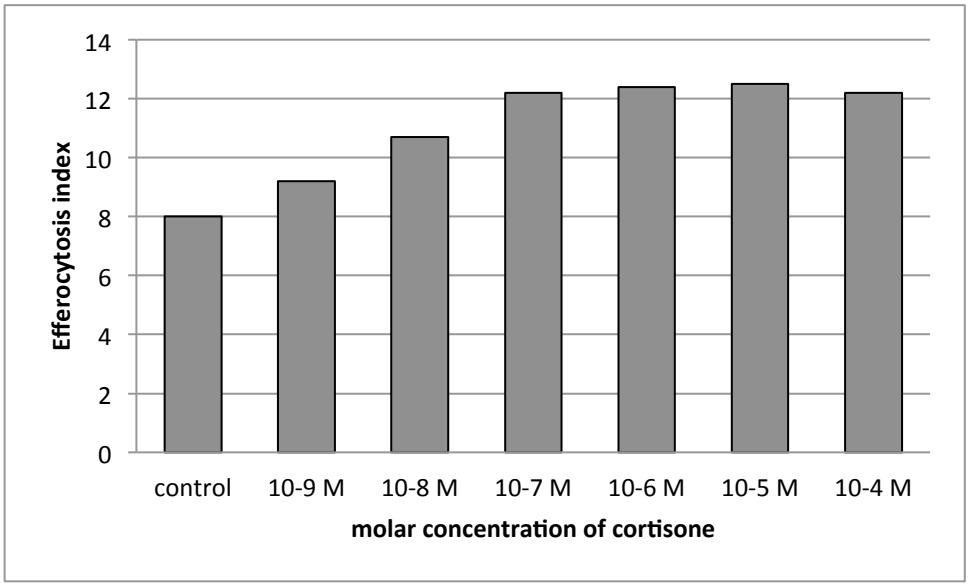
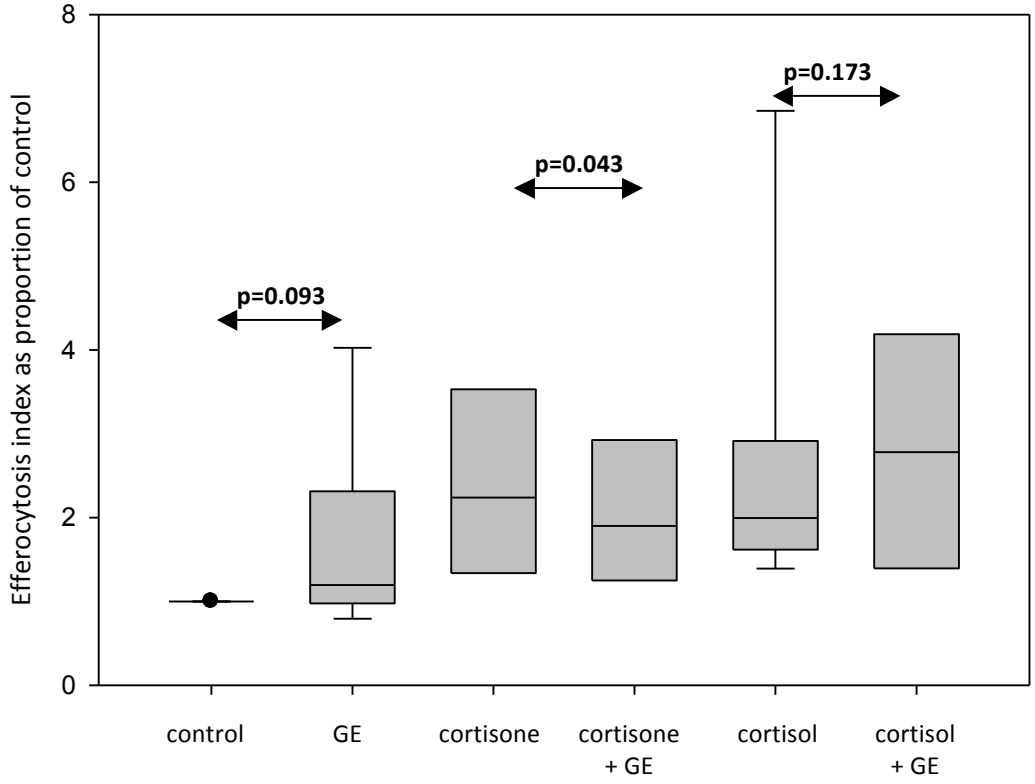


Figure 6.4 The effect of blockade of 11β-HSD enzyme activity by glycyrrhetinic acid on steroid influenced efferocytosis



Results are expressed as proportion of control

6.4.2 Effect of salbutamol on efferocytosis

Stimulation of alveolar macrophages with increasing concentrations of salbutamol caused the up-regulation of efferocytosis of apoptotic polymorphic neutrophils in a dose dependent manner (Friedman's ANOVA: n=6, $c^2(3)=12.864$, n=9, p=0.005). Between groups analysis showed differences in the EI of cells between control (median=2.26) and salbutamol concentrations at 10^{-5} molar (median EI= 8.15, Z(6)=-2.023, p=0.043), 10^{-6} molar (median EI=7.10, Z(6)= -2.578, p=0.010) and 10^{-7} molar (median EI=5.50, Z(6)=-2.201, p=0.028). Differences were not detected between EI of cells incubated in 10^{-7} molar and 10^{-6} molar salbutamol (Z(6)=-1.153, p=0.249), but were present between the

10^{-7} and 10^{-5} (Z(6)=-2.201, p=0.028). EI was also greater in cells incubated in 10^{-5} compared to 10^{-6} molar salbutamol (Z(6)=-2.207, p=0.027). *See figure 6.5.*

Up-regulation of efferocytosis by salbutamol can be abrogated by the co-incubation of alveolar macrophages with a beta-blocker. Salbutamol treated alveolar macrophages were co-stimulated with a 10^{-6} molar concentration of propranolol. No difference between control and propranolol treated cells was found (median EI =5.15 vs 5.35 Z(6)=-0.106, p=0.916). The addition of propranolol to salbutamol (10^{-6} molar) stimulated cells caused a down-regulation of efferocytosis (median EI= 8.25 vs. 6.95, Z(6)=-2.201, p=0.028), strongly suggesting that the up-regulation of efferocytosis caused by the β-adrenoceptor agonist salbutamol is mediated through the β-adrenoceptor. *See figure 6.6.*

Figure 6.5 Effect of stimulation with salbutamol on efferocytosis of apoptotic neutrophils by alveolar macrophages (Friedman's ANOVA: $\chi^2(3)=12.864$, n=9, p=0.005)

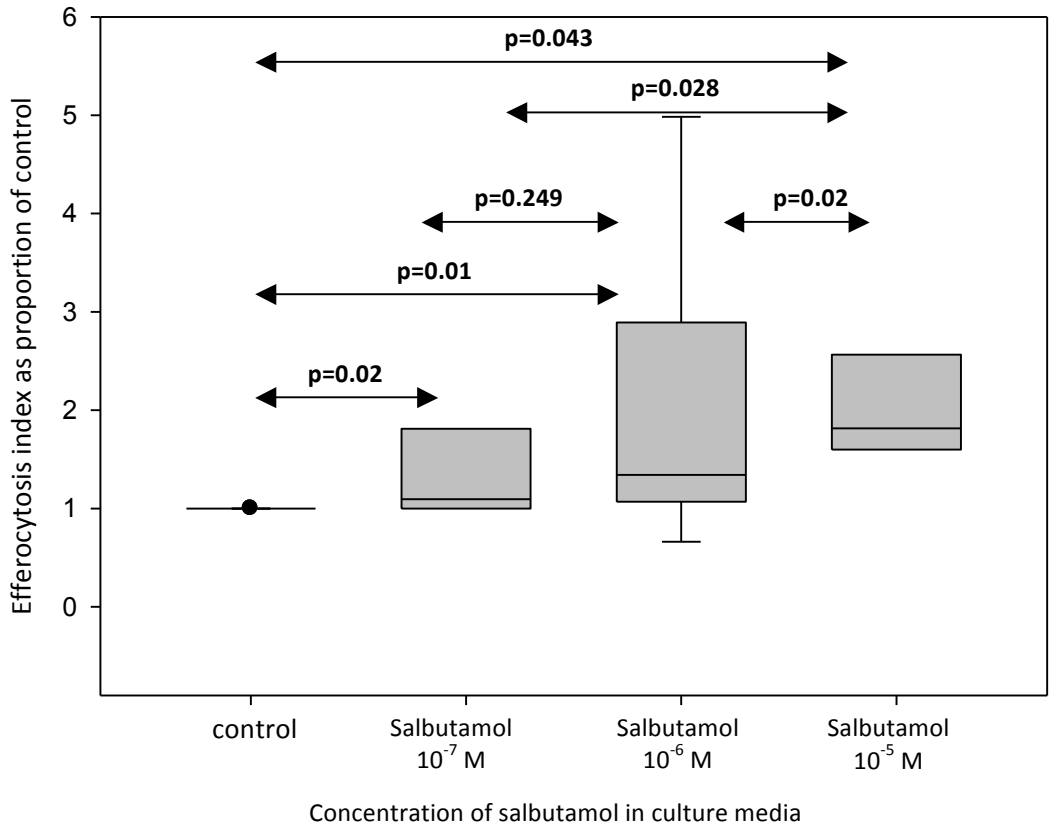
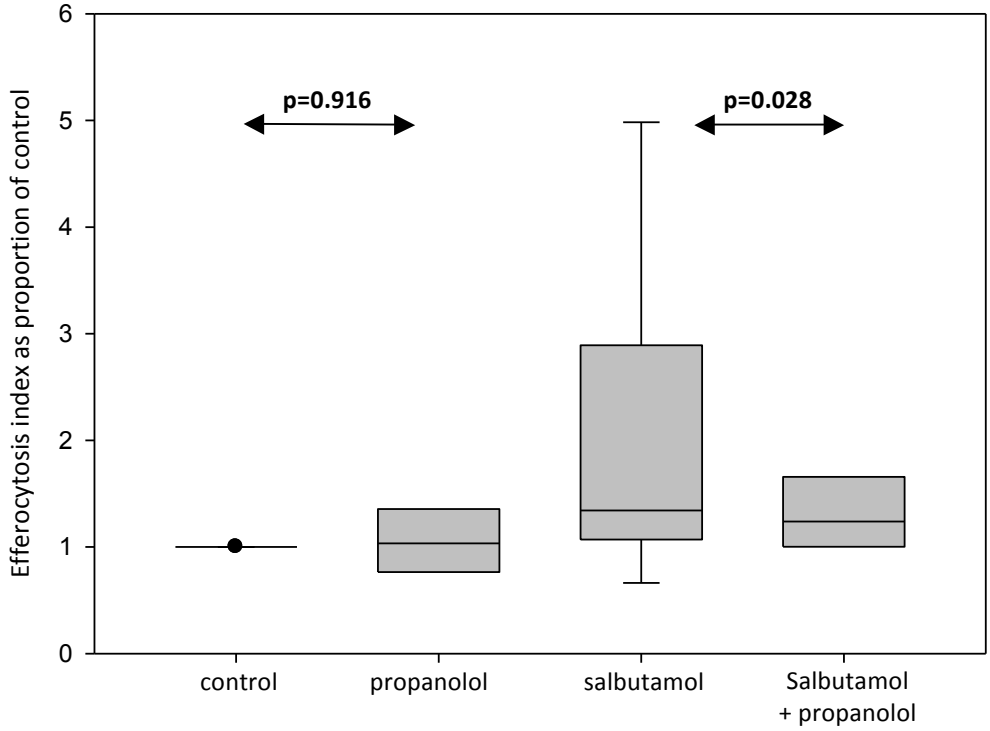


Figure 6.6 Effect of co-stimulation of alveolar macrophages with salbutamol and propanolol on efferocytosis of apoptotic neutrophils

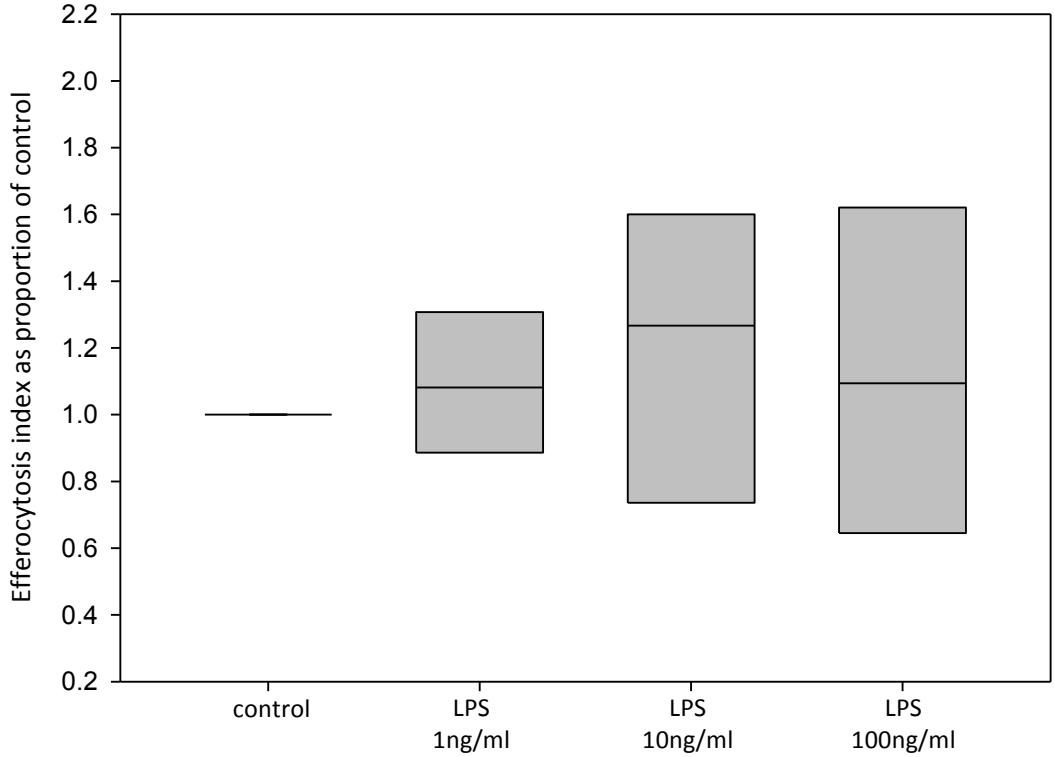


Salbutamol treated groups were incubated in a 10^{-6} molar concentration of salbutamol with and without a 10^{-6} molar concentration of propanolol. Results are expressed as proportion of control efferocytosis index

6.4.3 Effect of LPS on efferocytosis

To determine the effect of LPS on the efferocytosis of apoptotic neutrophils by alveolar macrophages; macrophages were incubated either un-stimulated, or in ascending concentrations of 1ng/ml, 10ng/ml or 100ng/ml LPS. Although increases in median EI values were observed (median EI=6.74, 6.23, 8.82 and 7.36 respectively), the differences between these groups failed to meet the pre-defined criteria of significance (Friedman's ANOVA: n=6, $\chi^2(3)= 1.0$, p=0.844). *See figure 6.7*.

Figure 6.7 The effect of incubation with LPS on efferocytosis of apoptotic neutrophils by alveolar macrophages (n=6, $\chi^2(3)= 1.0$, p=0.844)

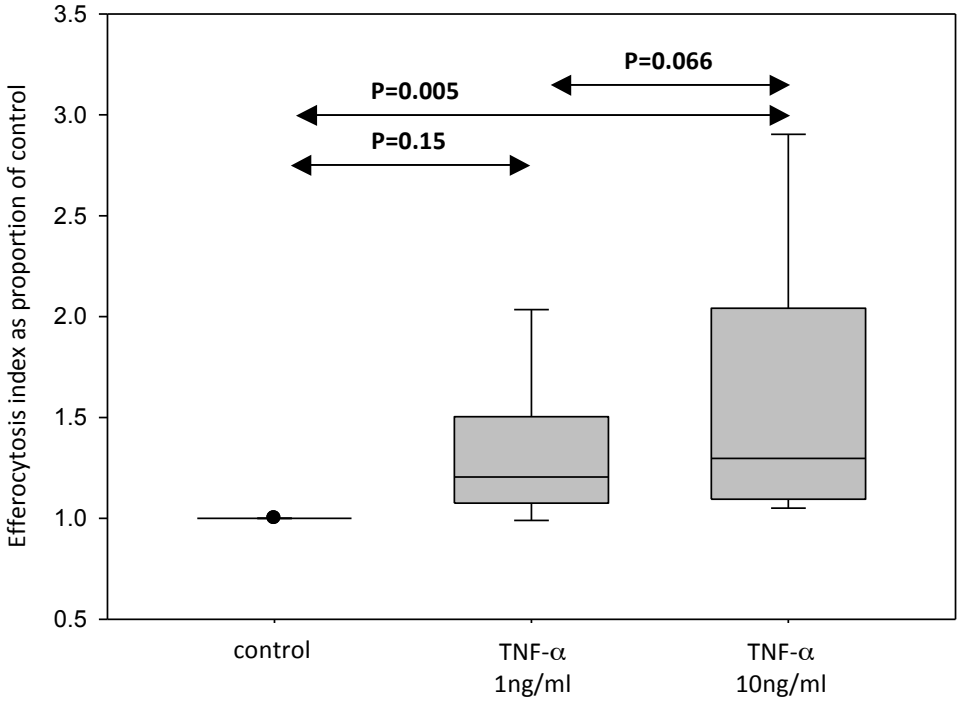


Results are expressed as proportion of control efferocytosis index

6.4.4 TNF increases efferocytosis of apoptotic neutrophils by alveolar macrophages

Stimulation of alveolar macrophages with the pro-inflammatory cytokine TNF-α caused an increase in the rate at which these cells engulfed apoptotic neutrophils in a dose dependent manner (Friedman test; n=9 $\chi^2(2)=11.556$, p=0.003). Between groups analysis showed differences between control and stimulation with TNF-α at both 1ng/ml (median EI= 1.86 vs 2.49, Z(9)=-2.429, p=0.015) and 10ng/ml (median EI=1.86 vs. 2.62, Z(9)=-2.803, p=0.005). Although between stimulation with 1ng/ml and 10ng/ml of TNF-α the difference in EI did not meet our pre-defined criteria for statistical significance (median EI=2.62 vs.2.49, Z(9)=-1.836, p=0.066). *See figure 6.8.*

Figure 6.8 TNF-α stimulation of alveolar macrophages causes an increase in rate of uptake of apoptotic neutrophils ($\chi^2(2)=11.556$, n=9, p=0.003)



Results are expressed as proportion of control efferocytosis index

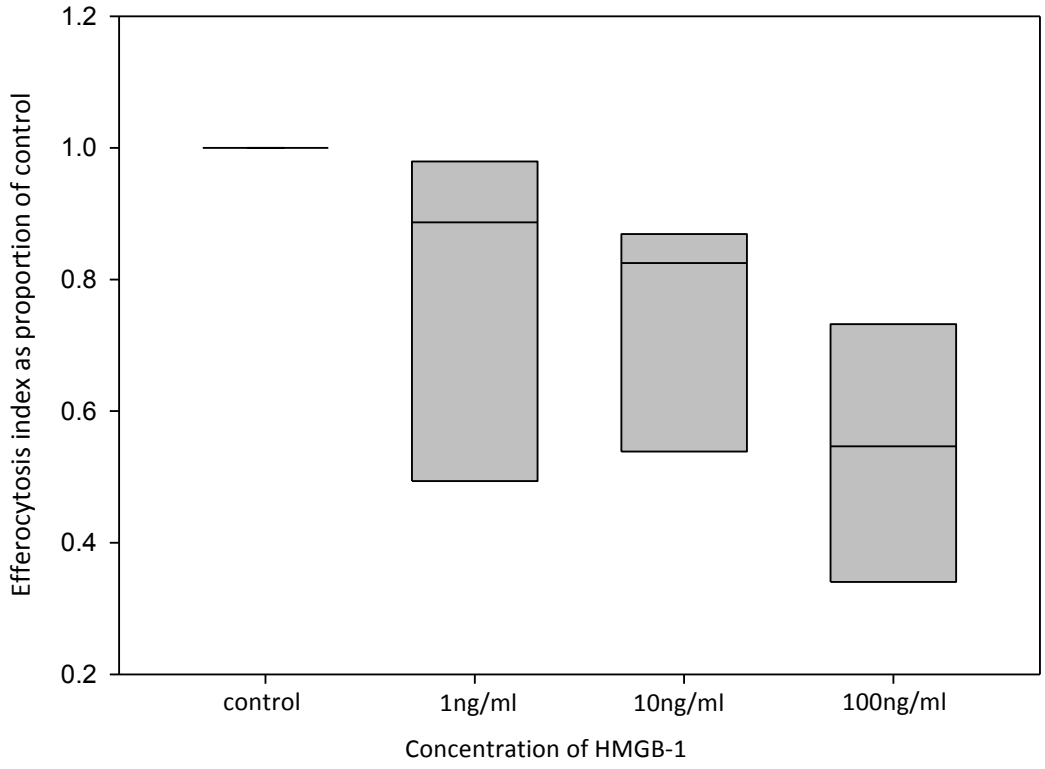
6.4.5 HMGB-1 decreases efferocytosis of apoptotic neutrophils by alveolar macrophages

Differences were detected between un-stimulated cells and those incubated with concentrations of 1, 10 and 100ng/ml (Friedman's ANOVA: n=6, $\chi^2(3)=10.2$, p=0.017). Between groups analysis was therefore performed, which showed that HMGB-1 at a concentration of 1ng/ml did not cause a demonstrable difference in the rate of efferocytosis compared to control (median EI= 7.57 vs. 5.54 Wilcoxon signed rank test: Z(6)=-1.572, p=0.116). However, decreases in efferocytosis compared to control were detected due to incubation with HMGB-1 at 10ng/ml (median EI= 7.57 vs. 4.95 Z(6)=-2.201, p=0.028) and 100ng/ml (median EI= 7.57 vs. 3.03, Z(6)=-2.023, p=0.043). the

differences between the EI of cells stimulated at 1ng/ml and 10ng/ml did not meet criteria for statistical significance, but the EI of cells incubated in 10ng/ml HMGB-1 was higher than that of cells incubated in 10ng/ml HMGB-1 (median EI= 4.95 vs. 3.03, Z(6)=-2.023, p=0.043). *See figure 6.9*.

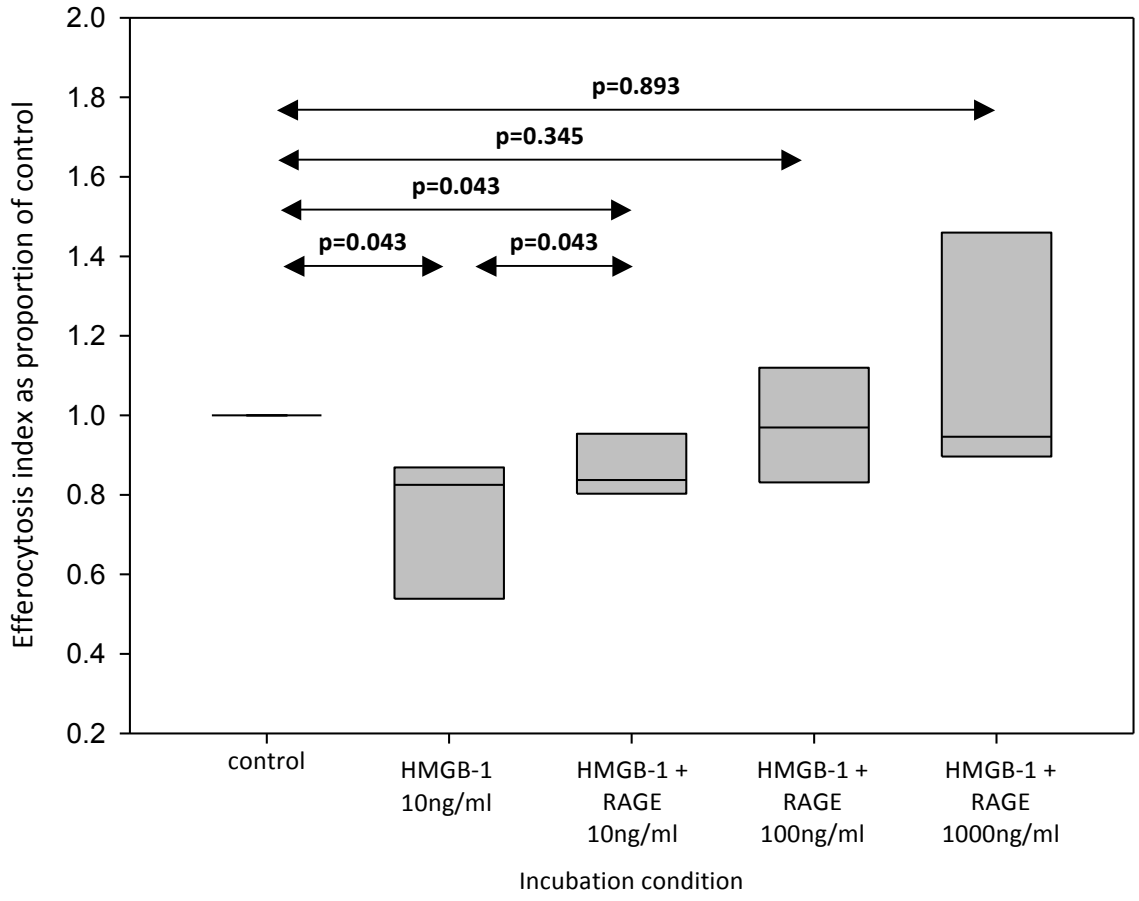
In order to confirm that the decrease in efferocytosis induced by HMGB-1 was due to the action of this mediator on cellular receptors; soluble RAGE was used as a "false" receptor in order to neutralise HMGB-1 prior to cell surface receptor binding. Cells were therefore incubated with HMGB-1 at 10ng/ml as above, and 10ng/ml, 100ng/ml and 1000ng/ml soluble RAGE. RAGE had no effect on efferocytosis itself at these concentrations on efferocytosis (Friedman's ANOVA; n=6, $\chi^2(3)=3.00$, p=0.392). As before HMGB-1 alone caused a decrease in efferocytosis (median EI= 8.49 vs. 5.34, Z(5)=-2.023, p=0.043). Although co-incubation with 10ng/ml RAGE caused an increase in EI from that of HMGB-1 alone (median EI= 7.11 vs. 5.33, Z(5)=-2.023, p=0.043), this was not enough to return HMGB-1 induced down-regulation of efferocytosis to that of control (median EI= 8.49 vs. 7.11, Z(5)=-2.023, p=0.043). Co-incubation of macrophages with 10ng/ml HMGB-1 and 100ng/ml RAGE returned the rate of efferocytosis to that of un-stimulated cells (median EI= 7.06 vs.8.49, Z(5)=-0.944, p=0.345), as did the co-incubation with 1000ng/ml RAGE (median EI= 8.49 vs. 8.19, Z(5)=-0.135, p=0.893). *See figure 6.10*.

Figure 6.9 The effect of stimulation with increasing concentrations of HMGB-1 on efferocytosis of apoptotic neutrophils by alveolar macrophages (n=6, $\chi^2(3)=10.2$, p=0.017)



Results are expressed as proportion of control efferocytosis index

Figure 6.10 The effect of co-incubation with RAGE on the down-regulation of efferocytosis of apoptotic neutrophils by alveolar macrophages caused by 10ng/ml HMGB-1



HMGB-1 + RAGE treated groups were co-incubated in a 10ng/ml concentration of HMGB-1 as well as the concentration of RAGE shown. Results are expressed as proportion of control efferocytosis index

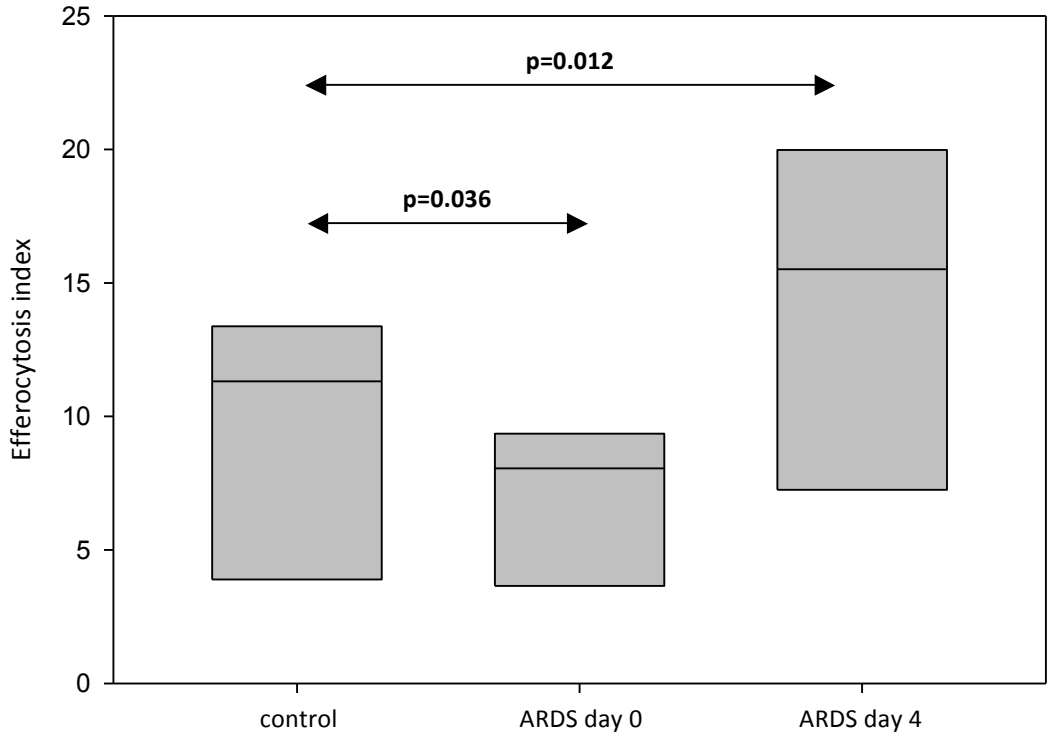
6.4.6 BALF from patients with ARDS and efferocytosis

To assess how the extra-cellular milieu in ARDS influenced efferocytosis we approximated ARDS conditions by incubating macrophages in a 50:50 mix of culture medium (RPMI) and BALF from patients with ARDS and normal controls. Differences were found in the rate of efferocytosis between these groups (Friedman's ANOVA: n=8, $\chi^2(2)=12.25$, p=0.002). Efferocytosis was decreased by BALF taken from patients at day 0

of ARDS compared to control (median EI= 8.05 vs. 11.32, Z(8)=-2.100, p=0.036), but increased by BALF taken at day 4 of ARDS (median EI=15.51 vs. 11.32, Z(8)=-2.521, p=0.012). *See figure 6.11*.

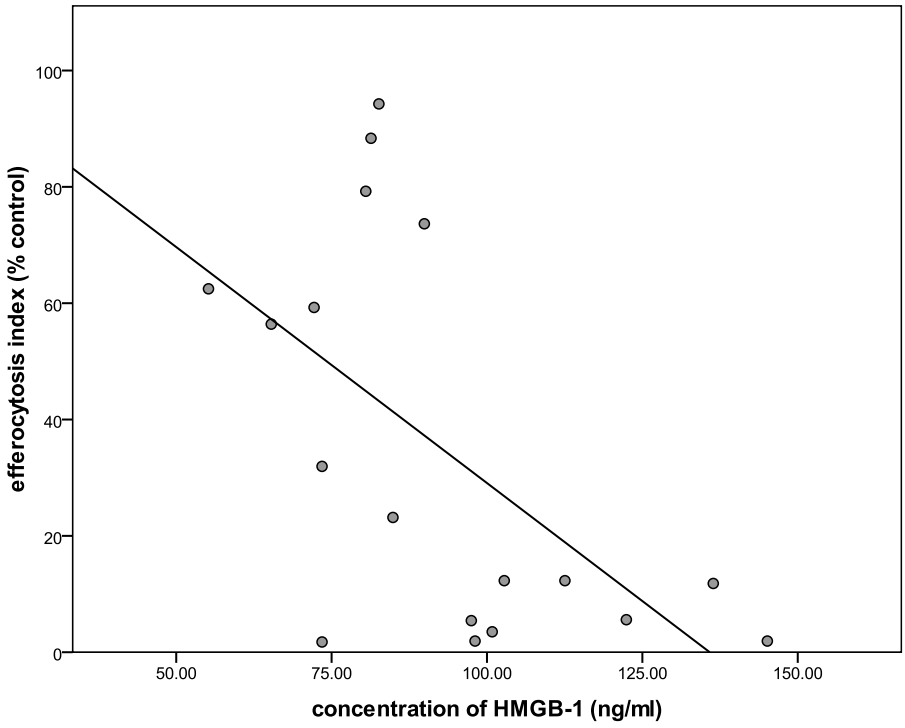
The change in efferocytosis index induced by BALF was converted to a percent control figure to control for inter-assay variability. The correlation between these values and the concentration of HMGB-1 in BALF was then calculated. A negative relationship between the concentration of HMGB-1 and the induced change in efferocytosis was thus established (n=18, ρ=-0.581, p=0.011). *See figure 6.12*.

Figure 6.11 The effect of incubation with BALF from patients with ARDS on efferocytosis of apoptotic neutrophils by macrophages (n=8, $\chi^2(2)=12.25$, p=0.002)



Alveolar macrophages incubated with BALF extracted from patients at either day 0 or day 4 of ARDS for 24 hours prior to efferocytosis assay.

Figure 6.12 Relationship between HMGB-1 concentration in BALF and BALF induced change in efferocytosis (n=18, ρ=-0.581, p=0.011)



6.5 Discussion

This investigation has provided new information on the factors that influence the efferocytosis of apoptotic neutrophils by alveolar macrophages. Firstly we have shown that glucocorticoids cause an increase in the rate of efferocytosis by AM. This effect was seen when using both cortisone and cortisol. These results support the findings of other research groups, who have shown in monocyte derived macrophages that glucocorticoids increase the uptake of apoptotic leucocytes.[75, 128, 227] This investigation is the first to show this using human alveolar macrophages.

In previous chapters we have demonstrated increased concentrations of cortisol are found in the alveolar space of patients at onset of ARDS. We have also shown that the inflammatory mediators which are present within the lungs of patients with ARDS increase the oxo-reductase activity of 11β-HSD in primary alveolar macrophages. The ratio of cortisol to cortisone is also increased in the lungs of patients with ARDS, suggesting that the net local activity of 11β-HSD is in the oxo-reductase rather than the dehydrogenation direction, thus increasing available cortisol. This anti-inflammatory response is a balance and limitation of the pro-inflammatory response seen in ARDS. In the current investigation when macrophages were co-incubated with cortisone and an antagonist of 11β-HSD, the up-regulation caused by cortisone was abrogated, showing that only cortisol was active in up-regulating efferocytosis. This experiment therefore shows that 11β-HSD activity will influence efferocytosis. Increased activity of the enzyme causes an increase in available cortisol, and an increase in efferocytosis. Tissue concentrations of active steroids are not merely dependent on circulating hormone levels. 11β-HSD is essential in determining the local concentration of active corticosteroids, and their effect on tissue function and pathology.[45] In this chapter it has been shown that a defect in 11β-HSD activity may prevent the up-regulation in efferocytosis capacity required to deal with the increased numbers of apoptotic cells seen within the lungs during ARDS. Previous investigations have shown that although the proportion of apoptotic neutrophils in the lungs of critically ill patients with and without ARDS are the same, as there is an increase in the total number of neutrophils the absolute numbers of apoptotic neutrophils increases.[235, 236] The failure to adequately up-regulate efferocytosis due to a defect in 11β-HSD activity will lead to neutrophil necrosis, the release of pro-inflammatory cell contents, and perpetuation of lung inflammation in ARDS.

Incubation of macrophages with BALF taken from patients at different time points of ARDS altered efferocytosis in contrasting ways. That different mediators cause different alterations in the rate of efferocytosis is perhaps unsurprising, and a balance between agents that promote and inhibit efferocytosis will determine the rate of this process. Incubation of AM in BALF from onset of ARDS suppresses efferocytosis, whereas incubation with BALF from later in ARDS up-regulates this process. It is unclear from these current investigations why the BALF taken from patients at onset of ARDS, which contained a higher concentration of cortisol, in fact decreased the rate of efferocytosis. We therefore investigated two inflammatory mediators thought to act at different points in the development of ARDS:

TNF-α is a pro-inflammatory cytokine produced by activated macrophages that mediates many aspects of macrophage function in inflammation.[239] Its concentrations are raised at the onset of ARDS and high concentrations and their persistence are associated with worse clinical outcomes.[10] In this investigation we found that that this pro-inflammatory cytokine promotes the anti-inflammatory process of efferocytosis, a finding that has previously been demonstrated in monocyte derived macrophages (MDMs).[123] However this has not been a consistent finding. Other investigations of the effect of pro-inflammatory cytokines on efferocytosis by MDMs have observed that TNF-α and LPS inhibit efferocytosis.[139, 142] In animal studies the administration of TNF-α decreases the rate of clearance of apoptotic neutrophils within the lung, and a clear mechanism for this suppression by oxidant production induced by TNF-α and mediated by the GTPase Rho, has been demonstrated by McPhillips et al.[139] Nevertheless our findings are that in primary alveolar macrophages TNF-α increases efferocytosis, and further work is required to explain the differences between our own findings and those of McPhillips.

HMGB-1 has roles both as an intracellular DNA binding protein and as an extracellular pro- inflammatory mediator.[240] It produces a biphasic inflammatory response, causing a later production of inflammatory mediators than LPS and TNF-α.[224, 241] In this investigation we have demonstrated that HMGB-1 causes a decrease in the rate of efferocytosis of apoptotic neutrophils by alveolar macrophages. HMGB-1 influences efferocytosis in a number of ways: HMGB-1 binds to exposed phosphatidylserine on the membrane of apoptotic cells, preventing the interaction of PS with macrophages.[140] HMGB-1 also acts directly on macrophages to inhibit the recognition and uptake of apoptotic cells through the a(v)b(3) integrin.[141] The present investigation supports HMGB-1 as an inhibitor of efferocytosis, and is the first investigation using primary human alveolar macrophages to demonstrate this influence of HMGB-1. Our finding of a relationship between induced changes in efferocytosis by BALF from patients with ARDS and HMGB-1 concentration in this BALF further strengthens the case for HMGB-1 in the regulation of efferocytosis in ARDS. HMGB-1 in the extra-cellular space comes primarily from two sources, its passive release from necrotic cells and its active secretion from activated macrophages.[224, 242, 243] Therefore in acute inflammation HMGB-1 appears to provide an autocrine negative feedback on macrophage efferocytosis of apoptotic cells.

Pharmacological manipulation of the immune cells in ARDS to enhance clearance of apoptotic cells is a potential therapeutic opportunity. Success of such therapies in reducing inflammation would depend on sufficient capacity for the safe disposal of the apoptotic cells being present within the inflamed tissue. The uptake of apoptotic cells is suppressed by oxidants such as the reactive oxygen species released by neutrophils, in a process acting through the Rho GTPase. Use of the anti-oxidant N-acetylcysteine increases neutrophil efferocytosis in animals in an LPS model of lung injury.[244] Statins are CoA reductase inhibitors that have recently provoked great interest as a potential

therapy in sepsis and ARDS. These drugs inhibit Rho and have been seen to enhance the clearance of apoptotic neutrophils.[245] Finally administering fish oil may have a role in promoting efferocytosis, that warrants further research.[143]

In conclusion this study has demonstrated that the glucocorticoids increase efferocytosis of apoptotic neutrophils by primary alveolar macrophages, and that this augmentation of cell uptake is influenced by 11β-HSD steroid metabolism. Further, it has been shown that regulation of efferocytosis is altered by components present in the alveolar space through the course of ARDS. Manipulation of this process may provide new opportunities to aid the resolution of this inflammation and improve outcomes in ARDS.

7 Alveolar Macrophage HSD metabolism in ARDS

7.1 Abstract

Introduction: ARDS is a severe lung inflammation that occurs in critically ill patients. Despite high endogenous concentrations of anti-inflammatory glucocorticoids, markers of inflammation in ARDS remain high. Exogenous glucocorticoids, while being seen to decrease inflammatory mediators, do not improve patient outcomes. 11β-hydroxysteroid dehydrogenase (11β-HSD) iso-enzymes interconvert the inactive cortisone to the active hormone cortisol, and altered activity of these enzymes is one potential mechanism for glucocorticoid resistance in ARDS. We examined the activity of 11β-HSD in alveolar macrophage taken from patients with ARDS to determine whether changes in the activity of this enzyme were present within these key effector cells.

Methods: Invasively ventilated patients with ARDS, and both at-risk and normal subjects, were included. Bronchoscopy was performed and broncho-alveolar lavage fluid collected at onset of ARDS and repeated at day 4 for patients with ARDS. Alveolar macrophages were extracted. The 11β-HSD activity in these cells was measured by tritiated thin layer chromatography. qPCR for 11β-HSD iso-enzymes was also performed. Cortisol and cortisone concentration in BALF and plasma was measured using mass spectrometry.

Results: In alveolar macrophages from patients with ARDS 11β-HSD metabolism of cortisone to cortisol is lower at onset of disease than that of normal subjects (median=1.534 vs.0.102 pg/million cells/hour, $p<0.001$), and those patients at risk of ARDS (median=0.853 vs.0.102 pg/million cells/hour, $p=0.006$). Conversion of cortisone to cortisol is also higher at day 4 than at onset of ARDS (median=0.393 vs.0.102 pg/million cells/hour, $p=0.001$). Cortisol production was also greater in normal subjects when compared to that of patients at day 4 of ARDS (median=1.534 pg/million

cells/hour vs.0.393 pg/million cells/hour, p<0.001). Cortisol to cortisone conversion was also higher in the cells of patients at onset of ARDS than in the at risk (0.475 vs. 0.186 pg/million cells/hour, p=0.002) and normal groups (0.413 vs. 0.186 pg/million cells/hour, p=0.007).

Conclusions: Despite the increase in 11β-HSD oxo-reduction seen in alveolar macrophages exposed to inflammatory stimuli in vitro, the cells present within the lungs of patients with ARDS have decreased 11β-HSD oxo-reductase activity. This will lead to a decreased availability of cortisol within these cells, and diminished promotion of anti-inflammatory cell function.

7.2 Introduction

ARDS is a severe inflammatory condition of the lung, common in critically ill patients. During this disease the hypothalamus- pituitary- adrenal (HPA) system is activated to produce corticosteroid hormones to limit the severity and duration of the inflammatory response.[8, 26] Previous investigations have found higher concentrations of endogenous glucocorticoids within the plasma of critically ill patients, [32] and in this thesis we have shown that concentrations of cortisol are higher in the plasma and alveolar space of patients with ARDS. Nevertheless ARDS is characterised by severe inflammation and continued inflammation corresponds to adverse outcome in these patients.[10] Because of the inflammatory nature of ARDS corticosteroids have been investigated as a potential disease modifying agent.[80, 81, 83-85, 103, 246] and results have shown that administering exogenous steroids can reduce the levels of pro-inflammatory mediators seen within the lungs of patients with ARDS.[9, 16] However, clinical outcomes of these investigations have

been inconsistent, and starting exogenous steroids late in the course of ARDS may in fact be harmful.[85] Meta-analysis has not been able to recommend this treatment,[98] and there remains considerable uncertainty on the role that these agents play in the management of ARDS.[247] These factors suggest an acquired tissue resistance to glucocorticoids in ARDS, that has previously been recognised in other inflammatory conditions.[16, 29, 31, 36, 37] Study of potential glucocorticoid resistance has focussed on the glucocorticoid receptor as the site of resistance,[36] but histopathology of patients who have died with ARDS has shown an increase in the expression of 11β-hydroxysteroid dehydrogenase type 2 both in the alveolar wall and in CD68 positive cells (cells of monocyte lineage)[70] in the alveolar lumen of patients with ARDS.[69] This iso-enzyme inactivates cortisol and its increased expression raises the possibility of steroid deactivation sharing responsibility for tissue glucocorticoid resistance in ARDS.

In peripheral tissues the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) inter-converts cortisol and cortisone. The active steroid cortisol is produced from cortisone by the type 1 iso-enzyme, which uses the co-factor NADPH to catalyse this oxo-reduction reaction. 11β-HSD type 1 can also catalyse the reverse reaction of cortisol to cortisone. Cortisol is also deactivated to cortisone by the type 2 iso-enzyme in a dehydrogenase reaction, using the co-factor NADH. 11β-HSD type 2 can only act to deactivate cortisol to cortisone; it cannot act in the reverse direction.[48] Altered levels of activity of this enzyme has been implicated as a mechanism by which specific tissues respond differently to inflammatory stimuli,[63] and thus would provide an explanation for the relative tissue resistance to glucocorticoids observed in the lung of patients with ARDS.

The resolution of ARDS must necessarily involve the clearance of spent and apoptotic neutrophils recruited to the lungs in this condition.[23] Macrophages within the lung are

the phagosomes responsible for this.[138] Macrophages are CD68 positive cells which are steroid sensitive.[70, 75, 210] In ARDS the cells which are resident within the lung are supplemented by other macrophages activated and recruited to the lung from the population of monocytes circulating in blood. Previous studies have shown that glucocorticoids increase the propensity of macrophages to take up apoptotic cells,[128, 227] and any change in steroid availability is therefore likely to impact on macrophage ability to take part in the resolution of lung injury.

We undertook a series of experiments to characterise the metabolism of steroids by the enzyme 11β-HSD within alveolar macrophages extracted from patients with ARDS. We hypothesised that the local metabolism of corticosteroids by the enzyme 11β-hydroxysteroid dehydrogenase is altered in patients with severe lung injury, and that this is associated with worse clinical outcomes and disease severity in these individuals.

7.3 Methods

This investigation is a prospective observational study of 11β-HSD glucocorticoid metabolism within alveolar macrophages, and the concentration of steroids within the alveolar space and plasma of patients with ARDS. The methods used in this chapter are in line with those set out in chapter 3 and are summarised below.

7.3.1 Subject recruitment

Invasively ventilated patients with ARDS were recruited on the day of diagnosis of ARDS. These patients and patients at risk of developing ARDS together with normal subjects were recruited to a bronchoscopic study as part of the BALTI-2 sub-study or the ARDS resolution study. ARDS was diagnosed by the European-American consensus conference

(EACC) criteria.[3] Patients defined as being at risk of ARDS were those meeting criteria for the systemic inflammatory response syndrome (SIRS),[215] but not meeting the EACC criteria for ARDS. Bronchoscopy was performed and broncho-alveolar lavage fluid (BALF) collected at onset of ARDS (day 0) and after 4 days (day 4). Blood was collected at the same time points.

7.3.2 Data collection and measurements

The aetiology and severity of lung injury of subjects was recorded using the GOCA stratification. Clinical and radiological data was collected daily from patients. severity of illness was measured using the acute physiology and chronic health evaluation score (APACHE-2) at enrolment to the study, and subsequent clinical progress was measured using sequential measurement of the sequential organ failure assessment tool (SOFA), the simplified acute physiology score (SAPS-2), and the Murray lung injury score (LIS) both at onset of ARDS and at the point of the day 4 bronchoscopy.

7.3.3 Laboratory techniques

Alveolar macrophages were isolated from BALF using density gradient techniques. BALF and plasma was frozen at -80^{o}C for subsequent analysis. Cortisol and cortisone concentrations were measured in BALF and plasma using mass spectrometry. The cortisol: cortisone ratio was calculated from these results. Colourmetric urea assays were performed on both BALF and plasma samples and the urea concentrations then used to calculate dilution factors for the BALF. Epithelial lining fluid (ELF) steroid concentrations were estimated by multiplying the BALF concentrations by this dilution factor. This estimate cannot be used for the cortisol to cortisone ratio, as it returns the BALF ratio only; the dilution factor being both a numerator and a denominator in the

calculation of the ELF cortisol: cortisone ratio. Viability of cells during assay was confirmed using celltiter aqueous assay (promega, UK).

11β-hydroxysteroid dehydrogenase oxo-reductase (conversion of cortisone to cortisol) and dehydrogenase (conversion of cortisol to cortisone) activity in alveolar macrophages was measured using a tritiated thin layer chromatography assay. RNA was collected from the cells after completion of the assay, and RT reaction followed by qPCR performed using β-actin as control to calculate an RQ value for each sample.

7.3.4 Statistical analysis

Statistical analysis was done using the statistical software SPSS (IBM, Somers, New York, USA). Normality was tested using the Kolmogorov-Smirnov test. If data was normally distributed further analysis was done using the parametric techniques t-tests and ANOVA. If data was not normally distributed analysis was done using the non-parametric techniques, Mann Whitney and ANOVA by ranks (Kruskal Wallis), related samples were tested using the Wilcoxon signed ranks test. Linear associations were tested using Pearson's correlation coefficient for parametric data, and Spearman's correlation coefficient for non-parametric data. All significance tests are done using an α-value of 0.05, using two-tailed tests, unless stated otherwise within the text.

7.4 Results

7.4.1 Patient characteristics

A total of 27 patients were enrolled in the bronchoscopic sub-study of the BALTI-2 trial. Of these 19 underwent bronchoscopy and had alveolar macrophages collected at onset

of ARDS, and 19 also underwent bronchoscopy and alveolar macrophage collection at day 4. Alveolar macrophage 11β-HSD activity data at both time points is available for 18 patients. Due to the limitations within the nature of the activity assays, not all enzyme activity could be measured for each patient on each occasion. Numbers of results available are in table 7.1. 5 further patients were enrolled in the resolution of inflammation in ARDS study, to act as an "at risk" comparison group. The aetiology of ARDS is recorded in table 7.2.

No difference was observed between the at risk and ARDS groups in the overall severity indices (SOFA score, SAPS-II, or APACHE -2 score). In keeping with the group selection, a difference was observed in the lung injury score and the P: F ratios between these two groups. The characteristics of these two groups, as defined by the GOCA stratification are detailed in table 7.3.

Table 7.1 Numbers of results of 11β-HSD assays available

	11β-HSD activity	
	Oxo-reductase activity	Dehydrogenase activity
Day 0	19	15
Day 4	19	18
Matched day 0 plus day 4	18	14

Table 7.2 predisposing factors for ARDS in patients with ARDS and at risk of ARDS

Predisposing condition	**ARDS patients**	**"At risk" patients**
Pneumonia	13 (48%)	1 (20%)
Sepsis	9 (33%)	3 (60%)
Aortic aneurysm repair	1 (5%)	-
Chest trauma	2 (7%)	1 (20%)
other	2 (7%)	-

Table 7.3 Comparisons of GOCA patient characteristics between ARDS and at risk groups

	ARDS (n=27)	At risk (n=5)	P value
Gas Exchange			
Average(SD) P:F ratio kPa	17.0 (3.9)	31.5 (11.3)	<0.01
PaO_2/FIO_2 > 40 kPa	0	1	
PaO_2/FIO_2 26.8 - 40 kPa	0	1	
PaO_2/FIO_2 13.46 - 26.8 kPa	23	3	<0.01
PaO_2/FIO_2 <13.46 kPa	4	0	
Spontaneous breathing, no PEEP	0	0	
Assisted breathing, PEEP 0-5 cm H_2O	6	2	NS
Assisted breathing, PEEP 6-10cm H_2O	13	3	
Assisted breathing, PEEP > 10cm H_2O	5	0	
Organ failure (Non-pulmonary)			
0 organ	5	2	
1 organ	9	0	NS
2 organs	9	2	
3 organs	4	1	
Cause			
Unknown	0	0	
Indirect lung injury	11	3	NS
Direct lung injury	16	2	
Other parameters			
Age	61.93 (16.53)	65.20 (13.29)	0.68
sex	16:11	3:2	
APACHE II	24.9 (4.94)	24.8 (4.97)	0.972
SAPS II	66.4 (10.64)	61.8 (8.29)	0.368
SOFA	11.58(2.10)	10.60 (2.61)	0.366
LIS	2.80 (0.45)	1.1 (0.77)	<0.001

An overall difference was demonstrated in the P: F ratios of patients between patients at onset of ARDS, at day 4 of ARDS and the at risk patients (ANOVA by ranks, H=22.957, df=2, p<0.001). Between group analysis was therefore performed. A difference was detected between the P: F ratios of patients at day 0 of ARDS and those at day 4 of ARDS (median= 17.05 vs. 22.95kPa, U=118.5, p<0.001). A similar difference was observed

between patients at onset of ARDS and those at risk of developing this condition (median= 17.05 vs. 26.00kPa, U=0.000, p<0.001). No difference was detected between the patients at day 4 of ARDS and the at risk group (median= 22.95 vs. 26.00kPa, U=34.00, p=0.101).

The different P: F ratios between the clinical groups are in part responsible for the different lung injury scores (LIS) observed between these groups (ANOVA on ranks, H=12.63, p=0.002). As expected the LIS is greater in the patients at onset of ARDS compared to those in the at risk group (median LIS =2.84 vs. 1.0, U=0.000, p<0.001). The LIS at day 4 of ARDS remained higher than that of the patients in the "at risk" group (median LIS =2.67 vs. 1.0, U=9.5, p=0.001). The LIS did not decrease by day 4 of ARDS compared to that at day 0 (median LIS =2.83 vs. 2.67, U=287, p=0.340). These results are as expected for these different groups of patients, and as such allow meaningful comparisons between these groups.

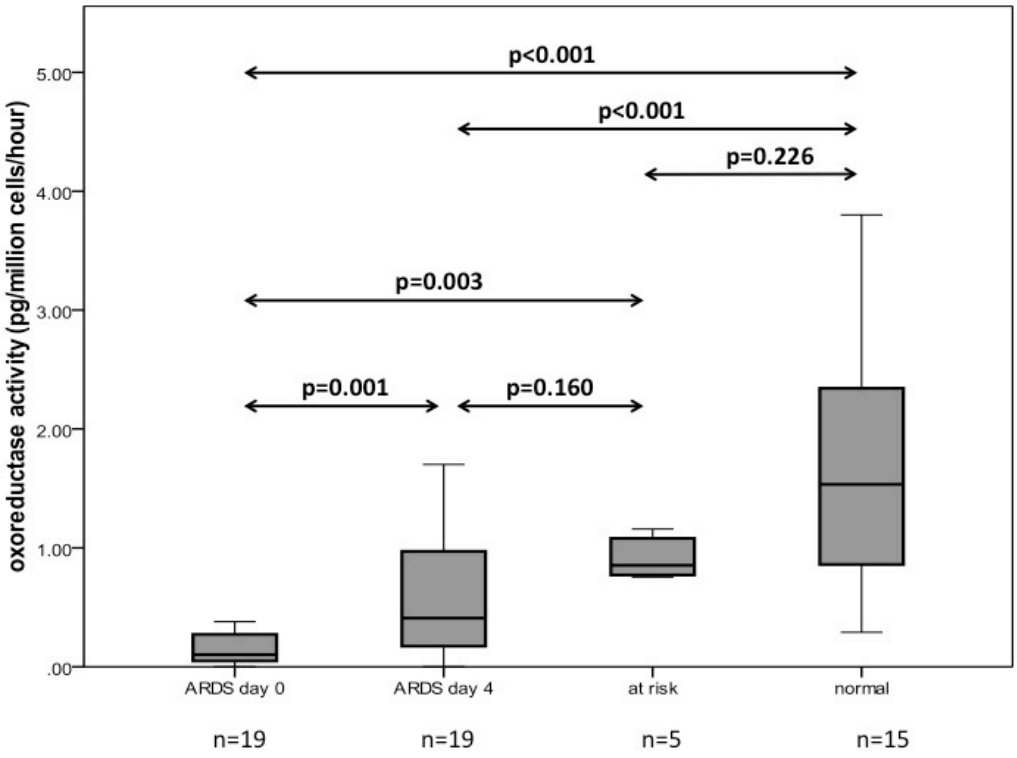
7.4.2 Alveolar macrophage conversion of cortisone to cortisol

When comparing the 11β-HSD oxo-reductase activity (conversion of cortisone to cortisol) in alveolar macrophages taken from either normal subjects, patients at onset of ARDS, after 4 days of ARDS, or patients at risk of developing ARDS, an overall difference between these groups was observed (ANOVA by ranks, H(3)=27.068, p<0.001). *See Figure 7.1*. Between groups analyses was therefore performed. Observed differences are as follows: Conversion of cortisol to cortisone in the alveolar macrophages of normal subjects was greater than that of patients at onset of ARDS (median=1.534 vs.0.102 pg/million cells/hour, U(34)=14.0, p<0.001). Cortisol production was also greater in

normal subjects when compared to that of patients at day 4 of ARDS (median=1.534 vs.0.393 pg/million cells/hour, U=40.0, p<0.001). The 11β-HSD oxo-reductase activity was higher at day 4 than at onset of ARDS (Wilcoxon signed rank test: median=0.393 vs.0.102 pg/million cells/hour, Z(18)=-3.201, p=0.001).

A difference was also observed when comparing the alveolar macrophage activation of cortisone to cortisol in cells taken from those patients at risk of ARDS, and patients at onset of ARDS (median=0.853 vs. 0.102 pg/million cells/hour, U(24)=8.0, p=0.003). However at day 4 of ARDS no difference was demonstrated between patients with ARDS and the "at risk" group (median=0.853 vs. 0.393 pg/million cells/hour, U(24)=27.0, p=0.160). No difference was present between the "at risk" group and normal subjects (median=1.534 vs.0.853 pg/million cells/hour, U(20)=24.0, p=0.266).

Figure 7.1 Box plot of alveolar macrophage 11β-HSD oxo-reductase activity at day 0 and day 4 of ARDS, as well as normal subjects and patients at risk of ARDS



7.4.3 Alveolar macrophage conversion of cortisol to cortisone

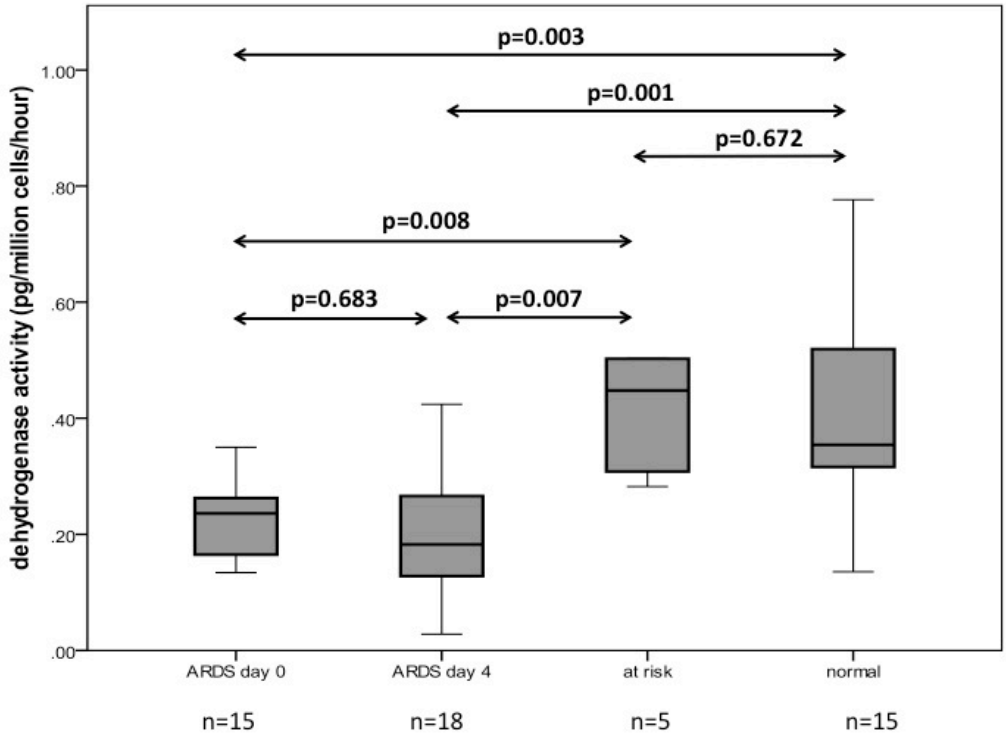
An overall difference was present between the normal subjects, patients at onset of ARDS, after 4 days of ARDS, and patients at risk of developing ARDS in the dehydrogenase activity of 11β-HSD (ANOVA by ranks, H(3)=15.02, p=0.002). *See Figure 7.2*. Between groups analysis was therefore performed. The inactivation of cortisol to cortisone by 11β-HSD in alveolar macrophages was significantly reduced in patients at onset of ARDS when compared to normal subjects (0.413 vs. 0.186 pg/million cells/hour, U(30)=42.0, p=0.003). At day 4 of ARDS there was also a decrease in 11β-HSD dehydrogenase activity when compared to normal subjects (0.413 vs. 0.2 pg/million cells/hour, U(33)=47.0, p=0.001). However, no difference was detected in 11β-HSD

dehydrogenase activity between patients at onset of disease and at day 4 of this condition (Wilcoxon signed ranks test: median=0.186 vs. 0.2 pg/million cells/hour, Z(14)=-0.408, p=0.683).

A difference in 11β-HSD dehydrogenase activity was demonstrated between those subjects at risk of ARDS, and patients at both time-points of ARDS. The greatest difference was seen between the "at risk" group and the day 0 of ARDS group (0.475 vs. 0.186 pg/million cells/hour, U(20)=8.0, p=0.008), with similar differences seen between the "at risk" and ARDS day 4 groups (0.475 vs. 0.2 pg/million cells/hour, U(23)=10.0, p=0.007).

No difference in 11β-HSD dehydrogenase activity was observed between normal subjects and patients at risk of developing ARDS (0.413 vs. 0.475 pg/million cells/hour, U(20)=32.0, p=0.672).

Figure 7.2 Box plot of alveolar macrophage 11β-HSD dehydrogenase activity at day 0 and day 4 of ARDS, as well as normal subjects and patients at risk of ARDS



7.4.4 11β-HSD activity and subject characteristics

No relationship was demonstrated between the alveolar macrophage 11β-HSD oxo-reductase or dehydrogenase activity and the gender, age, or aetiology of ARDS at either day 0 or day 4. *See tables 7.4, 7.5, and 7.6*.

Table 7.4 Differences in the 11β-HSD activity between male and female subjects

	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		**Mann-Whitney test**	
	Male (n, range)	**Female (n, range)**	**U**	**P value**
Day 0	0.14 (12, 0.01-0.97)	0.05 (7,0.01-0.88)	29.5	0.299
Day 4	0.22 (12, 0.01-1.70)	0.24 (7, 0.08-3.48)	41.0	0.967
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	Male (n, range)	**Female (n, range)**		
Day 0	0.47 (10, 0.13-0.69)	0.41 (5, 0.14-0.27)	20.0	0.594
Day 4	0.20 (11, 0.12-0.52)	0.18 (7, 0.03-0.65)	31.5	0.536

Table 7.5 Correlations between subject age and 11β-HSD activity

11β-HSD activity (pg/million cells/hour)		**Spearman's correlation coefficient (n)**	**P value**
Oxo-reductase	**Day 0**	-0.219 (19)	0.368
	Day 4	-0.047 (19)	0.850
dehydrogenase	**Day 0**	-0.355 (15)	0.195
	Day 4	-0.135 (18)	0.593

Table 7.6 Differences in the 11β-HSD activities between subjects with a direct and indirect cause of lung injury

	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		**Mann-Whitney test**	
	Direct (n, range)	**Indirect (n, range)**	**U**	**P value**
Day 0	0.09 (12, 0.01-0.38)	0.15 (7, 0.01-0.97)	34.5	0.536
Day 4	0.18 (12, 0.01-1.70)	0.35 (7, 0.08-3.48)	41.0	0.967
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	Direct (n, range)	**Indirect (n, range)**		
Day 0	0.44(10, 0.13-0.27)	0.24 (5, 0.17-0.69)	10.0	0.075
Day 4	0.18 (11, 0.12-0.65)	0.15 (7, 0.03-0.52)	33.5	0.659

7.4.5 11β-HSD activity and severity scores

No relationship was detected between the LIS, the SOFA score, the SAPS-2 score or the APACHE-2 score, and their simultaneously measured oxo-reductase 11β-HSD activity at either day 0 or day 4. *See table 7.7*. However a negative relationship was present between the APACHE-2 score and the 11β-HSD dehydrogenase activity at onset of ARDS (n=15, ρ=-0.671, p=0.006). This was reflected in a similar relationship between the day 0 SOFA score and the dehydrogenase activity (n=15, ρ=-0.571, p=0.026). *See figures 7.3 and 7.4 and table 7.8*.

Table 7.7 Correlations between alveolar macrophage 11β-HSD oxo-reductase activity and severity indices

		Spearman's correlation coefficient (n)	**P value**
APACHE-2	Day 0	0.142 (19)	0.562
LIS	Day 0	-0.425 (19)	0.070
	Day 4	-0.308 (19)	0.199
SAPS-2	Day 0	-0.145 (19)	0.552
	Day 4	-0.139 (19)	0.571
SOFA	Day 0	0.321 (19)	0.180
	Day 4	-0.076 (15)	0.756

Table 7.8 Correlations between alveolar macrophage 11β-HSD dehydrogenase activity and severity indices

		Spearman's correlation coefficient (n)	**P value**
APACHE-2	Day 0	-0.671 (15)	0.006**
LIS	Day 0	-0.199 (15)	0.478
	Day 4	-0.035 (18)	0.889
SAPS-2	Day 0	0.445 (15)	0.096
	Day 4	0.086 (18)	0.733
SOFA	Day 0	-0.571 (15)	0.026**
	Day 4	0.140 (18)	0.578

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 7.3 Scatter plot of APACHE-2 score and alveolar macrophage 11β-HSD dehydrogenase activity at onset of ARDS (n=15, ρ=-0.671, p=0.006)

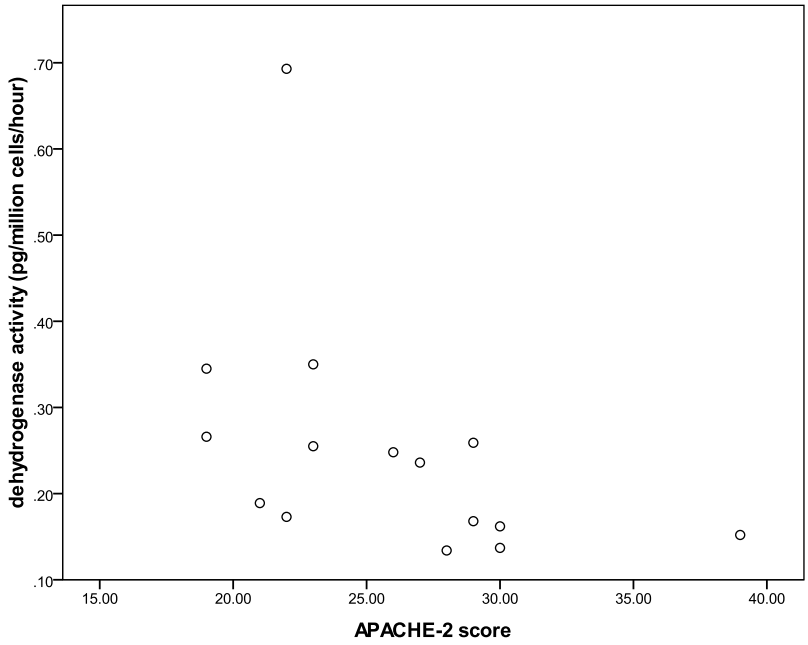
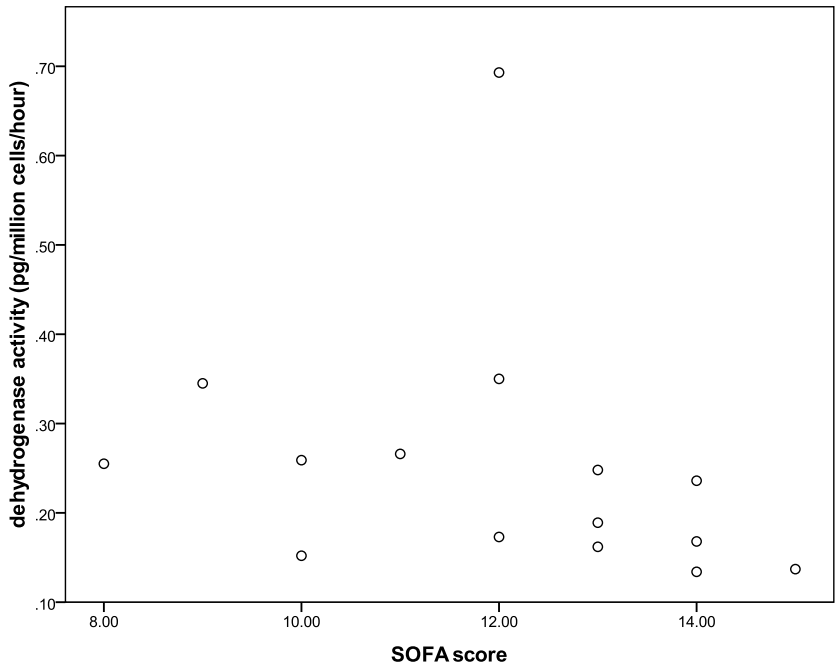


Figure 7.4 Scatter plot of SOFA score and alveolar macrophage 11β-HSD dehydrogenase activity at onset of ARDS (n=15, ρ=-0.571, p=0.026)



7.4.6 11β-HSD activity and change in severity score

To test for differences in 11β-HSD activity between those patients with ARDS which had improved, and without an improvement, patients were divided by changes in severity indices. To create comparably sized groups for analysis of SOFA and SAPS-2 scores improvement an arbitary whole number value close to the median improvement in these scores was used to divide patients into "improvers and non-improvers". These values were an improvement in SOFA score by 3 points and an improvement in SAPS-2 score by 10 points.

No difference in the 11β-HSD oxo-reductase or dehydrogenase activity was present between those subjects whose LIS had shown an improvement and those who had not at either time point studied. *See table 7.9*.

Those patients whose global health status had improved after 4 days to give an improvement in the SOFA score by 3 or more points had a lower median 11β-HSD dehydrogenase activity at day 4 than those patients who had failed to respond to this degree (median conversion of cortisol to cortisone: 0.133 vs. 0.206 pg/million cells/hour, U(18)=17.0, p=0.043). *See Figure 7.5*. However no other difference in the oxo-reductase or dehydrogenase activity was present in patients whose SOFA score had improved by 3 or more points, or by any degree, at either day 0 or day 4. *See table 7.10.*

Table 7.9 Alveolar macrophage 11β-HSD activity and improvement in LIS

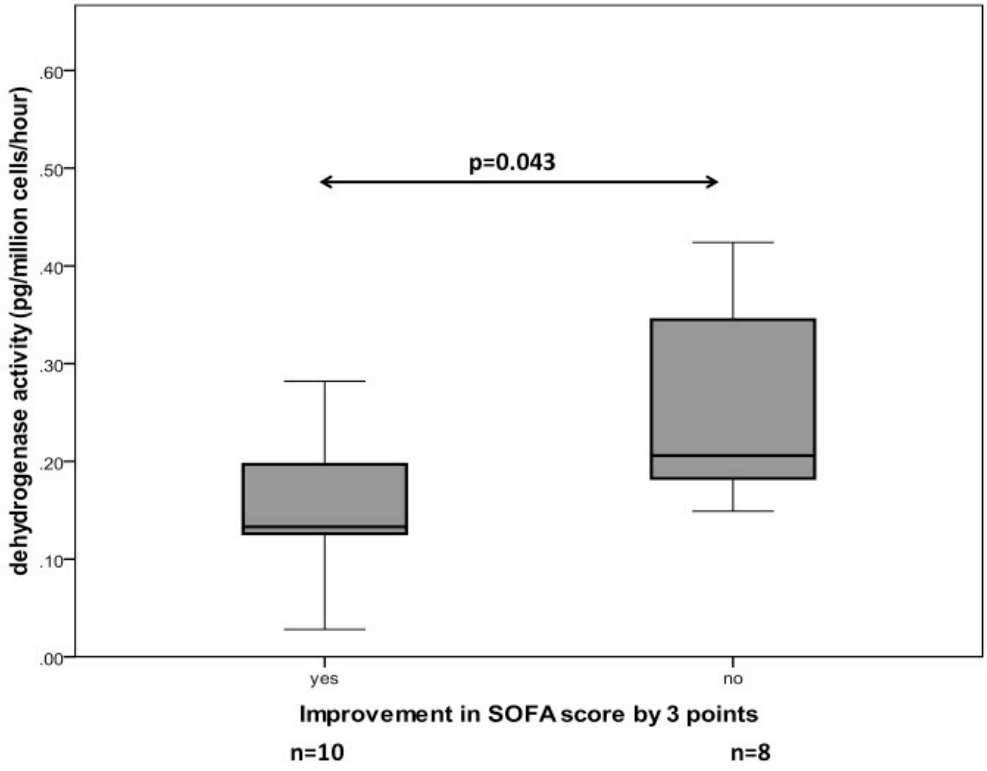
Improvement in LIS				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		Mann-Whitney test	
	yes (n, range)	no (n, range)	U	P value
Day 0	0.165 (8, 0.04-0.97)	0.065 (11, 0.01-0.83)	25.0	0.129
Day 4	0.764 (9, 0.11-3.48)	0.306 (10, 0.01-1.70)	26.0	0.121
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	yes (n, range)	no (n, range)		
Day 0	0.165 (6, 0.14-0.35)	0.255 (9, 0.13-0.69)	15.0	0.181
Day 4	0.197 (9, 0.03-0.65)	0.177 (9, 0.12-0.42)	34.0	0.605

Table 7.10 Alveolar macrophage 11β-HSD activity and improvement in SOFA score

Improvement in SOFA score by 3 or more points				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		Mann-Whitney test	
	yes (n, range)	no (n, range)	U	P value
Day 0	0.145 (11, 0.04-0.97)	0.062 (8, 0.01-0.88)	27.0	0.177
Day 4	0.332 (11)	0.614 (8, 0.04-3.48)	33.0	0.39 5
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	yes (n, range)	no (n, range)		
Day 0	0.171 (8, 0.01-1.45)	0.255 (7, 0.16-0.35)	17.0	0.232
Day 4	0.133 (10, 0.13-0.69)	0.206 (8, 0.15-0.65)	17.0	0.043*
Any improvement in SOFA score				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		Mann-Whitney test	
	yes (n, range)	no (n, range)	U	P value
Day 0	0.091 (16, 0.01-0.97)	0.324 (3, 0.04-0.88)	18.0	0.559
Day 4	0.437 (16, 0.01-1.70)	0.378 (3, 0.04-3.48)	24.0	1.0
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	yes (n, range)	no (n, range)		
Day 0	0.219 (12, 0.13-0.69)	0.236 (3, 0.16-0.26)	16.0	0.840
Day 4	0.177 (15, 0.03-0.52)	0.266 (3, 0.20-0.65)	7.0	0.076

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 7.5 Box plot of 11β-HSD dehydrogenase activity at day 4 of ARDS in patients with and without an improvement in SOFA score by 3 or more points



7.4.7 Alveolar macrophage 11β-HSD activity and mortality

There was no difference in the alveolar macrophage 11β-HSD oxo-reductase activity at either day 0 or day 4 between those patients who survived until hospital discharge. Also, there was no difference in the dehydrogenase activity between these groups. Similarly no difference was detected between those subjects who survived to hospital discharge in the oxo-reductase activity and the dehydrogenase activity of 11β-HSD at either day 0 or day 4 of ARDS. Between those subjects who survived 28 days from onset of ARDS and those who did not, again no differences were detected in either direction of 11β-HSD activity at either time point. *See table 7.11*.

Table 7.11 Differences in 11β-HSD activity and mortality

Hospital survival				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		**Mann-Whitney test**	
	survivors (n, range)	**Non-survivors (n, range)**	**U**	**P value**
Day 0	0.161 (4, 0.07-0.32)	0.079 (15, 0.01-0.97)	35.5	0.447
Day 4	0.378 (4, 0.33-1.23)	0.540 (15, 0.01-3.48)	38.0	0.657
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	survivors (n, range)	**Non-survivors (n, range)**		
Day 0	0.255 (3, 0.14-0.19)	0.205 (12, 0.13-0.69)	19.0	0.336
Day 4	0.191 (4, 0.13-0.65)	0.181 (14, 0.03-0.52)	30.5	0.408
ICU survival				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		**Mann-Whitney test**	
	survivors (n, range)	**Non-survivors (n, range)**	**U**	**P value**
Day 0	0.203 (10, 0.01-0.97)	0.065 (9, 0.01-0.88)	25.5	0.129
Day 4	0.437 (11, 0.04-1.45)	0.241 (8, 0.01-3.48)	30.0	0.272
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	survivors (n)	**Non-survivors (n)**		
Day 0	0.176 (7, 0.14-0.69)	0.255 (8, 0.13-0.27)	15.0	0.181
Day 4	0.197 (10, 0.13-0.65)	0.177 (8, 0.03-0.42)	28.5	0.375
28 day survival				
	Median 11β-HSD oxo-reductase activity (pg/million cells/hour)		**Mann-Whitney test**	
	survivors (n, range)	**Non-survivors (n, range)**	**U**	**P value**
Day 0	0.161 (8, 0.05-0.88)	0.079 (11, 0.01-0.97)	23.0	0.530
Day 4	0.393 (8, 0.28-3.48)	0.464 (11, 0.01-1.70)	27.0	0.810
	Median 11β-HSD dehydrogenase activity (pg/million cells/hour)			
	survivors (n, range)	**Non-survivors (n, range)**		
Day 0	0.162 (6, 0.14-0.27)	0.252 (9, 0.13-0.69)	7.0	0.136
Day 4	0.175 (7, 0.13-0.65)	0.183 (11, 0.03-0.52)	24.5	0.721

7.4.8 11β-HSD activity and glucocorticoid concentrations

No significant relationship was detected between the cortisol concentration in plasma or ELF and either the dehydrogenase or oxo-reductase activity of 11β-HSD. A relationship was established between the cortisone concentration in ELF at day 0 and the day 0 alveolar macrophage 11β-HSD oxo-reductase activity (n=15, ρ=0.550, p=0.033). *See figure 7.6.* No other relationship between cortisone and the activity of this enzyme could be established. No relationship was demonstrably present between enzyme activity and the cortisol: cortisone ratio. *See table 7.12 and 7.13*.

Table 7.12 Correlations between 11β-HSD oxo-reductase activity and glucocorticoid concentrations in plasma, BALF and ELF

Day 0 AM 11β-HSD oxo-reductase activity correlations			
		Spearman's correlation coefficient	p value
Plasma	cortisol	0.215(16)	0.424
	cortisone	0.349 (16)	0.185
	cortisol: cortisone ratio	0.243 (16)	0.365
BALF	cortisol	0.150 (15)	0.593
	cortisone	-0.013 (15)	0.965
	cortisol: cortisone ratio	0.091 (15)	0.747
ELF	cortisol	0.416 (15)	0.123
	cortisone	0.550 (15)	0.033*
Day 4 AM 11β-HSD oxo-reductase activity correlations			
		Spearman's correlation coefficient	p value
Plasma	cortisol	-0.315 (18)	0.203
	cortisone	-0.284 (18)	0.254
	cortisol: cortisone ratio	-0.257 (18)	0.303
BALF	cortisol	-0.197 (18)	0.433
	cortisone	-0.171 (18)	0.499
	cortisol: cortisone ratio	-0.209 (18)	0.404
ELF	Cortisol	-0.077 (18)	0.760
	cortisone	0.168 (18)	0.505

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Figure 7.6 Scatter plot of 11β-HSD oxo-reductase activity and ELF cortisone concentration at onset of ARDS (n=15, ρ=0.550, p=0.033)

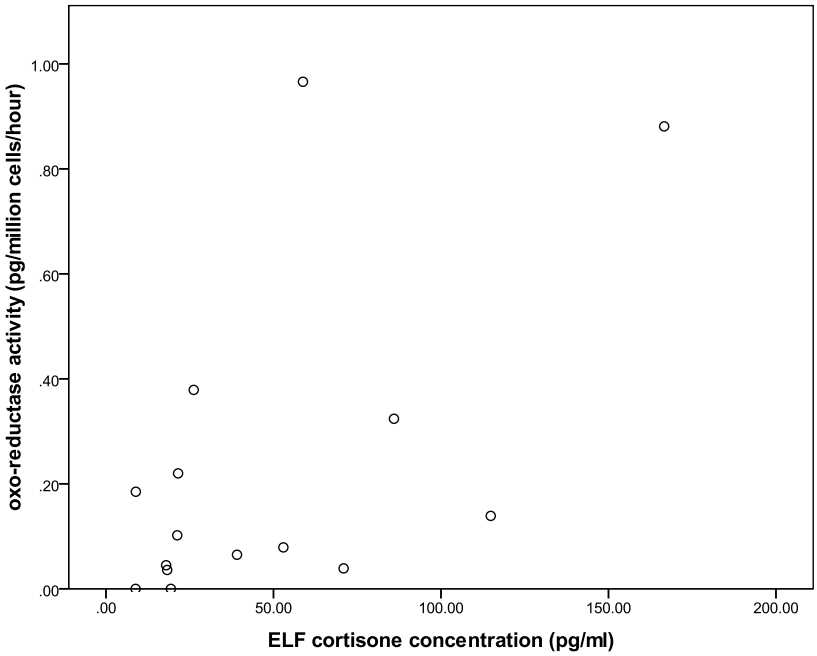


Table 7.13 Correlations between 11β-HSD dehydrogenase activity and glucocorticoid concentrations in plasma, BALF and ELF

Day 0 AM 11β-HSD dehydrogenase activity correlations			
		Spearman's correlation coefficient	p value
Plasma	cortisol	-0.341 (13)	0.255
	cortisone	0.336 (13)	0.262
	cortisol: cortisone ratio	-0.346 (13)	0.247
BALF	Cortisol	-0.497 (12)	0.101
	cortisone	0.091 (12)	0.779
	cortisol: cortisone ratio	-0.517 (15)	0.085
ELF	cortisol	-0.371 (12)	0.236
	cortisone	-0.196 (12)	0.542
Day 4 AM 11β-HSD dehydrogenase activity correlations			
		Spearman's correlation coefficient	p value
Plasma	cortisol	-0.315 (18)	0.203
	cortisone	-0.284 (18)	0.254
	cortisol: cortisone ratio	-0.257 (18)	0.303
BALF	cortisol	-0.315 (18)	0.433
	cortisone	-0.171 (18)	0.499
	cortisol: cortisone ratio	-0.209 (18)	0.404
ELF	Cortisol	-0.077 (18)	0.760
	cortisone	0.168 (18)	0.505

7.4.9 11β-HSD RNA expression and enzyme activity

To test the hypothesis that increased enzyme activity in these cells was the result of increased production of these proteins correlations between enzyme activity and 11β-HSD iso-enzyme RNA RQ values were calculated for cases where data existed for both these measures (n numbers are recorded with results). Positive relationships were hypothesised between the oxo-reductase activity and 11β-HSD type 1 qPCR RQ value, and between the dehydrogenase activity and the type 2 iso-enzyme RQ value. Due to these hypotheses, one-tailed significance tests were used.

No clear relationship was established between the enzyme activity and expression of RNA coding for either of these iso-enzymes. Nor was the direction of relationships interpretable from these results, with both non-significant positive and negative correlation coefficients recorded. *See table 7.14*.

Table 7.14 Correlations between alveolar macrophage 11β-HSD activity and 11β-HSD iso-enzyme RNA expression

RQ value		11β-HSD oxoreductase activity	11β-HSD dehydrogenase activity
11β-HSD 1 day 0	Spearman's ρ	0.226	0.275
	p value	0.218	0.171
	N=	14	14
11β-HSD 1 day 4	Spearman's ρ	-0.350	-0.364
	p value	0.178	0.123
	N=	9	12
11β-HSD 2 day 0	Spearman's ρ	0.071	-0.357
	p value	0.433	0.193
	N=	8	8
11β-HSD 2 day 4	Spearman's ρ	0.300	-0.314
	p value	0.312	0.272
	N=	5	6

7.5 Discussion

The principle finding of this study is that the oxo-reduction of cortisone to cortisol in the alveolar macrophages extracted from patients with ARDS is reduced when compared to that of both normal subjects and patients with a critical illness who are at risk of developing ARDS. The lower activity in patients at onset of ARDS compared to subjects at risk of developing ARDS, together with the fact that no difference in the oxo-reductase activity was seen between normal and at risk groups suggests that this is a phenomenon that is specific for ARDS and not one that is associated merely with critical illness. The production of cortisol from cortisone also increases between day 0 and day 4 of ARDS, such that by day 4 the activity is not demonstrably different than that seen in at risk patients, suggesting that increasing production of cortisol is associated with evolving ARDS.

The findings recorded in this chapter are inconsistent with the results of our previous experiments. In chapter 5 we reported that the pro-inflammatory mediators IL-1β and TNF-α increased 11β-HSD oxo-reductase activity in primary alveolar macrophages. We have also described in chapter 4 that increased concentrations of cortisol are present in the lungs of patients at the onset of ARDS. Both of these findings suggest an increase in 11β-HSD type 1 activity, rather than the decrease in activity we have seen in the current investigation. The oxo-reductase results described here are at first consideration more in keeping with the findings of Suzuki et al that there is an increase in the 11β-HSD type 2 expression and activity in the lungs of patients with ARDS.[69] However we have also not shown an increase in the 11β-HSD type 2 activity in the cells under investigation, cells that fit the description of the CD68 positive cells in the alveolar lumen described by Suzuki. In this investigation, the alveolar macrophage 11β-HSD dehydrogenase activity

follows the same general pattern as the oxo-reductase activity, being lower in those patients with ARDS at both time points than the at-risk and normal subjects. The explanation for these findings is most likely due to the experimental method used to measure enzyme activity. To evaluate the rate of dehydrogenase activity a surplus of cortisol is added to the cells in order to measure its conversion to cortisone, whilst ensuring first order reaction kinetics. Therefore increased activity of the type 1 enzyme may also cause an increase in the dehydrogenase activity observed in these experimental conditions, due to the bi-directional nature of this iso-enzyme's catalysis.[50] It is likely that the observed change in the dehydrogenase activity in the same manner as the more active oxo-reduction is likely to represent increased activity of the type 1 iso-enzyme, rather than synchronous up-regulation of the type 2 iso-enzyme.

Previous investigations in other cell types have shown that stimulation with inflammatory cytokines increases 11β-HSD type 1 enzyme activity by increasing enzyme expression.[54, 62-65] In this investigation we were unable to show a link between the expression of 11β-HSD iso-enzyme RNA and activity of the enzyme itself. This finding may suggest that in ARDS it is not an increase in the expression of this enzyme that determines the activity in these cells, and other factors such as the availability of co-factors may be responsible. These co-factors are further discussed below. The small numbers of patients we were able to include in our RNA analysis does not allow definitive conclusions to be drawn however, and further work is required.

Both iso-enzymes of 11β-HSD require co-factors to function,[48] and the type-1 iso-enzymes direction of reaction is determined by the presence of its NADPH co-factor, and the enzyme that produces it.[50] An explanation for the contrasting results between our in-vitro experiments and the enzyme activity of patients with ARDS is the availability of

cofactors for the reactions that 11β-HSD catalyses. The equilibrium constant for 11β-HSD type 1 at pH=7.0 is 0.03, implying that the reaction will preferentially move towards cortisone.[49] It is only the relative surplus of the co-factor NADPH provided by the enzyme hexose-6-phosphate dehydrogenase (H6PDH) that ensures the in vivo predominance of the oxo-reductase direction of the reaction.[50] Should the activity of H6PDH be compromised and the availability of NADPH diminish this would lead to a switch from oxo-reduction to dehydrogenation by 11β-HSD type 1. Other cell types demonstrate the possibility of this phenomenon: Omental adipose stromal cells exhibit a switch from a predominantly dehydrogenase activity of 11β-HSD type 1 to a predominantly oxo-reductase as they undergo differentiation to adipocytes, without an increase in the overall expression of the enzyme.[55]

One further potential explanation for cells taken from patients with ARDS having decreased 11β-HSD oxo-reductase activity is that the cells tested in these different experiments represent different populations of macrophages. The cells stimulated with inflammatory mediators in chapter 5 were resident type macrophages; in ARDS however new macrophages are recruited to the lungs, which have an inflammatory phenotype and a distinct cell marker profile from resident cells. A positive relationship has been demonstrated between the proportion of immature type macrophages present in the lung and disease severity.[118] A plausible explanation for our contrasting findings is that these newly recruited cells either have a constitutionally lower 11β-HSD type 1 activity, or have a different sensitivity to mediators that we were unable to demonstrate in this set of experiments. 11β-HSD type 1 is not expressed in cells of the monocyte lineage until their differentiation to macrophages,[76] when the ability to take up apoptotic cells also develops.[75, 76] The lower 11β-HSD oxo-reductase activity in macrophages of patients

with ARDS may therefore represent the presence of immature cells with lower phagocytic potential.

An important consideration is whether the changes in 11β-HSD activity we have observed have a relationship with organ and systemic steroid concentrations. Steroid metabolism within AM is likely to alter the available active steroid available within these particular cells, but only if this change in metabolism is present within a larger number of cell types would we observe associations with plasma and alveolar steroid concentrations. No convincing relationship between alveolar macrophage 11β-HSD activity and steroid concentration in the plasma or alveolar space was present, with only an association between the day 0 oxo-reductase activity and cortisone concentration in the ELF. Reasons for the lack of a relationship between the enzyme activity and plasma and BALF glucocorticoid concentrations may be due to small sample size, and the lack of this link should be interpreted cautiously. However a further explanation would be that we have only studied a single cell type that does not in any way represent the bulk of cellular activity within the lung. Suzuki et al described changes in the expression of 11β-HSD type 2 in the alveolar wall, as well as the CD68 positive cells in the alveolar lumen. We cannot comment on the 11β-HSD activity in other cells at this point. Nevertheless, in this investigation we have demonstrated that differences in steroid metabolism exist in the alveolar macrophages of patients with ARDS that are independent of the systemic increases in steroid concentrations seen in critical illness.[207]

The effect of the decrease in 11β-HSD oxo-reductase activity by alveolar macrophages would be to decrease the concentration of active glucocorticoid available to alveolar macrophages. The consequences of this would be to decrease the anti-inflammatory actions of these cells[129] and in so doing potentially perpetuate inflammation within the

lung, worsening the clinical condition and outcomes for these patients. We did not demonstrate a link between mortality, the only outcome measure directly measured in this study and 11β-HSD enzyme activity. However, given the small cohort of patients it is not surprising that such a relationship was not demonstrated, and therefore cannot be discounted.[152] In this thesis we have shown that glucocorticoids increase the efferocytosis of apoptotic neutrophils by alveolar macrophages. A finding that has previously been reported in monocyte derived macrophages.[75] We have also shown that 11β-HSD activity is required for cortisone to induce an increase in efferocytosis. Without an increase in the rate of efferocytosis apoptotic neutrophils will undergo a further secondary necrotic process, release pro-inflammatory mediators and perpetuate inflammation.[132] Investigations into tissue glucocorticoid resistance in ARDS and critical illness have previously focussed on the glucocorticoid receptor.[16, 248] In this study we have provided another possible mechanism by which the response to glucocorticoids may be blunted in ARDS, i.e. the absence of pre-receptor amplification of glucocorticoids in inflammatory cells within the lung

In conclusion, this study has shown that despite inflammatory mediators increasing the 11β-HSD oxo-reductase activity of resident alveolar macrophages in vitro, the macrophages present within the lungs of patients with ARDS have a decreased ability to convert cortisone to cortisol. This deficit in the pre-receptor amplification of the glucocorticoid anti-inflammatory signal may be responsible for some of the resistance to the anti-inflammatory effects of endogenous steroids, and the perpetuation of lung inflammation in ARDS.

8 RAGE as a biomarker in ARDS

8.1 Abstract

Introduction: The acute respiratory distress syndrome (ARDS) is diagnosed imperfectly by clinical criteria. The receptor for advanced glycation end products (RAGE) is a marker of alveolar damage and its plasma concentration is raised in ARDS. We investigated whether RAGE levels in the plasma or broncho-alveolar lavage fluid (BALF) could be used as a diagnostic marker or a monitor of disease progression in ARDS

Methods: invasively ventilated adult patients with ARDS and critically ill patients at risk of ARDS, together with normal controls were included. Bronchoscopy was performed and blood collected at recruitment to the study and repeated at day 4 in the patients with ARDS. RAGE concentrations were measured by ELISA.

Results: 67 patients with ARDS, 17 patients at risk of ARDS and 5 normal patients were included in this study. Plasma RAGE concentrations were higher at onset of ARDS than in normal and at risk groups (median =2629.94, 391.67 and 845.54 pg/ml, p=0.001). Plasma RAGE concentrations at day 4 of ARDS were no different to normal levels (median=1032.59 vs. 845.54pg/ml, p=0.211). BALF RAGE concentrations were raised at day 0 of ARDS compared to at risk and normal patients (median= 5609.99, 651.26 and 684.97pg/ml, p=0.016). BALF RAGE concentration at day 4 of ARDS was no different from day 0 (median= 2774.14pg/ml p=0.361). Using the AECC criteria as established diagnostic standard, at enrolment to the study ROC analysis of plasma RAGE had an area under the curve (AUC) of 0.910 (95%CI: 0.832 to 0.989), and BALF RAGE concentration an AUC of 0.915 (95% CI=0.846 to 0.984).

Conclusion: Neither BALF nor plasma RAGE can be recommended to monitor ARDS progression. Plasma and BALF RAGE are however good diagnostic tests for ARDS. Their use should be validated against the pathological changes of diffuse alveolar damage.

8.2 Introduction

The acute respiratory distress syndrome (ARDS) is a difficult disease to investigate. The diagnostic criteria include patients with diverse medical backgrounds,[3] a factor that may be in part responsible for the number of pharmacological therapeutic trials that have failed to show a patient benefit.[96] Relatively small numbers of patients are available for study enrolment and difficulties in recruitment make large trials difficult to conduct. This and infrequent mortality outcomes have provided a motivation to develop surrogate clinical end-points, such as ventilator free days.[152] Although biological markers (biomarkers) have previously been investigated as surrogate end points in the investigation of ARDS, many of those so far described are limited in their use as they are also markers of systemic as well as pulmonary inflammation.[13, 158] A validated biomarker specific to the lung inflammation of ARDS could be measured serially, and guide pharmacological and ventilatory therapy. It would also corroborate the physiological parameters in the diagnosis of ARDS and in so doing focus investigative effort on a more precisely defined and stratified population of patients, as well as providing a surrogate end-point for future trials.

Recently some attention has been paid to the receptor for advanced glycation end products (RAGE) as a potential biomarker of ARDS.[157] RAGE is a member of the immunoglobulin superfamily of proteins and is present endogenously as both a cell surface receptor, and in a modified form as a secreted protein.[164, 166] Particularly pertinent to the investigation of ARDS is that RAGE is expressed constitutively in the lung and is a marker of alveolar type 1 cell damage.[181, 185] Previous investigations have seen RAGE concentrations increased in the plasma of patients with ARDS, but limited information on how RAGE levels change through the course of ARDS, and RAGE concentrations in the alveolar space is available. This information is key to this molecules utility as a biomarker.[181, 183]

The aim of this study is to determine whether RAGE concentrations in the blood or alveolar space warrants further investigation as a biomarker in the treatment and investigation of ARDS.

8.3 Methods

This investigation was designed as a prospective observational study of RAGE as a potential biomarker for the investigation of ARDS. Experimental methods are described in detail in chapter 3 and are summarised below:

8.3.1 Patient recruitment and data collection

ARDS was diagnosed by the European-American consensus conference (EACC) criteria.[3] Patients with ARDS were recruited as part of either the BALTI-1 study or the bronchoscopic sub-study of the BALTI-2 trial. Subjects defined as being at risk of ARDS were patients undergoing invasive ventilation who met criteria for the systemic inflammatory response syndrome (SIRS),[215] but did not meet the EACC criteria for ARDS. These subjects were enrolled in either the resolution of inflammation in ARDS investigation, or the BALTI-1 investigation. Patient demographics and clinical data were collected and the Murray lung injury score (LIS), SAPS-2, SOFA and APACHE-2 clinical severity scores were calculated. Patients with ARDS were ventilated with a protocol-guided pressure controlled lung protective ventilatory strategy with tidal volumes of 6ml/kg.

8.3.2 Sample processing and laboratory technique

For patients with ARDS, bronchoscopy and broncho-alveolar lavage fluid (BALF) collection was performed on the day of diagnosis of ARDS (day 0) and repeated after 4 days (day 4). For normal subjects and patients at risk of ARDS, BALF collection was performed at enrolment to the study. Plasma was extracted from blood collected at the same time points. RAGE concentrations were measured in both plasma and BALF using commercially available ELISA kits (R&D Systems, Minneapolis UK). Protein concentrations were also measured using a commercially available assay (Biorad laboratories, Hercules, California, USA). The passage of protein through the alveolar membrane was then estimated by calculating the protein permeability index using the formula below:[214, 249]

Protein permeability index= (BALF protein (mg/l)/plasma protein (mg/l))*100

8.3.3 Data analysis

Statistical analysis was done using the statistical software SPSS (IBM, Somers, New York, USA). Normality was tested using the Kolmogorov-Smirnov test. If data was normally distributed further analysis was to be done using the parametric techniques t-tests and ANOVA. If data was not normally distributed analysis was done using the non-parametric techniques, Mann Whitney and ANOVA by ranks. Linear associations were to be tested using Pearson's correlation coefficient for parametric data, and Spearman's correlation coefficient for non-parametric data.

8.4 Results

A total of 67 patients with ARDS were enrolled as part of either the BALTI-1 study, or the bronchoscopic sub-study of the BALTI-2 investigation. RAGE concentrations were measured in the plasma of 54 of these patients at onset of ARDS, and 41 patients at day 4 of ARDS. We were able to measure RAGE concentrations in BALF of 43 patients at day 0 and 34 patients at day 4 of ARDS. In addition 17 patients at risk of ARDS and 5 normal patients were recruited as part of the BALTI-1 investigation and the resolution of ARDS investigation. Comparisons between the at-risk group and the patients with ARDS are shown in table 4.1 and the predisposing factors for ARDS are detailed in table 4.2. The higher LIS and lower P:F ratio in the ARDS group is a reflection of the diagnosis of ARDS. Plasma and BALF RAGE concentrations from each group were tested for normality of distribution using the Kolmogorov-Smirnov test. The ARDS plasma and BALF data at day 0 and day 4 violated the assumptions of normality, and therefore further analysis was conducted through non-parametric techniques. *See table 8.3*.

Table 8.1 Characteristics of at-risk and ARDS patients enrolled in the study. Data shown are mean and (standard deviation).

	ARDS (n=67)	**At risk (n=17)**	**Significance value**
P:F ratio (kPa)	15.57 (5.21)	31.52 (11.29)	p<0.001*
LIS score	2.85 (0.52)	1.0 (1.0)	P<0.001*
SOFA score	12.64 (3.10)	11.0 (3.0)	p=0.057
APACHE-2 score	24.15 (5.92)	25.0 (8.0)	P=0.686
Age	62.30 (16.25)	65.0 (3.0)	P=0.205

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

Table 8.2 Predisposing factors for ARDS amongst study recruits

Predisposing condition	ARDS patients	"At risk" patients
Pneumonia	24 (36%)	5 (29%)
Sepsis	31 (46%)	10 (59%)
Aortic aneurysm repair	4 (6%)	-
Chest trauma	3 (4%)	2 (12%)
Pancreatitis	1 (1%)	-
TRALI	1 (1%)	-
other	3 (4%)	-

Table 8.3 Tests of normality of data (Kolmogorov-Smirnov tests)

		df	statistic	Significance value
Plasma	**Day 0**	54	0.247	p<0.001*
	Day 4	41	0.160	p=0.010*
BALF	**Day 0**	43	0.312	p<0.001*
	Day 4	34	0.249	p<0.001*
At risk of ARDS	**plasma**	17	0.287	p=0.001*
	BALF	17	0.308	p=0.025*
Normal patients	**plasma**	5	0.237	P=0.200
	BALF	5	0.356	P=0.351

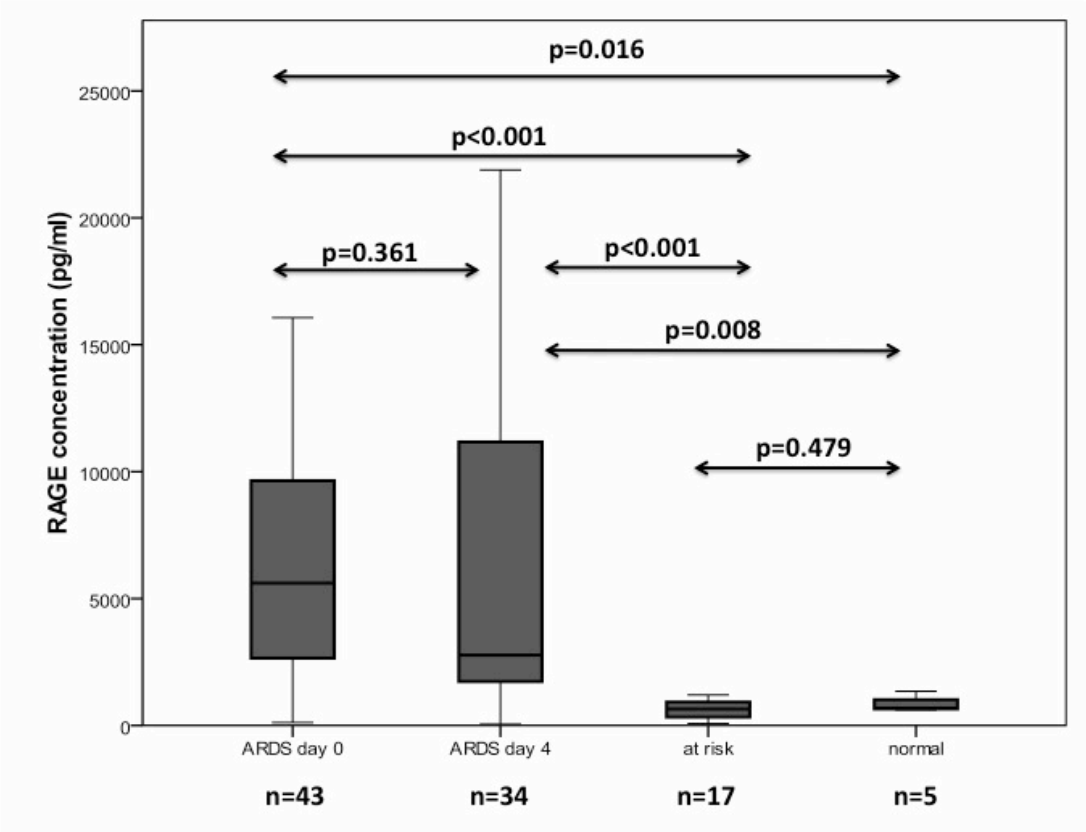
(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

8.4.1 RAGE concentration in BALF

RAGE concentrations in the BALF of patients with ARDS were higher at day 0 of disease than those present in the BALF of patients at risk of ARDS (median= 5609.99 vs. 651.26 pg/ml, U(60)=60.0, p<0.001), and in the BALF of normal subjects (median=5609.99 vs. 684.97, U(48)=13.0, p=0.016). Similarly, concentrations of RAGE in the BALF of patients at day 4 of ARDS were higher than those in the BALF of the at risk patients (median= 2774.14 vs. 651.26 pg/ml, U(51)=56.0, p<0.001), and in the BALF of normal subjects (median=2774.14 vs. 684.97pg/ml, U(39)=7.0, p=0.008). No difference in the RAGE of concentration of BALF was present between those patients at risk of ARDS and normal subjects (median= 651.26 vs. 684.97pg/ml, U(22)=18.0, p=0.479). No difference in the BALF concentrations of RAGE were present between day 0 and day 4 of ARDS

(median=5609.99 vs. 2774.14pg/ml, U(77)=642.0, p=0.361). The difference in BALF RAGE concentrations between day 0 and day 4 of ARDS was also tested using a repeated measures test (Wilcoxon matched pairs test) to determine whether a significant decline in the RAGE concentration was present for individuals; again no difference was found (Z=-0.571, p=0.568). *See figure 8.1*.

Figure 8.1 Differences in the concentrations of RAGE in the BALF of patients at day 0 and day 4 of ARDS, as well as patients at risk of ARDS and normal subjects

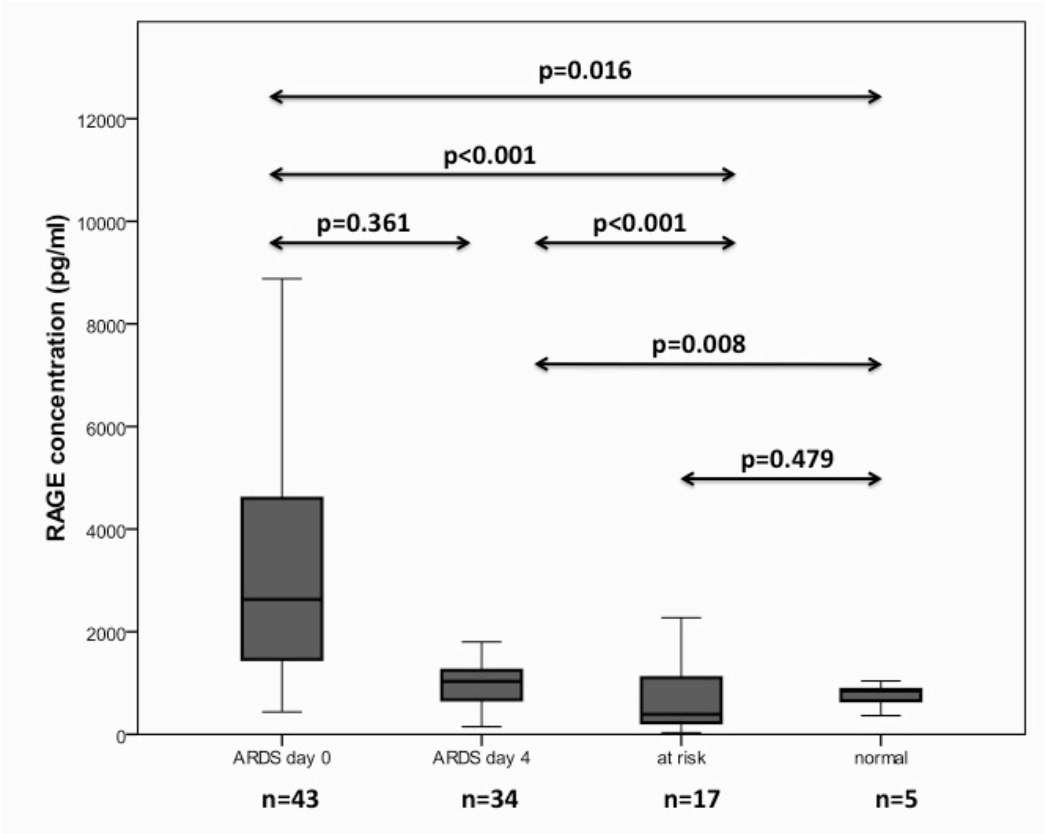


8.4.2 RAGE concentrations in plasma

Similarly to the results seen in BALF, the plasma concentrations of RAGE were higher at onset of ARDS than those seen in the plasma of normal subjects (median=2629.94 vs. 845.54 pg/ml, U(59)=22.0, p=0.001), and those at risk of ARDS (median=2629.94 vs. 391.67, U(71)=41.0, p<0.001). However, whilst no difference was seen in BALF RAGE concentrations between day 0 and day 4, the plasma RAGE concentrations at day 0 were

higher than those at day 4 of ARDS (median= 2629.94 vs. 1032.59 pg/ml, U(95)=380.0, p<0.001). Plasma RAGE concentrations at day 4 were not demonstrably different than those measured in the plasma of normal subjects (median=1032.59 vs. 845.54 pg/ml, U(46)=66.0, p=0.211) or those patients at risk of developing ARDS (median=1032.59 vs. 391.67 pg/ml, U(58)=95.0, p=0.063). There was no difference in the plasma RAGE concentrations between normal subjects, and patients at risk of ARDS (median=845.54 vs. 391.67, U(22)=12.0, p=0.284). *See figure 8.2*.

Figure 8.2 Box plot showing differences in the concentrations of RAGE in the plasma of patients at day 0 and day 4 of ARDS, as well as patients at risk of ARDS and normal subjects



8.4.3 Rage concentrations and patient characteristics

No difference in the RAGE concentration was detected between male or female subjects in the BALF or plasma at day 0 or day 4 of ARDS. *See table 8.4*. No relationship was found between the age of patients with ARDS and their RAGE concentration in either

BALF or plasma at either day 0 or day 4 of ARDS. *See table 8.5*. There was no demonstrable difference between the RAGE concentrations in the plasma or BALF of patients who had either a direct or an indirect aetiology for their ARDS. *See table 8.6*.

Table 8.4 Differences in plasma and BALF RAGE concentrations at day 0 and day 4 of ARDS between male and female patients

	Day 0 median RAGE concentration (pg/ml)		**Mann-Whitney test**	
	Male (n, range)	**Female (n, range)**	**U**	**P value**
plasma	2410.5 (32,438.51-15305.89)	3670.6 (22, 436.68-24499.13)	249.0	0.070
BALF	6669.7 (26, 833.40-50148.44)	4718.0 (17, 117.09-77067.15)	162.0	0.143
	Day 4 median RAGE concentration (pg/ml)			
	Male (n, range)	**Female (n, range)**		
plasma	767.3 (26,147.51-2349.10)	1149.2 (17, 266.44-3317.53)	144.0	0.112
BALF	2824.2 (20, 844.89-33676.37)	2505.1 (14, 51.41-12626.78)	106.0	0.245

Table 8.5 Relationship between RAGE concentration in BALF and plasma of patients at day 0 and day 4 of ARDS and patient age

		Spearman's correlation coefficient (n)	**P value**
Day 0	**Plasma**	-0.124 (54)	0.373
	BALF	0.042 (43)	0.791
Day 4	**Plasma**	0.002 (41)	0.990
	BALF	-0.43 (34)	0.809

Table 8.6 Differences in plasma and BALF RAGE concentrations between subjects with a direct and indirect cause of lung injury at day 0 and 4 of ARDS

	Day 0 median RAGE concentration (pg/ml)		**Mann-Whitney test**	
	Direct (n, range)	**Indirect (n, range)**	**U**	**P value**
plasma	3302.0 (31, 436.68-24499.13)	2095.7 (23,438.51-20098.69)	258.0	0.085
BALF	6727.9 (21, 982.33-77067.15)	5502.2 (22, 1376.10-117.09-48867.97)	171.0	0.145
	Day 4 median RAGE concentration (pg/ml)			
	Direct (n, range)	**Indirect (n, range)**		
plasma	1032.6 (25,147.51-3317.53)	1063.6 (16, 386.47-1752.46)	79.0	0.033
BALF	4935.4 (20, 1376.10-33676.37)	1784.9 (14, 51.41-20778.28)	183.5	0.663

8.4.4 RAGE concentrations and severity indices

Correlations between the concentrations of RAGE in both plasma and BALF at day 0 and day 4 of ARDS were calculated. A positive relationships was identified between the concentration of RAGE in the plasma of patients at diagnosis of ARDS and the lung injury score (n=54, ρ=0.288, p=0.035). No relationships between the day 0 plasma RAGE concentration and any of the other severity indices were demonstrated. A negative relationship between RAGE concentration in BALF at day 0 of ARDS and the SOFA score was also found (n=43, ρ=-0.429, p=0.004). No other relationship was found.

No relationship was found at day 4 between RAGE concentration in BALF or plasma and any of the severity indices. *See table 8.7*.

Table 8.7 Correlations between severity indices and concentrations of RAGE in plasma and BALF at day 0 and day 4 of ARDS

			LIS	SOFA	SAPS-2	APACHE -2
Day 0	plasma	Spearman's "ρ"	0.288*	0.227	0.108	0.099
		p =	0.035	0.099	0.439	0.475
		n =	54	54	54	54
	BALF	Spearman's "ρ"	-0.131	-0.429*	0.071	0.004
		p =	0.402	0.004	0.649	0.977
		n =	43	43	43	43
Day 4	plasma	Spearman's "ρ"	0.081	0.160	0.135	-0.117
		p =	0.615	0.318	0.400	0.467
		n =	41	41	41	41
	BALF	Spearman's "ρ"	-0.099	0.101	-0.220	-0.313
		p =	0.576	0.568	0.212	0.071
		n =	34	34	34	34

(*indicates results that have met pre-defined criteria for statistical significance (α=0.05))

8.4.5 RAGE concentration and improvement in severity score

RAGE concentrations were compared between those patients with and without an improvement in their LIS and SOFA score between day 0 and day 4 of ARDS. No difference was found in the RAGE concentrations at either day 0 or day 4, in BALF or plasma, between those patients whose LIS or SOFA score had improved and those who had not shown an improvement in these severity indices. *See table 8.8*.

Table 8.8 RAGE concentrations in patients with and without an improvement in severity scores at day 0 and day 4 of ARDS

		Any improvement in LIS		Any improvement in SOFA score	
		Yes	No	Yes	No
Day 0					
Plasma RAGE conc. (pg/ml)	Mdn.	3124.7	2484.3 (33)	2528.3 (28)	3277.4 (26)
	N	21	33	28	26
	range	676.99-24499.13	436.68-20098.69	438.51-24499.13	436.68-20098.69
	U	336.0		285.0	
	P value	0.852		0.171	
BALF RAGE conc. (pg/ml)	Mdn.	4597.7 (18)	6727.9 (25)	5394.4 (23)	6290.3 (20)
	N	18	25	23	20
	range	117.09-50148.44	881.20-77067.15	117.09-77067.15	473.89-14418.66
	U	165.0		224.0	
	P value	0.140		0.884	
Day 4					
Plasma RAGE conc. (pg/ml)	Mdn.	1026.1 (19)	1042.6 (22)	1029.3 (28)	1149.2 (13)
	N	19	22	28	13
	range	147.51-3317.53	178.25-2349.10	147.51-1800.25	441.57-3317.53
	U	183.5		142.5	
	P value	0.505		0.272	
BALF RAGE conc. (pg/ml)	Mdn.	1870.6 (16)	4989.0 (18)	4384.6 (23)	1837.3 (11)
	N	16	18	23	11
	range	51.41-33676.37	1322.44-27356.66	51.41-33676.37	992.26-21888.45
	U	98.0		101.0	
	P value	0.117		0.363	

8.4.6 RAGE concentrations and mortality

No difference was found in RAGE concentrations in either plasma or BALF of ARDS patients when they were divided by either ICU mortality, 28 day mortality or hospital mortality. *See table 4.8*.

Table 8.9 Differences in RAGE concentrations at day 0 and day 4 of ARDs between patients divided by ICU survival, 28 day survival and hospital survival.

		ICU survival		28 day survival		Hospital survival	
		Alive	**Dead**	**Alive**	**Dead**	**Alive**	**Dead**
Day 0							
Plasma RAGE conc. (pg/ml)	Mdn.	2561.9	2965.0	3218.5	2481.7	3351.1	2481.7
	N	22	32	20	34	16	38
	range	438.51-24499.13	436.68-20098.69	676.99-10040.76	436.68-24499.13	676.99-9749.40	436.68-24499.13
	U	331.0		332.0		279.0	
	P value	0.712		0.886		0.636	
BALF RAGE conc. (pg/ml)	Mdn.	4718.0	5789.5	5018.2	5789.5	4597.7	5969.0
	N	19	24	19	24	14	29
	range	833.40-50148.44	117.09-77067.15	473.89-74626.39	117.09-77067.15	833.40-50148.44	117.09-77067.15
	U	216.0		225.0		183.0	
	P value	0.769		0.942		0.604	
Day 4							
Plasma RAGE conc. (pg/ml)	Mdn.	1064.9	777.0	1039.3	1032.6	1026.1	1066.9
	N	22	19	18	23	15	26
	range	147.51-3317.53	266.44-2349.10	147.51-3317.53	266.44-2349.10	147.51-3317.53	178.25-2349.10
	U	196.0		200.0		192.0	
	P value	0.734		0.854		0.947	
BALF RAGE conc. (pg/ml)	Mdn.	3779.6	2273.2	6695.7	2330.0	5229.8	2401.6
	N	18	16	15	19	12	22
	range	1218.28-33676.37	51.41-16763.67	992.26-33676.37	51.41-21888.45	1376.10-33676.37	51.41-27356.66
	U	114.0		114.0		109.0	
	P value	0.313		0.336		0.423	

8.4.7 RAGE as a diagnostic marker of ARDS

8.4.7.1 BALF

In order to test whether RAGE concentrations in plasma and BALF could be used as a diagnostic tool in the investigation of ARDS we used receptor operating characteristic analysis (ROC). Using the AECC criteria as diagnostic, RAGE concentrations in BALF at both day 0 and day 4 of ARDS together had very a good diagnostic capability, with an area under the curve (AUC) of 0.912 (n=97, 95% CI=0.849 to 0.975). *See figure 8.3*. When using data only from BALF taken at day 0 of ARDS, the ROC curve showed a slightly improved AUC of 0.915 (n=63, 95% CI=0.846 to 0.984). *See figure 8.4.* A cut off RAGE concentration of 1360pg/ml in BALF that would provide an 81.8% sensitivity and 90.0% specificity as a test for these patients meeting the EACC criteria for diagnosis of ARDS at both day 0 and day 4 of ARDS. Using this RAGE criteria there is a clear association

between higher RAGE concentration and diagnosis of ARDS ($\chi^2(1)=37.04$, $p<0.001$). *See figure 8.5*. Using the same 1360pg/ml cut-off RAGE concentration, for patients only at day 0 of ARDS (i.e. when first meeting AECC criteria) the same specificity is given (90.0%), but the sensitivity is reduced to 79.1%. *See figure 8.6*. Using this time point only and the same RAGE criteria then again high RAGE concentrations in BALF are associated with the diagnosis of ARDS ($\chi^2(1)=26.59$, $p<0.001$).

Figure 8.3 ROC curve analysis of the utility of BALF RAGE as a biomarker in the diagnosis of ARDS (n=97, AUC=0.912, 95% CI=0.849 to 0.975)

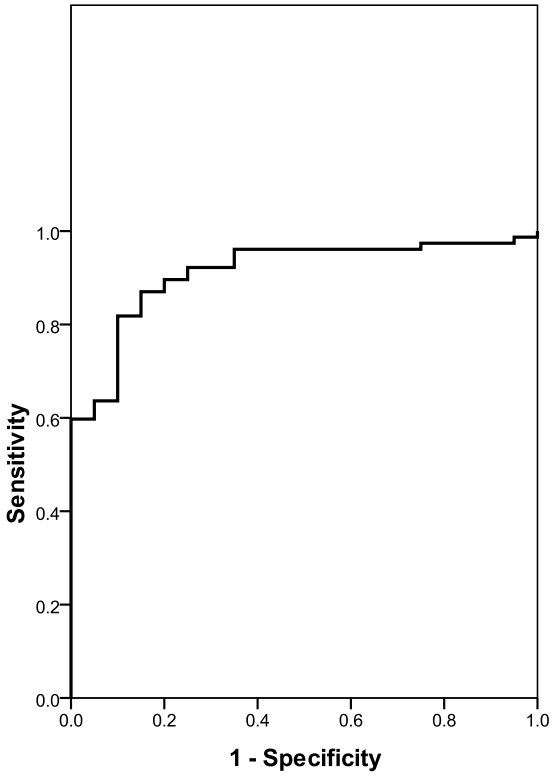


Figure 8.4 ROC curve analysis of the utility of BALF RAGE at day 0 of ARDS as a biomarker in the diagnosis of ARDS (n=63, AUC=0.915, 95% CI=0.846 to 0.984)

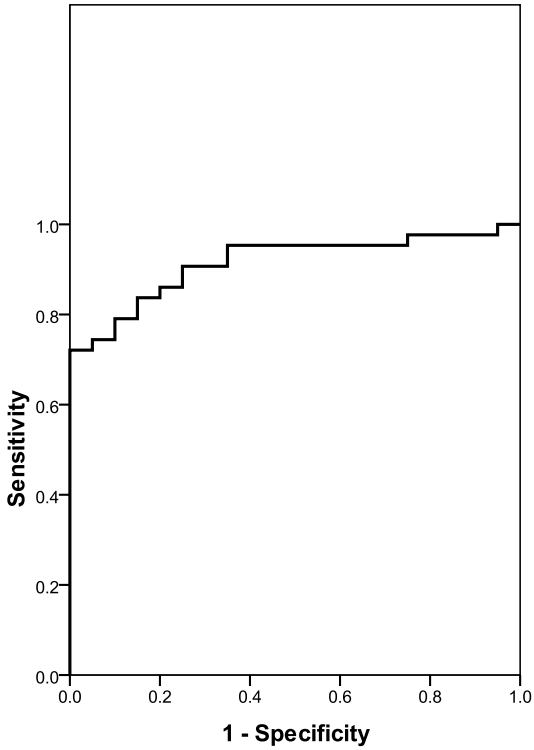


Figure 8.5 Scatter plot of RAGE concentrations in the BALF of patients diagnosed with ARDS by AECC criteria, and patients not meeting these criteria (red line at 1360pg/ml).

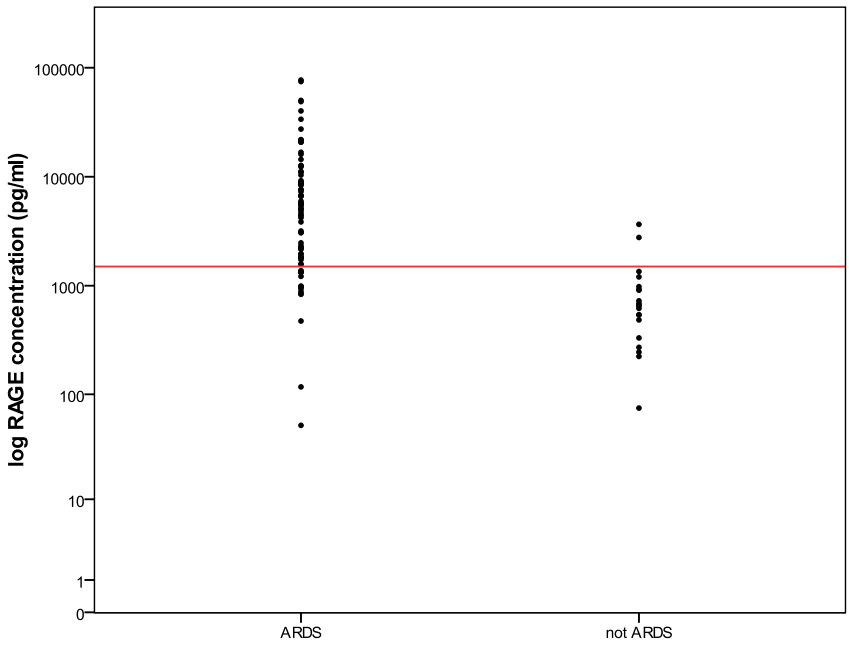
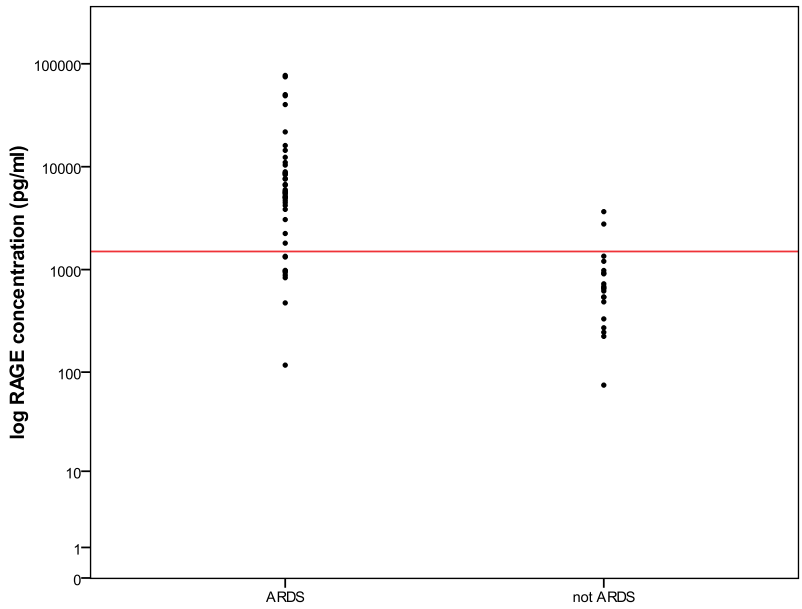


Figure 8.6 Scatter plot of RAGE concentrations in the BALF of patients at day 0 of ARDS by AECC criteria, and patients not meeting these criteria (red line at 1360pg/ml).



8.4.7.2 Plasma

ROC analysis was also used for plasma RAGE concentrations using the AECC criteria for diagnosis. When data from day 0 and day 4 of ARDS was used together the AUC was 0.819 (n=108, 95% CI=0.706 to 0.931). *See figure 8.7*. This improved to an AUC of 0.910 when data from day 0 of ARDS only was used. *See figure 8.8*. A RAGE concentration cut-off of 1610pg/ml gives a sensitivity of 56.8% and a specificity of 92.3%, using data from both day 0 and day 4 of ARDS. *See figure 8.9*. Plasma RAGE concentrations above this point are associated with the diagnosis of ARDS ($\chi^2(1)=11.05$, p=0.001). When only data from onset of ARDS are used, sensitivity is 77.8% and specificity remains at 92.3%. *See figure 8.10*. The association between the plasma RAGE concentration at study enrolment and diagnosis of ARDS is stronger than that using combined day 0 and day 4 results ($\chi^2(1)=22.39$, p<0.001).

Figure 8.7 ROC curve analysis of the utility of plasma RAGE as a biomarker in the diagnosis of ARDS (n=108, AUC=0.819, 95% CI=0.706 to 0.931)

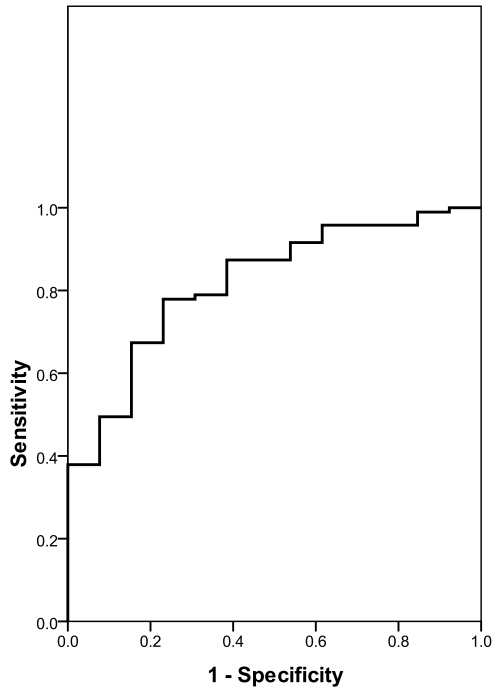


Figure 8.8 ROC curve analysis of the utility of plasma RAGE at day 0 of ARDS as a biomarker in the diagnosis of ARDS (n=67, AUC=0.910, 95% CI=0.832 to 0.989)

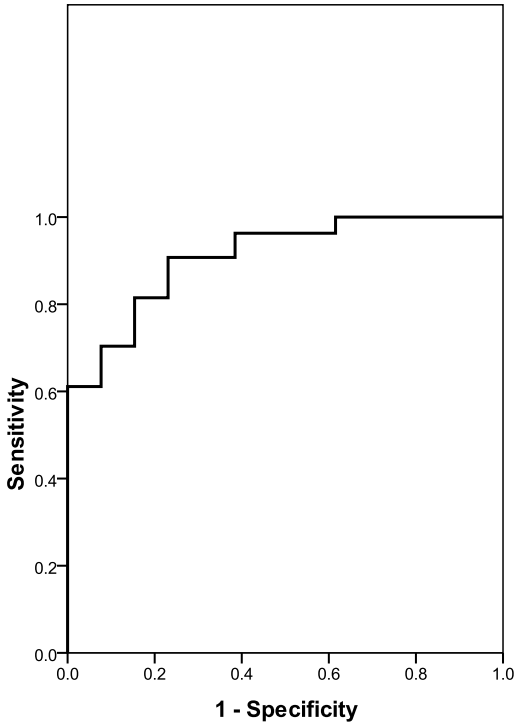


Figure 8.9 Scatter plot of RAGE concentrations in the plasma of patients diagnosed with ARDS by AECC criteria, and patients not meeting these criteria (red line at 1650pg/ml).

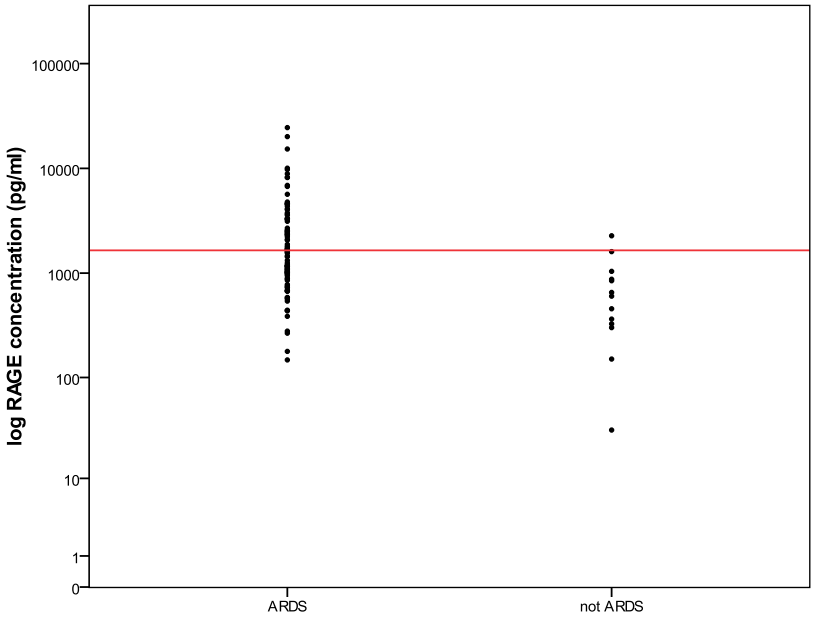
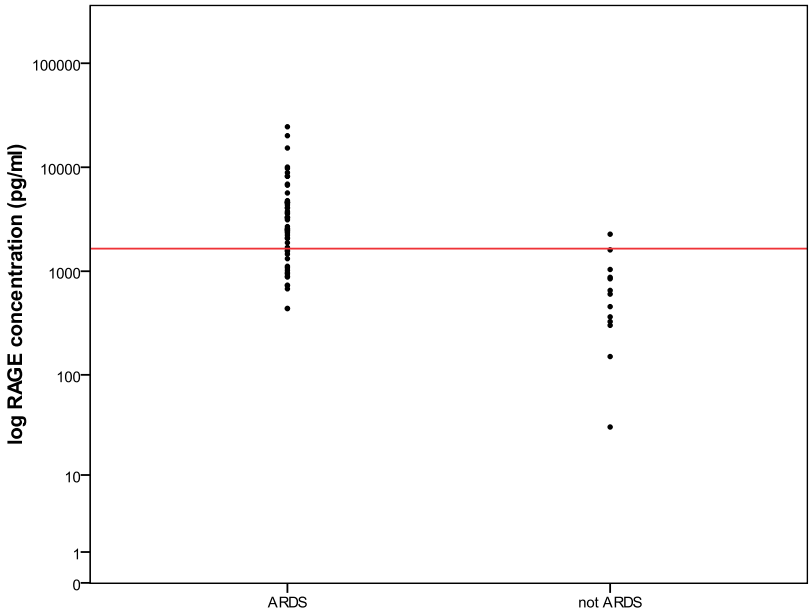


Figure 8.10 Scatter plot of RAGE concentrations in the plasma of patients at day 0 of ARDS as diagnosed by AECC criteria, and patients not meeting these criteria (red line at 1650pg/ml).



8.4.8 RAGE and cellular inflammation

In order to establish whether a relationship existed between cellular lung inflammation and concentration of RAGE in plasma and BALF, correlations were calculated between the RAGE levels and neutrophil concentration in BALF at day 0 and day 4 of ARDS. At onset of ARDS no relationship could be established between the BALF neutrophil concentration and BALF RAGE concentrations (n=39, ρ=0.130, p=0.431), or plasma RAGE concentrations (n=47, ρ=0.078, p=0.602). At day 4 of ARDS there was still no relationship between the BALF neutrophil concentration and either BALF RAGE (n=32, ρ=-0.031, p=0.866), or plasma RAGE levels(n=37, ρ=-0.048, p=0.778)

8.4.9 RAGE and protein permeability index

The protein permeability index is associated with the diagnosis of ARDS, and patients with higher indices have a worse patient outcome.[249] In order to assess the relationship between RAGE concentrations in plasma and BALF and alveolar membrane dysfunction, correlations were calculated between the RAGE levels and the protein permeability index. A positive linear relationship was found between the RAGE concentration in BALF and the protein permeability index at both day 0 (n=40, ρ=0.349, p=0.027), and day 4 of ARDS (n=30, ρ=0.678, p=0.004). *See figure 8.11 and 8.12*. No relationship was seen between the RAGE concentration in plasma at either day 0 (n=50, ρ=0.207, p=0.148) or day 4 (n=34, ρ=-0.107, p=0.549) of ARDS and the protein permeability index.

Figure 8.11 Relationships between BALF RAGE concentration and the protein permeability index at day 0 of ARDS (n=40, ρ=0.349, p=0.027).

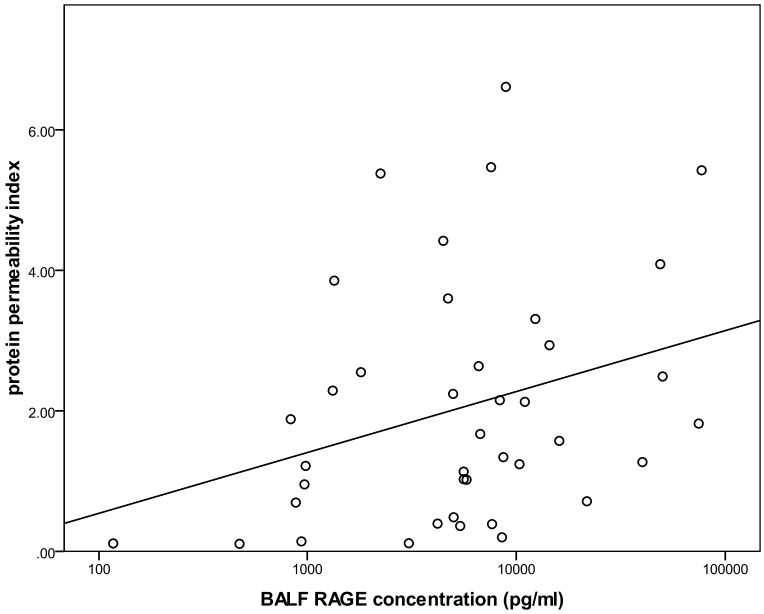
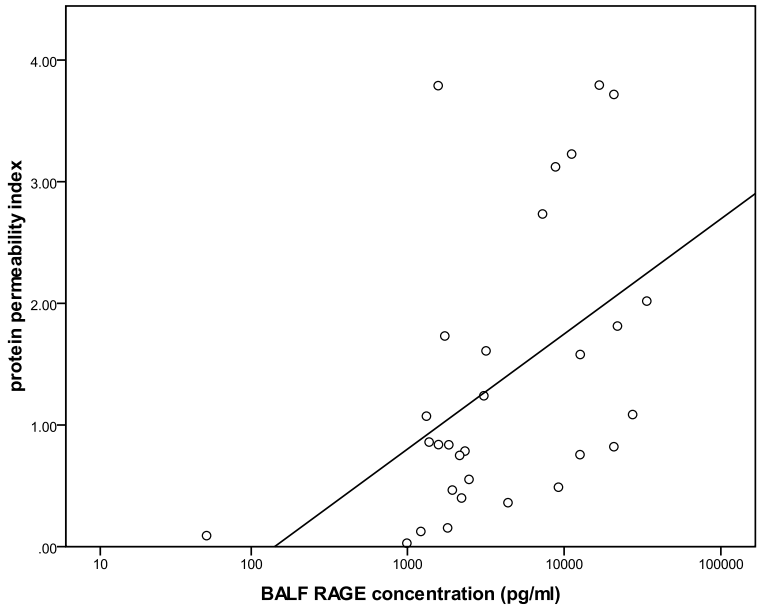


Figure 8.12 Relationships between BALF RAGE concentration and the protein permeability index at day 4 of ARDS (n=30, ρ=0.678, p=0.004).



8.5 Discussion

This investigation has confirmed that RAGE concentrations are increased in both the BALF and the plasma of patients with ARDS. RAGE concentrations in plasma were diminished by day 4 of ARDS compared to day 0, but as they were no higher than those seen in patients without ARDS, the use of plasma RAGE concentrations to monitor resolution of ARDS is not possible. Although RAGE concentrations in BALF at day 4 of ARDS remain high there was no decrease in the RAGE concentrations in line with clinical evaluation of the patient. For this reason we cannot recommend using RAGE concentrations in either BALF or plasma to monitor the progression of ARDS.

Importantly, patients with a critical illness and systemic inflammatory response, but who do not have ARDS, had RAGE concentrations that were no different from normal. This supports the use of RAGE as a marker that is specific to lung inflammation. Other investigations have shown biomarkers raised in ARDS, but have not been specific to the lung inflammation.[13, 158] In their retrospective analysis, Determann and colleagues used samples from a ventilator acquired pneumonia study to investigate potential diagnostic markers of ARDS, and found that soluble RAGE had limited utility, with an AUC of only 0.63 on ROC analysis. This study involved fewer patients than the current investigation and measured soluble rather than all forms of RAGE, factors which may explain the discrepancy between results.[161] These authors suggested Clara cell protein as an alternative diagnostic marker of ARDS, a molecule that does have support from other investigators.[250]

Plasma RAGE levels that are high at onset of ARDS have returned to normal by day 4. The presence of a raised plasma RAGE concentration therefore seems to be associated with early ARDS. Although it's rapid decline over the subsequent days precludes its use as a measure of ARDS recovery, this effect may help in other ways: it would make characterisation of early ARDS rather than later stages of the disease possible, and act as a potential diagnostic criteria, analogous to the use of troponin in myocardial infarction.[153] This information could better target enrolment into studies, and patients recruited into studies split into RAGE positive and RAGE negative groups for subsequent analysis. Early therapy in critically unwell patients with sepsis has been established to be of benefit,[251, 252] and this may be the case in lung injury. If a tool such as RAGE could be used to identify early disease better than the current AECC criteria, this may allow

targeted therapy in the earlier phases of ARDS. Temporal variation in the effects of treatment of ARDS have previously been found; the ARDSnet therapeutic study of steroids found that patients started on exogenous glucocorticoids late in the course of disease had a worse outcome than those in the earlier part of their illness.[85]

The absence of a gold standard in the diagnosis of the pathological process of ARDS is a major hurdle for the development of a useful biomarker for ARDS. The most frequent pathological finding for ARDS is diffuse alveolar damage (DAD), however the sensitivity of the AECC criteria at detecting patients with DAD is only 75%, dropping to 61% in patients with a respiratory cause for their ARDS.[253] Conversely many patients with DAD do not fit the AECC criteria for ARDS.[254] This would suggest that the AECC criteria require revision that include a biological marker of inflammation, a role that RAGE is well placed to fill. Our ROC analysis shows that RAGE has very good predictive capabilities, especially at onset of disease. In this investigation we did not aim to measure RAGE concentrations against the presence of DAD. Therefore we cannot determine whether RAGE is a better predictor of the presence of this pathological appearance or not. We were however able to show that at onset of ARDS RAGE can be used as a marker with good specificity, sensitivity to predict the AECC diagnosis of ARDS.

We did not find relationships between RAGE concentrations and mortality, or improvements in the severity indices we measured in this investigation. Neither was there a link present between the cellular inflammation and RAGE concentrations. Although a relationship was present between the plasma RAGE concentration and the LIS, and the BALF RAGE concentration and the SOFA score, the values for these relationships may not be convincing (ρ=0.288 and ρ=-0.429 respectively). However, the absence of these relationships should not preclude the use of RAGE as a marker of ARDS. As patients with ARDS tend to die due to a precipitating illness, rather than from the effects of ARDS itself,[255, 256] expecting a biomarker of ARDS to reflect patient mortality seems over-optimistic. Indeed Calfee and colleagues did not show a relationship between RAGE and patient outcome when a protective ventilatory strategy was employed. The patients enrolled in the current study were ventilated using a lung protective strategy, and thus our findings support those of Calfee et al.[183]

The most immediate physiological consequence of ARDS inflammation is the alveolar membrane dysfunction, and subsequent pulmonary oedema and impairment of gas

exchange. Therefore, a biochemical marker of damage to this structure is a more pertinent measure of inflammation in ARDS than neutrophil numbers or global severity indices. In this respect the relationship between the protein permeability index and RAGE concentrations is both plausible and supportive of our hypothesis.

Causing lung injury by the use of mechanical ventilation is a pitfall of intensive care. In their study Calfee and colleagues did see links between patient outcomes and RAGE concentrations when higher volume ventilation was used: supporting the rationale that higher volume ventilation is injurious to the alveolar membrane and detrimental to the patient overall. It has been suggested that RAGE may have a role as a biomarker of ventilator induced lung injury (VILI); concentrations being used to guide ventilator settings.[157] RAGE concentrations measured immediately after a potential lung insult to predict the physiological manifestations of ARDS, the need for intensive care support and lung protection is another potential clinical role of this molecule that requires further research.

In conclusion, measurement of plasma or BALF RAGE at study enrolment to biologically confirm alveolar membrane damage could be used together with the clinically measured parameters that make up the AECC criteria. This would help to provide better defined cohorts of patients for investigation, and may help to diminish the number of clinical trials failing to show patient benefit. Further corroboration of our results is undoubtedly required, and confirmation using histological evidence of DAD would be the ideal.

9 Summary and conclusions

9.1 Overview of glucocorticoid metabolism in ARDS investigations

ARDS is a severe inflammatory condition of the lung that causes respiratory failure in critically ill patients. The inflammation in ARDS is refractory to the endogenous anti-inflammatory signal of glucocorticoids produced by the hypothalamus-pituitary-adrenal gland (HPA) system, and is perpetuated by long-lived pro-inflammatory neutrophils in the lung not being adequately disposed of as they undergo apoptosis. This thesis has addressed the presence of glucocorticoids within the lung in ARDS, the amplification of this anti-inflammatory signal by 11β-hydroxysteroid dehydrogenase (11β-HSD), and the relevance of these hormones and their metabolism to the clearance of apoptotic neutrophils and the resolution of ARDS.

This thesis has also addressed the need for the development of a biomarker for the investigation and clinical management of ARDS, and contains the report of an investigation into whether the receptor for advanced glycation end products (RAGE) could be used as such.

9.1.1 Alveolar glucocorticoids in ARDS

We have demonstrated that not only are corticosteroid concentrations raised in the plasma of patients who have ARDS, but that they are also raised within the alveolar space. This finding has been suspected, but not previously demonstrated. We have also shown that glucocorticoid concentrations had a negative relationship with both cellular inflammation and alveolar membrane permeability, suggesting that these hormones have anti-inflammatory, pro-resolution properties in this setting. Glucocorticoid concentrations in the plasma and the alveolar space are however linked to severity of the patient's critical illness, but not with severity indices specific to lung injury.

We have also shown that the cortisol: cortisone ratio within the lung is increased in patients with ARDS. While this latter parameter is not a completely satisfactory measure of the 11β–HSD activity within the lungs, in this instance it does show that even should there be an increase in 11β-HSD type 2 expression, as suggested by the histopathology studies of Suzuki et al, it does not seem to be impacting on steroid concentrations within the lung.

9.1.2 Alveolar macrophage 11β-HSD activity

Alveolar macrophages are key cells in the resolution process of ARDS whose function is influenced by glucocorticoids. They develop 11β-HSD type 1 expression as they differentiate from monocytes, simultaneously developing the ability to take up apoptotic cells in the process of efferocytosis. In keeping with findings of investigations of other cell types, primary alveolar macrophages extracted from healthy sections of lung have an increase in oxo-reductase activity (conversion of cortisone to active cortisol) when exposed to inflammatory stimuli such as TNF-α and IL-1β. We also demonstrated that when broncho-alveolar lavage fluid was used to model ARDS this also increased the 11β-HSD oxo-reductase activity of these cells. We were unable to show any convincing increase in the 11β-HSD dehydrogenase activity (inactivation of cortisol to cortisone) which would have signalled an increase in activity and expression of 11β-HSD type 2 in these cells.

9.1.3 Efferocytosis in ARDS

For the resolution of ARDS the inflammatory neutrophils that are present in abundance in the lungs of patients with ARDS must be safely disposed of by alveolar macrophages. Failure of this process leads to necrosis and the release of pro-inflammatory mediators. We have shown that glucocorticoids increase the efferocytosis of apoptotic neutrophils

by alveolar macrophages. 11β-HSD oxo-reductase activity amplifies the anti-inflammatory signal of glucocorticoids, and its activity determines the concentration of active steroid available. Blockade of 11β-HSD prevents cortisone induced increases in efferocytosis, and shows that activity of this enzyme will increase available cortisol, which would further increase the capacity for efferocytosis.

Efferocytosis is suppressed by BALF taken from patients at the onset of ARDS, but increases later in the course of this disease. HMGB-1 is a pro-inflammatory mediator released from activated macrophages and necrotic cells that is present in increased concentrations in the lungs of patients with ARDS. HMGB-1 suppresses efferocytosis, and its concentration in the BALF used to model ARDS is associated with the degree to which that BALF sample suppresses efferocytosis.

9.1.4 11β-HSD activity in alveolar macrophages from patients with ARDS

Despite our findings that healthy resident alveolar macrophages increase their 11β-HSD oxo-reductase activity in response to inflammatory stimuli, we have demonstrated that in alveolar macrophages taken from patients with ARDS the 11β-HSD oxo-reductase activity was decreased in comparison to the activity present in cells both from ventilated patients at-risk of ARDS and normal subjects. Such a deficit in 11β-HSD oxo-reductase activity will limit the potential of these cells to amplify the glucocorticoid signal, and the anti-inflammatory function of these cells. The 11β-HSD activity in these cells is not related to the glucocorticoid concentrations present in the alveolar space, and as such the activity in these cells is not reflecting the increased net conversion of cortisone to cortisol observed within the lung. Similar to the healthy resident cells, no increase in the 11β-HSD dehydrogenase activity was seen in these cells. We were unable to determine

whether increased transcription of 11β-HSD iso-enzymes was responsible for the observed differences.

9.1.5 Summary

Concentrations of cortisol in the lung are increased in ARDS, due to increased cortisol delivery from higher plasma concentrations, and increased amplification of the glucocorticoid signal by increased local 11β-HSD oxo-reductase activity within the lung. Our findings show that resident macrophages increase their 11β-HSD conversion of cortisone to cortisol in response to inflammatory stimuli in-vitro, and that glucocorticoids and 11β-HSD oxo-reductase activity increase the uptake of apoptotic cells by alveolar macrophages. However, alveolar macrophages from patients with established ARDS have decreased 11β-HSD oxo-reductase activity. The implications of this are that they will have a limited capacity for efferocytosis and a diminished anti-inflammatory potential.

9.2 RAGE as a biomarker in ARDS

RAGE has been previously investigated as a biomarker in lung injury, and fulfils many of the pre-requisites for this role. RAGE is an immunoglobulin type protein that is up-regulated in inflammation, it is specific to lung injury in critically ill patients, and can be easily measured in either BALF or plasma. This thesis investigated the use of RAGE as a marker of lung injury severity, diagnosis and progression of ARDS.

In keeping with RAGE being a marker of alveolar membrane injury RAGE concentrations in the BALF are associated with increases in the protein permeability of the alveolar membrane in ARDS patients. This investigation also showed that RAGE concentrations

are higher in the BALF and plasma of patients at the onset of ARDS than those in normal subjects or patients who are ventilated, with systemic inflammation, but without ARDS. However in the plasma at day 4 of ARDS RAGE concentrations are no higher than in at-risk patients, and in BALF have not declined from day 0 levels. RAGE concentrations in the BALF or plasma are not linked to severity of the patient's critical illness, or mortality measures. These factors mean that RAGE would make a poor monitor of disease progression or response to therapy. However in this investigation RAGE concentrations in both plasma and BALF had excellent diagnostic ability when compared to the AECC diagnostic criteria. The use of a threshold RAGE concentration as an inclusion criterion in the investigation of ARDS may assure pulmonary inflammation in trials involving anti-inflammatory therapy.

9.3 Further investigation

As is typical of translational research more questions have been generated in the course of this investigation. Two areas that require further investigation are:

1. Direct efferocytosis experiments similar to the ones reported in chapter 6 should be conducted using alveolar macrophages extracted from patients with ARDS. This would determine whether these cells do indeed have diminished capacity for efferocytosis as has been strongly suggested by these experiments.

2. The phenotype of macrophages present within the lungs of patients with ARDS and their steroid 11β-HSD metabolism and capacity for efferocytosis should be characterised, and linked to clinical markers and outcome measures. This would further improve our understanding of how the alveolar macrophage population within the lung

evolves and functions and allow the development of therapies to improve ARDS resolution.

3. A large cohort of patients should be enrolled to validate the use of RAGE in the investigation of ARDS, and computerised tomography (CT) data or autopsy specimens obtained to link RAGE concentration increases to either the histopathology changes of ARDS, and detailed radiological evidence of this disease, before this marker could be accepted as a tool for the investigation and treatment of ARDS.

9.4 Conclusions

ARDS remains an important cause of morbidity and mortality in critically ill patients. Investigation of this disease using RAGE as a diagnostic biomarker is a potential new tool in developing new treatments and guiding therapy. This thesis has demonstrated that glucocorticoid metabolism by 11β-HSD within alveolar macrophages promotes the clearance of apoptotic neutrophils, but that this enzymes activity is diminished in the macrophages of patients with ARDS. The influence of this enzymes activity on the resolution of ARDS requires further investigation. This will enhance our understanding of how lung inflammation can be controlled and the development of anti-inflammatory therapies.

10 Abbreviations used

AECC	American European consensus conference (on ARDS)
ALI	Acute lung injury
AM	Alveolar macrophage
APACHE-2	Acute physiology and chronic health evaluation score-version 2
ARDS	Acute respiratory distress syndrome
BALF	Broncho-alveolar lavage fluid
cAMP	cyclic adenosine mono-phosphate
CBG	cortisol binding globulin
CLP	caecal ligation and puncture
COPD	chronic obstructive pulmonary disease
CO_2	carbon dioxide
DNA	deoxyribose nucleic acid
ELF	Epithelial lining fluid
EI	efferocytosis index
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FiO_2	Fraction of inspired oxygen
HMGB-1	High mobility group box protein-1
HSD	hydroxysteroid dehydrogenase
H6PDH	hexose-6-phosphate dehydrogenase
ICU	Intensive care unit
I-κβ	Inhibitor-κβ
IPF	idiopathic pulmonary fibrosis
IL-1β	Interleukin-1β
LIS	Lung injury score
MDM	Monocyte derived macrophage
MLTC	Midlands lung tissue collaborrative
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide (NAD+ is dehydrogenated form)
NF-κβ	Nuclear factor-κβ
NHS	National Health Service

PAOP	pulmonary artery occlusion pressure
PaO_2	arterial oxygen pressure
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEEP	positive end-expiratory pressure
P: F ratio	the ratio of plasma oxygen pressure to inspired oxygen pressure
PMN	polymorphic neutrophil
PS	phosphatidyl serine
qPCR	quantitative PCR
RAGE	Receptor for advanced glycation end products
RNA	ribose nucleic acid
RPMI	Roswell Park Memorial Institute (cell culture media)
RQ	relative quantity (of DNA in qPCR assay)
SAPS-2	Simplified acute physiology score-2
SOFA	sequential organ system failure assessment
TNF-α	Tumour necrosis factor –α
TRALI	transfusion related acute lung injury
VILI	Ventilator induced lung injury
VFD	Ventilator free day

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