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IS VITAMIN D DEFICIENCY A MECHANISTIC DRIVER OF ACUTE LUNG INJURY?

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the degree of

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This thesis is dedicated to Kat, Neeva and Rahi for keeping me smiling and reminding me of life's simple pleasures.

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DECLARATION

No part of this thesis has previously been submitted for the award of any degree at the University of Warwick, or any other institution.

The work presented in this thesis is the work of Dr Dhruv Parekh. Areas of technical assistance from collaborators are outlined here and acknowledged within the text and at the end of each section

Parts of chapter 1 and chapter 3 have been published and parts of chapter 4 and 5 presented in abstract form prior to submission of this thesis. These are listed at the beginning of each relevant section and in the Appendix. This work was not published or presented prior to the beginning of the candidate's period of study for this degree at the University of Warwick.

ABSTRACT

The acute respiratory distress syndrome (ARDS) remains a major cause of morbidity and mortality in the critically ill patient. There are no effective strategies for identifying those most at risk or therapeutic interventions proven to prevent its occurrence. Vitamin D deficiency is common and has important functions besides calcium homeostasis with profound effects on human immunity. Preliminary data suggests in the high-risk sepsis and oesophagectomy groups that vitamin D deficiency may be a pre-existing risk factor and mechanistic driver of ARDS.

This thesis investigated in an animal model and *in-vitro* studies whether vitamin D influences the innate immune response to sepsis and resolution of neutrophilic injury. In addition, it reports a proof of concept phase II study to determine if vitamin D therapy in patients undergoing oesophagectomy is anti-inflammatory and protective of markers of lung injury

Vitamin D deficiency significantly increased the bacterial load, bacteraemia and translocation to the lung in a murine model of peritonitis. This was associated with a rise in tissue permeability locally and within the lung, reduced antimicrobial peptide and defective peritoneal macrophage phagocytosis. These data support pre-existing vitamin D deficiency as a determinant of the severity of bacteraemic sepsis.

In-vivo high dose vitamin D supplementation was a safe, well-tolerated pre-operative intervention with reduced biomarkers of alveolar oedema, capillary leak and macrophage efferocytosis. *In-vitro* culture with vitamin D increased macrophage efferocytosis and promoted monocyte differentiation to a pro-resolution phenotype. This suggests a potential mechanism for vitamin D on protecting barrier integrity and resolution of neutrophilic inflammation, a hallmark of ARDS.

This body of work demonstrates that vitamin D deficiency is a potential modifiable risk factor and should be identified and treated in patients at risk of sepsis and ARDS. Larger trials powered to evaluate the effect of vitamin D on preventing and improving clinical outcomes in sepsis and ARDS are warranted.

CHAPTER 1

INTRODUCTION

Parts of this chapter have been published:

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Acute lung injury.

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Inflamm Allergy Drug Targets 2013;12:253-61.

1.1 ACUTE RESPIRATORY DISTRESS SYNDROME

Acute lung injury (ALI) and the more severe acute respiratory distress syndrome (ARDS) are devastating clinical syndromes characterised by pulmonary inflammation, increased alveolar-capillary permeability and pulmonary oedema that typically causes acute respiratory failure refractory to oxygen therapy in the critically ill person.¹ Patients with ARDS usually require mechanical ventilation during the course of their illness and mortality remains high. Survivors of ARDS experience a significant reduction in health-related quality of life (HRQOL) and debilitating long-term sequelae including pulmonary, psychological and neuromuscular impairment.^{2,3}

Due to the wide presenting phenotype and heterogeneity of the syndrome, diagnosis remains challenging and developing therapies to treat and prevent it remain elusive despite promising positive pre-clinical studies. The advent of lung protective ventilation has resulted in reduced mortality in patients with ALI.⁴ However there are no current readily available tests that can clearly identify those who are at high risk of ALI, and no therapeutic interventions proven to prevent its occurrence.

1.1.1 Evolution of the definition

In 1967 Ashbaugh *et al.* published the first description of 12 patients with similar clinical physiology, radiography and pathology that was later described as the acute respiratory distress syndrome (ARDS).⁵ The 12 patients involved had acute respiratory distress requiring positive pressure

mechanical ventilation with the addition of positive end expiratory pressure (PEEP), cyanosis refractory to oxygen therapy, decreased lung compliance and diffuse pulmonary infiltrates on chest radiograph (CXR). The majority of patients were previously fit and well but mortality was high and post mortem examination of the 7 that died demonstrated widespread atelectasis, vascular congestion, intra-alveolar haemorrhage, severe pulmonary oedema and hyaline membrane formation. It is clear however, that patients with ARDS have been described before particularly in the context of battlefield trauma. Thus post-traumatic lung injury has been described as “wet lung” in World War 2, “shock lung” or “Da-Nang lung” after a particularly bloody battle during the Vietnam War.⁶

Numerous attempts have been made to quantify the degree of lung injury severity including a 4 point scoring system by Murray *et al* in 1988.⁷ Nevertheless, specific criteria for ARDS to identify patients were only established in 1994 by the American European Consensus Conference Committee (AECC).⁸ They include acute onset of hypoxemia, and bilateral infiltrates on chest radiograph in the absence of clinical evidence of left atrial hypertension. The severity of hypoxemia differentiates between ALI and ARDS (Table 1.1).

Table 1.1: American European consensus committee (AECC) 1994 diagnostic criteria for Acute Respiratory Distress Syndrome and Acute Lung Injury.⁸

- Acute onset
- Bilateral pulmonary infiltrates on chest radiograph
- Pulmonary Capillary Wedge Pressure <18 mmHg (2.4kPa) or the absence of clinical left atrial hypertension
- Acute Lung Injury: PaO₂/FiO₂ ratio < 300mmHg (40kPa)
- Acute Respiratory Distress Syndrome: PaO₂/FiO₂ ratio < 200mmHg (26.7kPa)

FiO₂, fraction of inspired oxygen; PaO₂, partial pressure of arterial oxygen

AECC diagnostic criteria are a crude screening tool but for many years were established as the most widely accepted method of identifying these patients both clinically and for research trials. They have provided a tool to diagnose, investigate potential treatment options and understand the epidemiology and disease process. However AECC criteria have been challenged over the years due to its limitations relating to specificity and reproducibility⁹ as well as the omission of the standardisation of ventilatory support in its assessment of hypoxaemia.¹⁰

Table 1.2: Berlin definition of Acute Respiratory Distress Syndrome.¹¹

Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest imaging	Bilateral opacities—not fully explained by effusions, lobar/lung collapse, or nodules
Origin of edema	Respiratory failure not fully explained by cardiac failure or fluid overload. Need objective assessment (e.g. echocardiography) to exclude hydrostatic oedema if no risk factor present
Oxygenation	
Mild	200 mm Hg < PaO ₂ /FIO ₂ < 300 mm Hg with PEEP or CPAP > 5 cm H ₂ O (may be non-invasive ventilation)
Moderate	100 mm Hg < PaO ₂ /FIO ₂ < 200 mm Hg with PEEP > 5 cm H ₂ O
Severe	PaO ₂ /FIO ₂ < 100 mm Hg with PEEP > 5 cm H ₂ O

CPAP, continuous positive airway pressure; FIO₂, fraction of inspired oxygen; PaO₂, partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure.

More recently, a new consensus definition, the Berlin definition for ARDS has been described and is currently the recommended definition of the condition.¹¹ The new definition maintains the 1994 AECC criteria of timing, chest imaging, hypoxaemia and origin of oedema. It attempts to improve diagnosis and prognostication by recommending the use of 3 categories of ARDS based on level of hypoxaemia (mild, moderate, severe and removing the term ALI, together with a minimum level of positive end-expiratory pressure (PEEP) (Table 1.2) . It also attempts to address some of the other limitations of the AECC criteria by: defining 'acute' as a period of onset of

ARDS within 7 days of known clinical insult; clarifying chest radiograph (CXR) criteria and removing pulmonary artery occlusion pressure as a measure of cardiogenic pulmonary oedema as this has been difficult to measure and shown to be poor at differentiating between cardiogenic and non-cardiogenic pulmonary oedema.^{12,13} The revised definition appears to have marginally improved predictive validity for mortality using a receiver-operator curve with an area under the curve from 0.53 to 0.57.¹¹ The severity and duration of ARDS have also been correlated to histological findings.^{14,15}

In view of the change in the definition of the condition during the period of the work presented in this thesis an attempt has been made to use the term ARDS. However on occasion when the term ALI is used it refers to the old AECC criteria definition (which does not require the use of PEEP or CPAP) and ARDS refers to the new Berlin definition.

1.1.2 Epidemiology and Outcome

The overall incidence of ARDS remains unclear due to limitations in the diagnostic criteria and heterogeneity of the populations and underlying causes, but the most recent studies in the USA utilising AECC criteria have suggested rates of 13-59 cases per 100,000 population per year for ARDS and 18-79 per 100,000 population per year for ALI.^{16,17} Studies in Europe have consistently reported lower rates between 4.9-13.5 cases per 100,000 person-years.¹⁸⁻²¹ Sigurdsson *et al* have recently reported a large retrospective study spanning 23 years in Iceland in which incidence of ARDS

doubled, but hospital mortality decreased.²¹ The same incidence of 7 cases per 100,000 population per year was seen in another recent large prospective study in Spain, however despite the use of lung-protective ventilation, overall intensive care unit (ICU) and hospital mortality was still reported as 43% and 48% respectively.²⁰

Overall, reported mortality rates vary between 36-44%²² but more recent clinical trials and reviews in ARDS suggest that this may be lower at 19-23%.²³⁻²⁶ This may be attributed to lung protective ventilation^{4,27,28}, conservative fluid strategies²⁹ and improvements in advanced supportive care, as well as avoiding potentially aggravating factors such as ventilator associated pneumonia, multiple blood transfusions and gastric aspiration.^{15,30} The majority of patients die of the underlying pathology rather than respiratory failure itself.³¹ Several factors have been associated with an increased risk of mortality in ARDS including: severity of arterial hypoxaemia¹¹ and increase in pulmonary dead space fraction³² as well as immune-incompetence, shock, sepsis, liver dysfunction, acute kidney injury and age over 60 years.^{19,31,33-35}

Although lung function parameters recover well in ARDS patients who survive there is significant residual physical limitations and poorer quality of life, with 46% reported to be unable to return to work within 12 months.^{2,3} Depression, post-traumatic stress and neurocognitive impairment is not uncommon leading to a significant impact on the individual, carers and

society as a whole.^{2,36} In a prospective 5 year follow-up study of 109 survivors of ARDS, Herridge et al found that although pulmonary function (spirometry, lung volumes and gas transfer) were near normal, decrements in quality of life and exercise capacity persisted at 5 years.² The authors suggest that this may be due to persistent ICU acquired weakness and neuropsychological impairment. A further recent prospective study of over 200 survivors has also shown substantial physical morbidity after ICU discharge, including impairments in muscle strength, physical function, and HRQOL.³⁷ Recovery of muscle strength generally occurred within 12 months after ALI, but muscle weakness contributed to significant impairments in physical function (measured by 6 minute walk test) and HRQOL that persisted to 24 months post-ALI.³⁷

1.1.3 Aetiology

Certain known risk factors such as sepsis, surgery or trauma lead to the development of ALI and ARDS. The mode of injury can be either direct or remote to the lung (Table 1.3) with pneumonia and sepsis being the most common causes of ALI/ARDS.^{6,20,21} Only a relatively small proportion of patients develop ALI, with research suggesting that genetic³⁸, demographic (age)^{19,31}, social (smoking³⁹, alcohol abuse⁴⁰) and other factors play a role in determining who develops ALI.^{16,17} Due to this broad range of precipitating conditions patients can present to any emergency, medical or surgical specialty with early recognition being the vital step in providing appropriate care. Considering the underlying aetiology is important, as the initiating

cascade of pathophysiological changes and prognosis may be different depending on the underlying cause of injury.⁶ This is extremely relevant if we are to be successful in developing strategies to identify patients at risk of developing ARDS. Furthermore this may represent a reason as to why a blanket approach to potential drug treatments has resulted in negative trials to date.

Table 1.3 Conditions associated with the development of Acute Respiratory Distress Syndrome.⁶	
Indirect	Direct
Sepsis	Pneumonia
Multiple trauma	Aspiration
Cardiothoracic bypass	Near drowning
Post-oesophagectomy	Smoke inhalation
Massive blood transfusion	Pulmonary contusion
Acute pancreatitis	Post lung transplantation

1.1.4 Early identification of ARDS

Epidemiologic investigations and clinical trials in ALI and ARDS have predominantly focused on mechanically ventilated patients in ICUs, but recent data suggests ~9% of patients in respiratory isolation wards meet the criteria for ALI at some point during their admission, and 2% met criteria for the diagnosis of ARDS.⁴¹ Furthermore, Levitt et al have shown that ALI can be diagnosed in the spontaneously breathing patient in the emergency department⁴² and subsequently Gajic and colleagues have prospectively

derived the Lung Injury Prediction Score (LIPS).⁴³ In this large multi-centre study of 5,584 patients admitted with one known risk factor for ALI the authors were able to identify patients who went on to develop ALI with an area under the receiver operator curve of 0.80 (95% confidence interval, 0.78–0.82). Consequently it is possible to identify a significant number of patients prior to established ALI and prior to the requirement of invasive ventilation. Indeed, the LIPS is currently being utilised in ongoing lung injury prevention studies of aspirin (LIPS-A)⁴⁴ and inhaled budesonide/formoterol (LIPS-B NCT01783821). Therefore identifying possible modifiable risk factors for ARDS may be invaluable in early identification and prevention of lung injury.

1.1.5 Treatment

1.1.5.1 Pharmacological Interventions

Despite almost 25 years of research many interventions to treat ARDS have failed in clinical trials despite promise in early phase studies. Given their established anti-inflammatory properties corticosteroids have unsurprisingly been the most studied drugs for the prevention and treatment for ARDS. Overall current evidence to support moderate-high dose steroids for treating early or late ARDS is limited⁴⁵ with concern of increased neuro-myopathic events.⁴⁶ However, one study that used prolonged low dose corticosteroids in early ARDS reported significant improvement in hypoxaemia and lung injury scores by day 7 of treatment.⁴⁷ However, there remains conflicting evidence

and uncertainty and adequately powered randomised trials are ongoing (NCT01731795 and NCT01284452).⁴⁸

Alveolar fluid clearance is a critical process in the resolution of ARDS. Experimental data suggest that β -adrenergic agonists could augment alveolar fluid clearance as well as improve alveolar/capillary barrier function and pulmonary mechanics.⁴⁹ Based on this evidence, the single-centre β -Agonist Lung Injury Trial (BALTI)⁵⁰ randomised 40 patients and showed a reduction in extravascular lung water (EVLW) in the IV salbutamol arm compared to placebo. This subsequently led to 2 large multi-centre randomised placebo-controlled trials. Both studies were terminated early. The ALTA Study (Albuterol Treatment for ALI)²³ failed to show a difference in ventilator free days between nebulised albuterol and placebo whilst the BALTI-2 trial found an excess in mortality in the group receiving IV salbutamol.²⁴ On the basis of these larger trials, β -agonists should be avoided in patients with ARDS due to their postulated harmful cardiac effects, inducing tachy-arrhythmias and lactic acidosis. Furthermore, more recently the BALTI-prevention trial has reported that although perioperative treatment with inhaled salmeterol was well tolerated it did not prevent ALI in patients undergoing oesophagectomy.⁵¹

Ketoconazole has the ability to block the synthesis of pro-inflammatory mediators such as thromboxane A₂ and leukotrienes as well as suppress alveolar macrophage procoagulant activity⁵², however again early positive

small studies did not translate to a larger study by the ARDSnet group which was stopped early due to lack of efficacy.⁵³

Neutrophils are believed to play a key role in the progression of ARDS⁵⁴ and excessive neutrophil elastase, a degranulation protease enzyme has the ability to degrade the lung extracellular matrix and has therefore been implicated in the pathogenesis of ARDS.^{48,55} However, a large multi-centre trial of the neutrophil elastase inhibitor, silvestat was stopped prematurely due to a negative trend in 180-day all-cause mortality,⁵⁶ which has subsequently been supported by a more recent meta-analysis.⁵⁷

Lisofylline, a synthetic methylxanthine decreases circulating free fatty acids, including linoleic acid and was thought to be a potential therapeutic strategy to mitigate against lipid-based oxidant injury.⁵⁸ However the LARMA⁵⁹ study was stopped early due to lack of efficacy and no difference in 28-day mortality and ventilator free days.

Statins (HMG CoA-reductase inhibitors) are well established in primary and secondary prevention of cardiovascular disease. *In-vivo* and *in-vitro* evidence suggests that they have pleiotropic anti-inflammatory actions including reducing cytokine levels, adhesion molecule expression, neutrophil proliferation and nitric oxide production.⁶⁰ A phase II clinical trial in patients with ARDS suggested potential benefit with reduced EVLW and improvements in secondary outcomes.⁶¹ Nevertheless, two large trials of

simvastatin in HARP-2⁶² and rosuvastatin in SAILS⁶³ did not show an improvement in clinical outcomes and in the latter may have contributed to hepatic and renal organ dysfunction.

Activated platelets play an important role in disease progression in ARDS. They sequester in the lung, releasing pro-inflammatory cytokines, surface adhesion factors and lipid mediators.⁶⁴ However, observational studies have been conflicting and the largest cohort found no protective effect of pre-hospital anti-platelet use and subsequent ARDS incidence.^{65,66} Studies are ongoing to investigate both the effect of aspirin on reducing inflammation in a human model of ARDS (ARENA, NCT01659307) and in the prevention of ARDS.⁴⁴

Neuromuscular blocking agents have recently been re-evaluated with favorable results. A recent trial has shown that cisatracurium given for 48 hours early in the course of severe ARDS ($\text{PaO}_2/\text{FiO}_2 < 150$) with low tidal volume (V_T) ventilation may improve outcomes without significantly increasing the incidence of muscle weakness.⁶⁷ Other potential therapies being evaluated include keratinocyte growth factor,⁶⁸ mesenchymal stem cells⁶⁹ and IFN β .⁷⁰

1.1.5.2 Ventilation

Since there are no current therapeutic treatments for ARDS, critical care support and mechanical ventilation is usually required. The ARDSnet ARMA study of 2000⁴ was instrumental in turning the tide on conventional high V_T ventilation for ARDS. This multi-centre, randomised, controlled trial compared high V_T therapy (12 mL/kg) to low V_T therapy (6 mL/kg) in mechanically ventilated patients with ALI/ARDS for 28 days. The trial was terminated early when the data demonstrated that lower V_T ventilator settings in ALI/ARDS patients led to a significant decrease in mortality by a relative 22%. Low V_T ventilation also led to an increase in ventilator-free days and a decrease in the number of days with systemic organ failure. Patients assigned to lower V_T ventilator settings initially required a higher PEEP to maintain arterial oxygenation and were more likely to develop respiratory acidosis, although the difference disappeared by day 7, suggesting that although lower tidal volumes require a higher respiratory rate to maintain adequate ventilation, its benefits on mortality outweigh transient acid/base and oxygenation imbalances.

These beneficial results seemed to hold across a wide spectrum of patients, including septic and non-septic patients, and also those with different degrees of lung dysfunction as assessed by respiratory system compliances. A lung protective ventilation strategy should therefore be initiated for all cases of ARDS. However, in critically ill patients without ARDS, or those undergoing surgery, there is less evidence regarding the benefits of lower V_T partly

because of a lack of randomised controlled trials. Observational studies suggest that the use of higher V_T in patients without ARDS at the initiation of mechanical ventilation increases morbidity and mortality.⁷¹⁻⁷³ A recent meta-analysis has suggested that among patients without lung injury, protective ventilation with the use of lower tidal volumes at onset of mechanical ventilation may be associated with decreased risk for developing ARDS.⁷⁴ Furthermore, this was associated with lower mortality, fewer pulmonary infections and less atelectasis. Recently, Lellouche *et al* also found that a V_T of more than 10mL/kg was risk a factor for organ failure and prolonged intensive care unit stay after cardiac surgery.⁷⁵ Interestingly, another group has shown higher V_T for even a few hours intra-operatively to be associated with postoperative respiratory failure.⁷⁶

The IMPROVE Study,⁷⁷ a double blind parallel group trial randomised 400 adults at intermediate or high risk of pulmonary complications undergoing elective abdominal surgery at 7 French academic centres to receive either: low V_T mechanical ventilation — 6-8 mL/kg ideal body weight; PEEP 6-8 cm H₂O; recruitment manoeuvres every 30 minutes or non-protective mechanical ventilation — V_T 10-12 mL/kg; PEEP and recruitment manoeuvres were not protocolled but could be provided at the anaesthetists discretion for clinical need. The primary outcome, a composite of major pulmonary (pneumonia, invasive or non-invasive ventilation for respiratory failure) and extra pulmonary complications (sepsis, severe sepsis, shock or death) in the first 7 days after surgery, occurred in 10.5% of patients

receiving lung-protective ventilation and in 27.5% of patients receiving non-protective ventilation. Non-invasive ventilation or intubation for acute respiratory failure occurred within 7 postoperative days in 5 vs 17% who received lung-protective ventilation.

1.1.5.3 Extracorporeal membrane oxygenation

Extracorporeal membrane oxygenation (ECMO) has been studied in a UK trial (CESAR) of 180 patients with refractory hypoxaemia. Patients were randomised to transfer to a tertiary care centre where 77% received ECMO versus control patients remaining at the referring centre who were treated with non-protocolised ventilator strategies. Compared with the control group, transferring adult patients with severe but potentially reversible respiratory failure to a single centre specialising in the treatment of severe respiratory failure for consideration of ECMO significantly increased survival without severe disability.⁷⁸ Considerable debate is ongoing as to whether this trial proves the benefit of ECMO per se or the benefits of managing severe respiratory failure in specialist centres of excellence.

1.1.5.4 Open lung strategies

The open lung strategy of ventilation involves the use of recruitment manoeuvres followed by increasing PEEP in combination with lung protective ventilation to prevent atelectrauma secondary to recurrent opening, overdistension and shearing of the alveoli secondary to atelectasis. Two trials have shown a benefit on ventilator free days, improved

oxygenation and the need for rescue interventions such as ECMO however no mortality benefit. However, a meta-analysis from 2010 of 2299 patients showed that higher levels of PEEP were associated with improved survival among ARDS but not ALI patients (i.e. if PF ratios <200mmHg) with a mortality benefit of 34% vs. 39% (adjusted relative risk 0.90; 95% CI, 0.81-1.00; p= 0.049).⁷⁹ An open lung approach is therefore safe and may be of benefit in moderate to severe ARDS.

High Frequency Oscillation Ventilation (HFOV) showed some initial promise with a meta-analysis in 2010 suggesting that it improves oxygenation, risk of treatment failure and 30-day mortality compared with conventional ventilation.⁸⁰ Subsequently, two large clinical trials^{81,82} have shown no benefit of HFOV and it may increase hospital mortality.⁸¹ In contrast, early prolonged prone position ventilation has recently been shown to improve oxygenation and outcomes in severe hypoxaemic ARDS ($\text{PaO}_2/\text{FiO}_2 < 150$).⁸³

1.1.5.5 Fluids

Another advance in supportive therapy reported by the National Heart Lung and Blood Institute ARDS Network are the results of a prospective, randomised clinical trial evaluating the use of a liberal versus conservative fluid strategy in patients with ALI. The latter resulted in a significant increase in ventilator-free days.²⁹ The conservative fluid management strategy used diuretics to target a central venous pressure less than 4 mmHg or a pulmonary artery occlusion pressure below 8 mmHg.⁸⁴

1.2 PATHOPHYSIOLOGY OF ARDS

Despite the varied precipitating events for ARDS the histological changes are identical and represent a final common pathway of response. ARDS is characterised in the acute phase by an overwhelming inflammatory process leading to diffuse alveolar epithelial and endothelial injury with alveolar flooding with protein rich exudates due to increased vascular permeability. It is this breakdown in the alveolar epithelial and endothelial barrier, inappropriate influx of inflammatory cells and platelets, uncontrolled inflammation and activation of the coagulation pathways that are the key pathophysiological elements of ARDS^{1,85}, illustrated in Figure 1.1.

Alveolar epithelial cell apoptosis and necrosis of Type I cells contribute to the pulmonary oedema⁸⁶ and exposes the basement membrane to further insult. Injury to Type II alveolar cells leads to impaired surfactant synthesis resulting in alveolar collapse and decreased lung compliance. An increase in extra-vascular lung water (EVLW) is also seen during this phase, as pulmonary capillary permeability increases.^{85,87,88} Endothelial and epithelial injury is accompanied by an influx of neutrophils into the interstitium and alveolar space. This neutrophil recruitment causes both alveolar and systemic release of chemokines (e.g. CXCL-8, ENA-78), pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF), acute phase reactants (e.g. CRP, lipocalin), proteases, reactive oxygen species (ROS) and matrix remodeling enzymes (e.g. MMP-9) leading to further tissue damage.^{1,6,54,85}

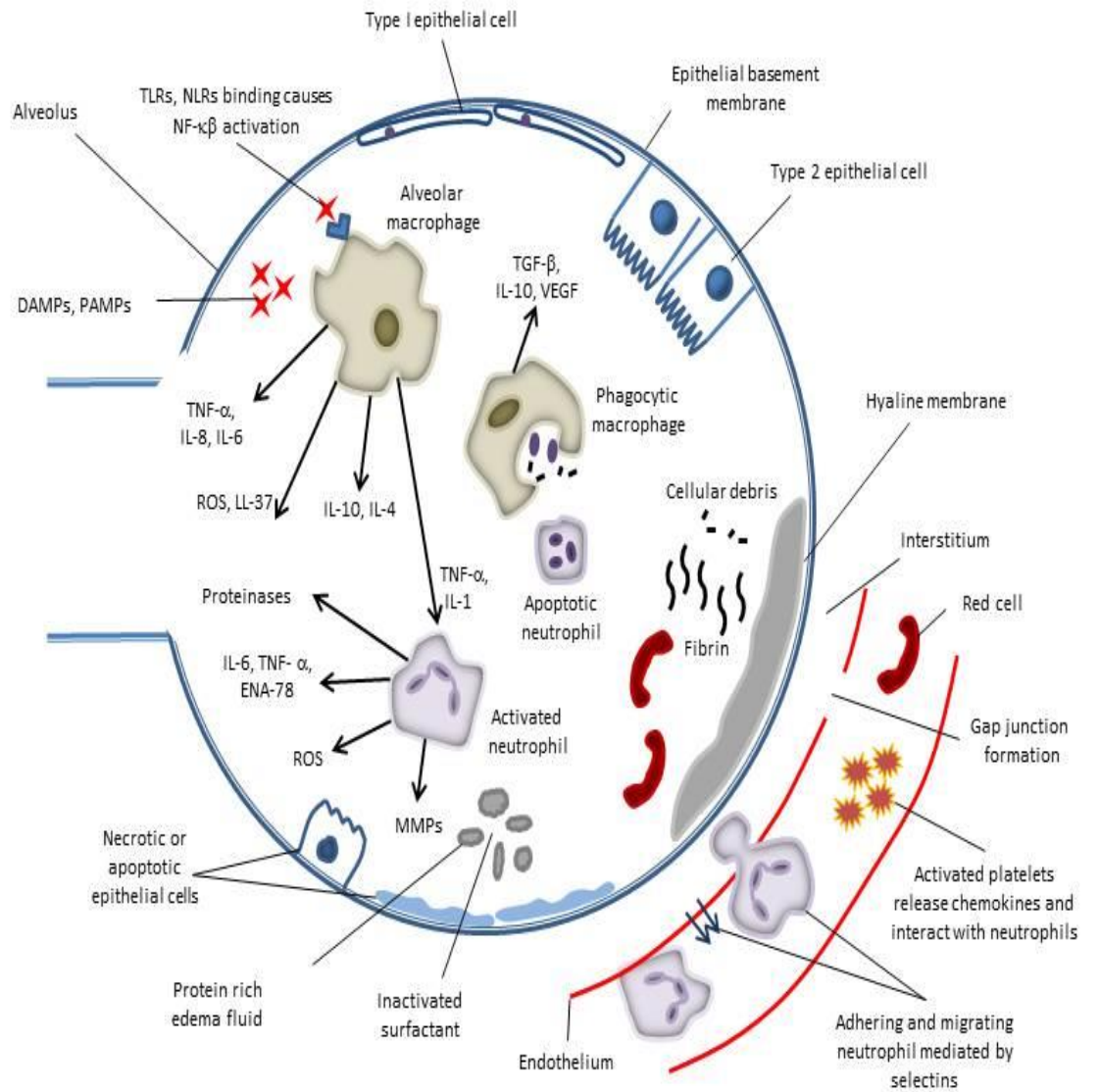


Figure 1.1: The Pathophysiology of ARDS.

There may then be further inflammatory cascade due to persistent primary stimulus such as pneumonia or due to systemic inflammation being amplified by the lung injury or potential secondary insults such as ventilation and blood transfusion. The subsequent course of ARDS is variable.^{6,85} In some patients there is reabsorption of alveolar oedema fluid and repair of the injured region of the alveolar epithelium, followed by clinical recovery from respiratory failure. However, in other patients alveolar oedema persists followed by organisation of hyaline membranes, collagen deposition and gradual appearance of intra-alveolar fibrosis and scarring.^{1,85,86}

1.2.1 The macrophage and lung injury

Alveolar macrophages are derived from peripheral blood monocytes and the bone marrow and on migration to the lung attain site-specific functions. They are normally recruited to the lung forming the first line of defense towards pathogen and particulate exposure acting to induce and perpetuate innate and adaptive immune responses. Evidence suggests that the diverse activity of macrophages is mediated by two distinct subsets playing critical roles in both initiation and recovery of inflammation.⁸⁹ Classically activated M1 macrophages are activated by cytokines (e.g. $\text{IFN}\gamma$, $\text{TNF}\alpha$) or by pathogen-associated molecular patterns or PAMPs, e.g. lipopolysaccharide (LPS) or danger-associated molecular patterns or DAMPs (e.g. heat shock proteins or high mobility box group protein 1, HMGB1) via toll-like receptors (TLRs), NOD-like receptors (NLRs) and other pattern recognition receptors leading to release of early response cytokines ($\text{TNF}\alpha$, IL-6, IL-1) in an NF-

κ B dependent way, as well as ROS and antimicrobial peptides.⁸⁹ This pro-inflammatory activity is counterbalanced by the alternatively activated M2 macrophage that is involved in down regulating inflammation and promoting wound repair by the release of anti-inflammatory cytokines (e.g. IL-10, IL-4). M2 macrophages are also involved in the phagocytosis of apoptotic neutrophils, supporting an anti-inflammatory, resolving and tissue remodelling phenotype by the release of TGF β , IL-10 and vascular endothelial growth factor (VEGF).^{90,91} Furthermore the mediators released by M2 macrophages have been shown to abrogate alveolar neutrophil recruitment and mediate the effects of regulatory T-cells (T_{reg}).⁹² Macrophages within the inflamed lung are in a state of dynamic flux and different phenotypes are activated by specific signals during inflammation, resolution and repair. This microenvironment therefore determines the macrophage response to lung injury.

The alveolar macrophage is therefore well placed for the detection of injurious pulmonary stimuli and initiation of the inflammatory cascade in ARDS. As they activate the host defense response they have an important role in the initiation of acute lung injury, and in early ARDS NF- κ B activation promotes the transcription of pro-inflammatory genes within alveolar macrophages.^{93,94} However, a key function of alveolar macrophages in ARDS is in the resolution of lung injury (Figure 1.2). The resolution of ARDS is dependent on the action of macrophages to ingest apoptotic neutrophils, a process termed efferocytosis, and prevent the release of their pro-

inflammatory intra-cellular contents during secondary necrosis.⁹⁵ In contrast to the uptake of other extracellular material providing a pro-inflammatory stimulus, the uptake of apoptotic cells is part of an anti-inflammatory process⁹⁶ and there is evidence that a defect in the process of efferocytosis is a cause of on-going inflammation in ARDS.⁹⁷⁻⁹⁹

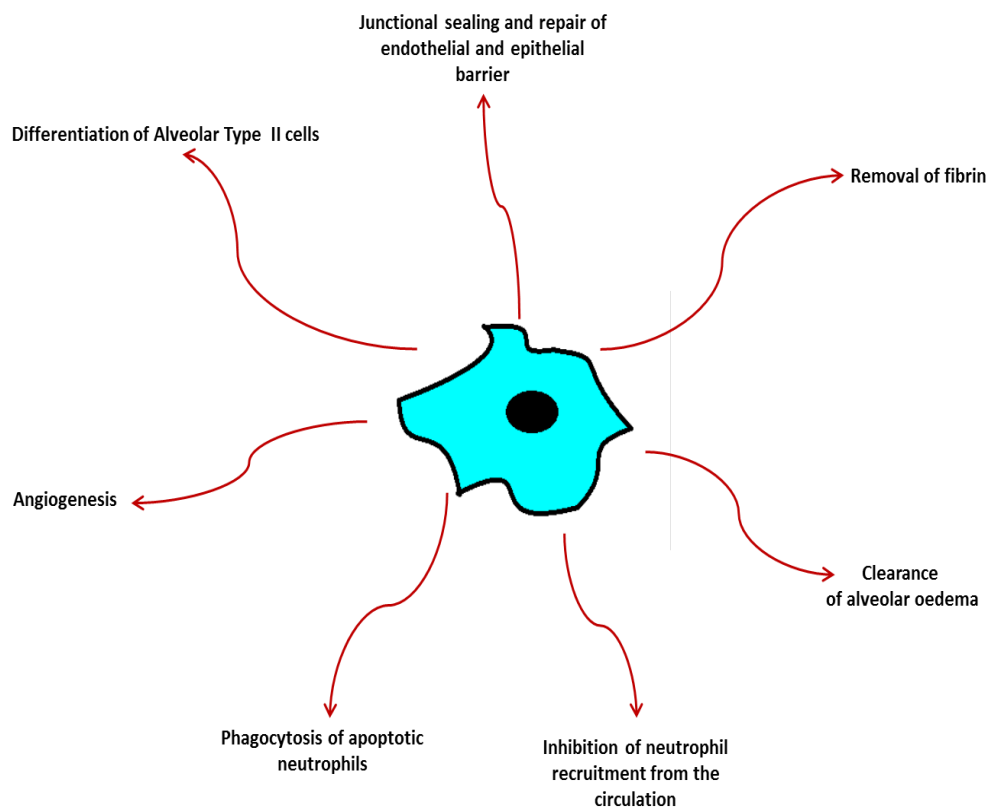


Figure 1.2: Macrophages resolve alveolar inflammation after Acute Respiratory Distress Syndrome.
(adapted from Herold *et al*)⁹⁰

1.3 MODELS OF ACUTE LUNG INJURY

1.3.1 Murine Models of ARDS

Rodent models have been invaluable in the study and understanding of acute lung injury. These have involved direct injury models in the form of intra-tracheal LPS to model pneumonia, intra-tracheal acid to model aspiration pneumonitis and high volume ventilation to reproduce ventilation induced lung injury and hyperoxic lung injury. Indirect lung injury models include caecal ligation and puncture, intravenous bacteria or LPS to simulate sepsis. Although each model can reproduce components of the pathological changes seen in ARDS none have been shown to create all the changes of neutrophil alveolitis, capillary endothelial and alveolar epithelial damage and hyaline membrane formation.¹⁰⁰ Despite these models having limitations they remain of considerable value in the study of ARDS as they allow genetic manipulation, investigation and testing of new therapies in a complex biological system. Adopting these models carefully to ensure they are pertinent to answering specific hypotheses ensures their scientific validity.¹⁰¹

1.3.2 Human Model of ARDS – One Lung Ventilation

To allow access to the oesophagus during surgery (using the transthoracic technique), one of the lungs is deflated and the subject is ventilated through the other lung. This is known as one-lung ventilation (OLV). There is a high postoperative incidence of ALI/ARDS¹⁰²⁻¹⁰⁶ following OLV and unlike most insults leading to lung injury the delivery of OLV is predictably timed, thereby allowing serial studies to be carried out throughout the period of stimulus

and development of the condition. Preoperative risk factors including age, respiratory function and cigarette smoking have been found to be related to the incidence of postoperative pulmonary complications.^{102,105,107-109} It is unclear at present why only a percentage of patients undergoing OLV develop lung injury or why the lung injury typically occurs 24-48 hours after the cessation of OLV.⁵¹ Our local data show that the development of lung injury is however associated with a doubling of in-hospital stay and elevated mortality.

Our group has extensively characterised the local and systemic inflammatory response to transthoracic oesophagectomy in patients undergoing OLV.⁵¹ After OLV, patients have a neutrophilic alveolitis, with a significant alveolar and systemic inflammatory response.^{110,111} This is associated with the release of markers of both endothelial and alveolar epithelial dysfunction, and an increase in the permeability of the alveolar barrier.^{112,113} This manifests clinically as increased extravascular lung water, and a fall in oxygenation.^{51,114}

Alveolar levels of surfactant protein D and bronchoalveolar lavage fluid (BALF) protein permeability index are highest in those who develop ARDS within 72 hours of OLV suggesting that perioperative alveolar epithelial damage is a risk factor for the subsequent development of ARDS. Immediate post-operative plasma markers of neutrophilic activation [myeloperoxidase, and matrix metalloproteinase-9 (MMP-9)] as well as the Receptor for

Advanced Glycation End-products (RAGE, a type I epithelial cell marker) are similarly raised in those who develop ARDS within 72 hours of OLV.⁵¹ Proposed causative mechanisms for this injury include the ischaemia/reperfusion insult suffered by the collapsed lung, as well as oxidative stress and barotrauma causing epithelial injury to the ventilated lung.^{115,116} These mechanisms are important in the pathogenesis of ARDS making OLV a valid model for studying the pathogenesis of ARDS in humans and exploring therapeutic strategies for preventing lung injury in a predefined subject population.^{1,117}

1.4 RATIONALE FOR VITAMIN D IN ARDS

1.4.1 Vitamin D biology

Vitamin D₃, or cholecalciferol, is mainly formed in the skin after exposure to sunlight (UVB). Synthesised or dietary vitamin D is hydroxylated in the liver by CYP27A1 to 25-hydroxyvitamin D₃ (25(OH)D₃) the major circulating form of vitamin D and widely accepted measure of vitamin D status.¹¹⁸ In the classical model (Figure 1.3) of vitamin D metabolism, 25(OH)D₃ undergoes further hydroxylation in the kidney to the biologically active 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] by the mitochondrial enzyme 25(OH)D-1-alpha (CYP27B1).¹¹⁸ When 25(OH)D₃ is sufficiently available, 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) is formed in the kidney which is further catabolised.⁸⁵ Vitamin D metabolites are bound in the circulation to vitamin D binding protein (DBP) which has a high affinity for 25(OH)D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ and therefore regulates free circulating concentrations of

vitamin D metabolites.¹¹⁹ Vitamin D is inactivated by a ubiquitous enzyme, 24-hydroxylase (CYP24A). The active metabolite, 1,25(OH)₂D₃ acts on the kidney, gut and bone to maintain calcium homeostasis. The effects of vitamin D are mediated through the vitamin D receptor (VDR) which is a ligand dependant transcription factor. VDR binds to its ligand 1,25(OH)₂D₃, dimerises with the retinoid X receptor (RXR) and attaches to specific genomic sequences termed vitamin D response elements (VDRE).⁸⁵ The transcription of VDR target genes also results in cell growth inhibition, induces apoptosis, controls proliferation and differentiation.^{85,118,120,121}

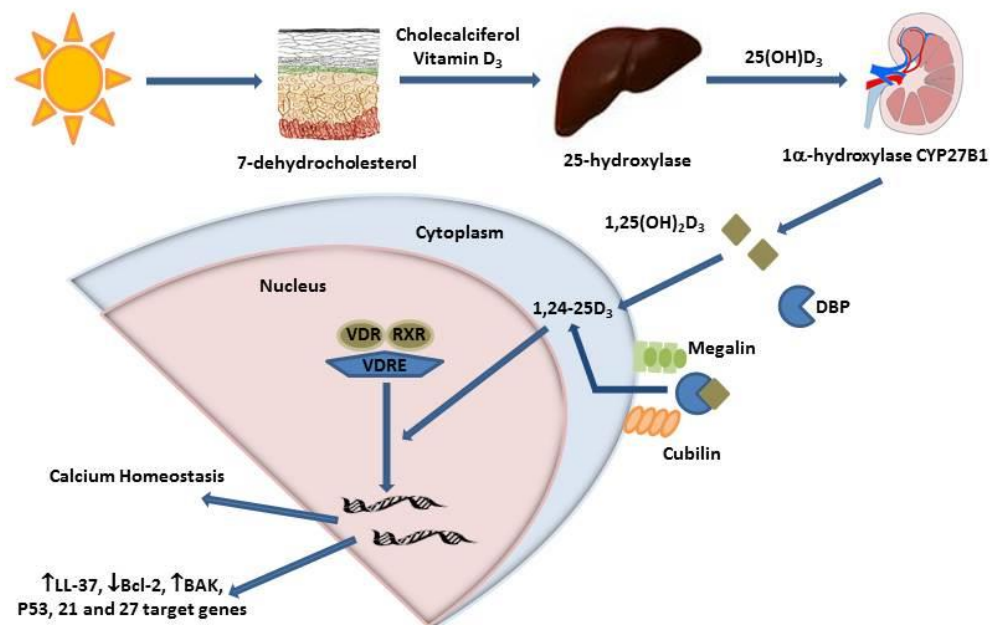


Figure 1.3: Schematic diagram of vitamin D biology.⁸⁵
(Adapted from Chishimba *et al*)¹²²

In recent years interest has shifted from the calcium homeostasis role to the non-classical actions of vitamin D since the biologically active metabolite 1,25(OH)₂D₃ can also be generated locally within tissues due to induction of extra-renal CYP27B1.⁸⁵ An increasing number of tissues have been found to express CYP27B1, including lung epithelial cells¹²³ and cells of the immune system including macrophages¹²⁴, lymphocytes¹²⁵ and dendritic cells.¹²⁶ DBP is a serum protein which too has immunomodulatory functions relevant in the lung, predominantly relating to macrophage activation and neutrophil chemotaxis.¹²² It has been suggested that local production of 1,25(OH)₂D₃ along with the presence of the VDR is evidence of a local paracrine/autocrine action in various tissues.^{120,127}

1.4.2 Vitamin D deficiency

Worldwide prevalence of vitamin D deficiency is on the increase.^{118,128} In a study of middle-aged adults in the United Kingdom, 40% had serum 25(OH)D concentrations above 30ng/L in the summer months but this fell to less than 17% in the winter.¹²⁹ According to several other studies, 40-100% of U.S and European elderly adults are deficient in vitamin D.^{118,129} Epidemiological studies have suggested a role for low vitamin D status in the risk of developing both viral and bacterial infection.^{130,131} A recent study has further demonstrated the anti-inflammatory effects of vitamin D in patients with pulmonary tuberculosis.¹³² In addition, asthma, chronic obstructive airways disease (COPD), interstitial lung disease and cystic fibrosis show correlations between severity and level of vitamin D deficiency.¹³³ Published data suggest

that vitamin D deficiency is also common in critically ill patients¹³⁴ and recent prospective studies suggest an association with increased morbidity and mortality.¹³⁵⁻¹³⁷

1.4.3 Potential effects on ARDS pathophysiology

Vitamin D has been linked to many pathophysiological processes within the lung. These potential pathological concepts linking vitamin D to ARDS are discussed in detail in this section and summarised in Table 1.4.

1.4.3.1 Barrier integrity

Barrier integrity of the alveolar epithelium is essential not only to prevent pulmonary oedema but also to facilitate removal of fluid from the air space. The epithelial cell-cell functional complex is formed by gap, tight and adherens junctions. 1,25(OH)₂D₃ has been shown to upregulate transcription of proteins required for the formation of connexin 43, claudin-1, -2 and E-cadherin in epithelial cells of the skin and intestine.¹³⁸⁻¹⁴¹ Although this has yet to be confirmed in the lung it suggests that vitamin D mediated action may play a role in stabilising the epithelial junction complex. 1,25(OH)₂D₃ at physiological sufficient levels can reduce the activation of lung microvascular endothelial cells, reducing adhesion molecule expression (ICAM-1 and ELAM-1), reducing iNOS expression and NO release, and attenuating platelet activating factor (PAF) induced neutrophil adhesion to pulmonary microvascular endothelial cells suggesting that sufficient vitamin D levels may attenuate/prevent an important initiating event of acute lung injury ¹⁴²

1.4.3.2 Innate immunity

Vitamin D is a potent stimulator of antimicrobial peptides in innate immunity¹⁴³ and the production of LL-37 (cathelicidin) and some defensins (human β -defensin-2) is dependent on sufficient circulating 25(OH)D₃.¹⁴⁴ 1,25(OH)₂D₃ has the ability to induce the release of LL-37 within the lung and LL-37 is stored at high concentrations in specific granules of neutrophils, and can also be produced by macrophages and epithelial cells. It can be detected in airway secretions and is upregulated in response to infection and inflammation.¹⁴⁵ In addition to direct antimicrobial capability, *in vitro* and *in vivo* studies suggest a broad range of activities that could modify innate inflammatory processes and adaptive immune responses. LL-37 can bind to and neutralize lipopolysaccharide (LPS), and functions as a chemoattractant for neutrophils, monocytes and T cells through a formyl peptide receptor.¹⁴⁵ Furthermore, respiratory epithelial cells can convert 25(OH)D₃ to 1,25(OH)₂D₃ and activate VDR responsive genes increasing the production of TLR co-receptor CD14 and *hCAP18* from which LL-37 is cleaved within 24 hours.¹⁴⁶ In the context of ARDS, LL-37 is elevated significantly in the BAL fluid of these patients in comparison with normal controls.¹⁴⁷ In terms of the pathophysiology of ARDS this could be important as LL-37 may drive epithelial repair responses as well as being an anti-microbial peptide.¹⁴⁸ Elevating local LL-37 may also be important as a downstream immunomodulator of vitamin D since it has recently been shown to reduce TLR agonist-mediated neutrophil-derived increases in IL-1 β , IL-6, IL-8 and TNF- α in addition to stimulating bacterial

phagocytosis.¹⁴⁹ Further studies are needed to determine if LL-37 is causative of inflammation or a response to injury in ARDS.

Vitamin D has been shown to stimulate the differentiation of precursor monocytes to mature phagocytic macrophages.¹⁵⁰ Localised synthesis of 1,25(OH)₂D₃ by normal human macrophages on stimulation with interferon gamma (IFN γ) is suggestive of an intracrine system for the action of vitamin D.^{143,151} Hydrogen peroxide secretion in human monocytes is also activated by 1,25(OH)₂D₃ resulting in increased oxidative burst potential.¹⁵² NF- κ B signalling and suppression of macrophage TLR expression by 1,25(OH)₂D₃ suggests that it may also be a regulator of macrophage inflammatory responses.^{153,154} Furthermore, 1,25(OH)₂D₃ has also been shown to reduce the production of inflammatory cytokines and chemokines (IL-8 and CXCL-10) from stimulated epithelial cells by modulating the NF- κ B signalling.^{155,156} Any or all of these actions are likely to be relevant in the lung and ARDS. Neutrophils also express the VDR¹⁵⁷ however, little is known about vitamin D regulation of neutrophil function.

Process/Cell Type	Role of vitamin D	Potential effect in ARDS	Relevant references
Antimicrobial peptide production	Increased LL-37 release by macrophages and epithelial cells	Promote epithelial repair responses and reduce downstream pro-inflammatory signals	<i>Shaykhiev et al¹⁴⁸</i> <i>Alalwani et al¹⁴⁹</i>
Monocyte differentiation	Stimulates differentiation to mature phagocytic macrophages	Anti-inflammatory actions and prevention of secondary necrosis of apoptotic neutrophils and persistence of inflammation	<i>Kreutz et al¹⁵⁰</i>
NF-κB signaling	Reduces NF- κ B signaling and TLR expression	Dampen inflammatory response	<i>Sadeghi et al¹⁵⁴</i> <i>Hansdottir et al¹⁵⁶</i>
Regulatory T-cells	Stimulates differentiation towards regulatory T-cell and away from Th17 cells	Promote resolution of ALI	<i>D'Alessio et al⁹²</i>
MMP driven tissue remodeling	Reduces MMP	Prevent initial destruction of basement membrane but potentially impair resolution	<i>O'Kane et al¹⁵⁸</i> <i>Albaiceta et al¹⁵⁹</i> <i>Yamashita et al¹⁶⁰</i> <i>Song et al¹⁶¹</i>
Neutrophil adhesion to endothelium	Reduces adhesion molecule expression on the endothelium, PAF and NO release	Prevent neutrophil migration and initiation of lung injury	<i>Chen SF et al¹⁴²</i>
Vitamin D Binding Protein (DBP)	Actin scavenging and macrophage Activation	Prevent vascular occlusion, tissue damage and organ dysfunction	<i>Dahl et al¹⁶²</i>
Autophagy	Promotes autophagy	Not known	<i>Wu et al¹⁶³</i>
Barrier integrity	Upregulate gap, adherens and tight Junctions	Prevent alveolar fluid influx and facilitate removal	<i>Palmer et al¹⁴⁰</i> <i>Kong et al¹⁴¹</i>

Table 1.4: Pathological concepts linking vitamin D and ARDS

1.4.3.3 Adaptive immunity

Recent data have implicated vitamin D in adaptive immunity because of its influence upon the differentiation of T cells between the regulatory T cell (T_{reg}) and the pro-inflammatory T helper 17 (T_h17) subsets.¹⁶⁴⁻¹⁶⁶ T_h17 cells are known to stimulate tissue inflammation and neutrophil chemotaxis, both of which are seen in ARDS, predominantly by IL-17 production. It also appears that expression of markers of T_{reg} cells (Foxp3) or T_h17 cells (IL-17) by T cells may not be stable and that there is a greater degree of plasticity in their differentiation than previously appreciated.¹⁶⁵ Recent evidence has further suggested T_{reg} cells are important in resolution of experimental ARDS.⁹² This suggests that local lung regulation of the balance between T_{reg} and T_h17 cells may be a determinant of resolution/persistence of neutrophilic inflammation, which is known to be associated with a poor prognosis in human ARDS.

1.4.3.4 Autophagy

Vitamin D also promotes autophagy, a cellular process that ensures the synthesis, degradation and recycling of intracellular macromolecules and inclusions in mononuclear cells.^{163,167} It represents an inducible response to stress in lung cells. Agents that trigger autophagy that are particularly relevant to lung cell biology include hypoxia, particle and cigarette smoke exposure, proinflammatory states, and conditions that promote ER stress or oxidative stress.¹⁶⁸ Autophagy has been shown to be both protective and injurious in a variety of different models, suggesting that its role in human

diseases is complex. Relatively few studies have been done in the lung and the functional importance of autophagy in ARDS is yet to be explored.

1.4.3.5 Metalloproteinases

Matrix metalloproteinases (MMPs) are upregulated in activated cells and can facilitate tissue remodelling and repair.¹⁵⁸ They can also control cytokine and chemokine processing, apoptosis and antimicrobial peptide cleavage and activation.¹⁶⁹ They are however a complex family of enzymes with MMP-2, MMP-7 and MMP-9 shown to be involved in alveolar epithelial repair in experimental models of lung injury^{158,170} and increased levels of lung injury in MMP-9 deficient mice exposed to ventilator induced lung injury.¹⁵⁹ In contrast MMP-3 deficient mice were protected against bleomycin induced lung injury¹⁶⁰ and MMP-8 has shown opposing effects depending on the model of lung injury employed. 1,25-(OH)₂D₃ has been reported to down-regulate the expression of protein for MMP-9 in airway smooth muscle cells from asthma patients¹⁶¹ and inhibit the expression and activity of a number of MMP during *in vitro* mycobacterium tuberculosis (MTB) infection.¹⁷¹ This limited data support a role for vitamin D in suppressing MMP. Although blockade of specific MMPs during the early stages of ARDS may be beneficial to prevent initial destruction of the basement membrane this may be detrimental to the resolution process.¹⁷² Thus, further investigation of the possible effect and mechanisms of vitamin D on MMP function is needed in appropriate ARDS models.

1.4.3.6 Vitamin D binding protein

Apart from its specific role in being the carrier protein for 25(OH)D₃, DBP exerts several other important biological functions, from actin scavenging to fatty acid transport and macrophage activation¹⁷³. Tissue injury and cell death release actin into the circulation. In the extracellular compartment, G-actin polymerizes into F-actin filaments. This may cause vascular obstruction and organ dysfunction. Severe cell or tissue loss lowers the DBP-serum level. The degree of reduction correlates with the development of organ dysfunction, respiratory failure, hematologic failure and sepsis¹⁶² and low concentrations of DBP have been reported in ARDS^{173,174}, further suggesting a role for the vitamin D axis in ARDS.⁶

1.4.4 Animal studies

The few animal studies to date investigating the role of vitamin D deficiency and its supplementation in ARDS have conflicting results. 1,25(OH)₂D₃ inhibited neutrophil but not monocyte recruitment by 40% in a hamster model of ALI, thought to be in part due to its inhibitory effects on IL-8.¹⁷⁵ It also ameliorated lung damage secondary to ischaemia reperfusion injury after femoral artery ligation in rats.¹⁷⁶ A further study has shown reduced interstitial inflammation and collagen deposition post radiotherapy in rats administered vitamin D.¹⁷⁷ More recently, 1,25(OH)₂D₃ has been shown to attenuate hyperoxia-induced lung injury in neonatal rats with 1,25(OH)₂D₃ significantly down-regulating the expression of TLR4, NF-κβ, and the inflammatory cytokines TNF-α, IL-1β, and IL-6.¹⁷⁸ Aspiration with seawater

has been shown to upregulate VDR expression in rat lungs and A459 cells. Pre-treatment with 1,25(OH)₂D₃ significantly inhibited the activation of NF- κ B and RhoA/Rho kinase pathways, improved lung histopathologic changes, reduced inflammation, lung oedema and vascular leakage.¹⁷⁹ Conversely, a study of LPS induced lung injury in vitamin D deficient mice found no difference in the degree of lung injury.¹⁸⁰ Issues centred on differing aetiologies of lung injury as well as methodology of when lung injury is measured or defined may go some way to explaining this apparent contradiction.

1.4.5 Clinical evidence

Primary studies and meta-analyses have identified significant associations between lower serum 25(OH)D₃ levels and impaired lung function¹⁸¹, decreased survival from lung cancer^{182,183}, increased rates of severe asthma exacerbations¹⁸⁴, tuberculosis (TB)¹⁸⁵, acute respiratory tract infections¹⁸⁶ and chronic obstructive airways disease¹⁸⁷. A recent study has shown that high vitamin D levels are associated with better lung function, less airway hyper responsiveness and improved glucocorticoid response in asthma¹⁸⁸ and others have shown an interesting potential application of vitamin D to overcome the poor glucocorticoid responsiveness in severe asthmatics by upregulation of IL-10 production from regulatory T-cells.¹⁸⁹ Clinical trials of steroids in ARDS have to date revealed conflicting results despite their potential overwhelming anti-inflammatory effect and could represent an element of glucocorticoid resistance as seen in other inflammatory

conditions.¹⁹⁰ Therefore low levels of vitamin D could be a contributory factor in steroid resistance seen in some patients with ARDS.

Exposure to sunlight has been known to help with the treatment of TB for more than 100 years, although it is only recently been found that macrophage release of cathelicidin, required for efficient macrophage killing of *mycobacterium tuberculosis*, requires co-activation of the VDR and TLR¹⁹¹. The TLR activation of macrophages and the epithelium by PAMPs to stimulate local vitamin D expression could theoretically be performed by DAMPs and non-pathogen injurious stimuli in a similar fashion, both having potential relevance in the multiple aetiologies involved in ARDS.

There are few human studies of vitamin D replacement in critically care patients. Cecchi *et al* reported vitamin D levels to be lower in sepsis patients than in trauma and age related controls but this was not related to outcome.¹⁹² In a recent case-control study reported by Barnett *et al* serum 25(OH)D₃ measured early after admission to intensive care was not associated with the development of acute lung injury, hospital or one-year mortality in critically ill patients with sepsis although lower levels were associated with higher one-year mortality in patients with severe trauma. In contrast in a large cohort of critically ill patients, it has been demonstrated that deficiency 25(OH)D₃ of at the time of critical care initiation is a significant predictor of all-cause patient mortality.¹³⁶ Findings that are also true in surgical patients admitted to critical care.¹⁹³

Sepsis has direct relevance to ARDS and the pathophysiological changes leading to multi-organ failure may be similar and therefore highly relevant. A study looking at vitamin D levels in patients with severe sepsis showed that these patients have a lower serum vitamin D level than intensive care control patients which was associated with lower plasma levels of LL-37, suggesting that this deficiency is of functional importance *in vivo*.¹⁹⁴ Conversely in a small randomized placebo controlled trial of calcitriol (1,25(OH)₂D₃) replacement in patients with severe sepsis or shock reported by Leaf *et al* did not show alterations in plasma LL-37 concentrations but did increase whole blood leucocyte LL-37 and anti-inflammatory IL-10 mRNA expression at 24 hours.¹⁹⁵ No differences in other cytokine or clinical outcomes were noted, however this was a study with small numbers and there is a debate as to whether the intracrine conversion of 25(OH)D₃ to 1,25(OH)₂D₃ is an active process required for the cellular responses to vitamin D.¹⁹⁶

A large retrospective study of over 1900 patients to critical care has recently reported that compared to patients with 25(OH)D₃ ≥30ng/mL, patients with lower 25(OH)D₃ levels had significantly higher cumulative adjusted odds of acute respiratory failure (≤10 ng/mL, OR=1.84 (95% CI 1.22 to 2.77); 11–19.9 ng/mL, OR=1.60 (95% CI 1.19 to 2.15); 20–29.9 ng/mL, OR=1.37 (95% CI 1.01 to 1.86)).¹⁹⁷ This excluded patients with congestive cardiac failure and utilised the Berlin definition of ARDS. Our group has recently shown that patients undergoing oesophagectomy are also deficient in vitamin D and the severity of deficiency (<20nmolL) is a risk factor for the development of post-

operative lung injury (37.5% vs 15%).¹⁹⁸ Amrein *et al* have recently reported a large randomised placebo controlled clinical trial of single high-dose enteral 540,000IU of cholecalciferol in ICU patients that did not reduce hospital length of stay, hospital mortality, or 6-month mortality. However, higher hospital mortality was observed in the pre-defined severe vitamin D deficiency subgroup (<12ng/mL).¹⁹⁹ Suggesting that it is patients with severe deficiency that are likely to benefit from the hypothesised effects of vitamin D. It also confirmed safety and demonstrated successful increase in levels of 25(OH)D₃ and 1,25(OH)₂D₃ over 7 days. There are currently no registered clinical trials of vitamin D replacement in patients with established ARDS, although a few trials of high dose cholecalciferol administration in critical care are in progress (NCT01372995 and NCT01896544).

1.5 SUMMARY

Despite many years of research, pharmacological therapies for ARDS remain elusive with numerous negative clinical trials. Apart from lung protective ventilation and judicious fluid therapy, management remains supportive. Although these strategies have improved the outcomes from ARDS, it still remains a significant burden and important cause of respiratory failure, morbidity and mortality in the critically ill. Improving strategies to identify patients at risk of ARDS has been highlighted as an important area of research to try and prevent the initiating insult and control the inflammatory cascade that ensues. The macrophage plays a pivotal role in initiating this cascade and promoting resolution.

The evidence presented supports a profound role of vitamin D in modulating immune responses, especially on macrophage differentiation and function. Vitamin D deficiency is widespread in critically ill patients and has been associated with increased risk of sepsis, respiratory failure and ARDS post oesophagectomy. However whether this is cause or effect is yet to be ascertained. Mechanistically vitamin D deficiency could lead to an impaired innate immune response to injury to the lung proceeded by an excessive adaptive immune response to the injury with increased production of inflammatory cytokines, exaggerated inflammation as well as impaired resolution and repair of damage. However, many questions remain unanswered and more *in-vitro*, *in-vivo* and human studies are required to ascertain the potential mechanisms of vitamin D deficiency in driving ARDS.

CHAPTER 2

AIMS

2.1 HYPOTHESIS

It was hypothesised that vitamin D deficiency is a mechanistic driver of ARDS and that replacement therapy could prevent its occurrence.

2.2 THESIS AIMS

This thesis is a report of a series of investigations studying the effect of vitamin D deficiency and its replacement on ARDS. The specific aims of this thesis are to:

- 1] To establish in a murine model of sepsis induced lung injury if vitamin D deficiency exaggerates innate immune response to sepsis.
- 2] Determine if vitamin D replacement therapy can reduce biomarkers of local and systemic inflammation in a human model of ARDS – in a phase II proof of concept study: vitamin D to prevent acute lung injury following oesophagectomy trial.
- 3] Study the effect of *in-vitro* and *in-vivo* vitamin D on macrophage efferocytosis and differentiation.

CHAPTER 3

GENERAL METHODS

Parts of this chapter have been published:

Parekh D, Dancer RC, Lax S, et al.

*Vitamin D to prevent acute lung injury following oesophagectomy (VINDALOO):
study protocol for a randomised placebo controlled trial.*

Trials 2013;14:100.

3.1 CLINICAL STUDIES

3.1.1 Ethical approvals

National research ethics for the vitamin D to prevent acute lung injury post oesophagectomy trial (VINDALOO) reported in this thesis was approved by the South Birmingham Research and Ethics Committee (REC 12/WM/0092). The trial is registered on the International Standard Randomised Controlled Trial Registry (ISRCTN27673620) and European Union database of randomised Controlled Trials (2012-000332-25). All participants provided written informed consent. Full trial protocol¹¹⁶ has been published and can be viewed in the Appendix.

3.1.2 Patient Recruitment

Patients for the oesophagectomy study (VINDALOO) were recruited from 3 large tertiary centres (Queen Elizabeth Hospital, Birmingham, Birmingham Heartlands Hospital and University Hospital of Coventry and Warwickshire). All 3 centres are regional specialist centres for the management of upper gastro-intestinal malignancies. Patients were identified from the upper gastrointestinal cancer multidisciplinary team meetings and subsequently recruited from a preoperative assessment outpatient clinic after a face to face meeting and obtaining informed written consent.

3.1.3 Midlands Lung Tissue Collaborative (MLTC)

The MLTC is an ongoing regional collaborative between the University of Birmingham, Glenfield Hospital, Leicester and Birmingham Heartlands

Hospital that utilises tissue from lung resections to investigate the molecular and functional mechanisms of human lung disease. National Research Ethics approval was provided by the North West Research Ethics Committee (REC 07/MRE08/42; UKCRN ID 6664). Eligible patients were those undergoing thoracic surgery for lobectomy or pneumonectomy. The surgery was conducted at Birmingham Heartlands Hospital Thoracic Unit as part of routine treatment for lung cancer. Patients provided informed consent for any tissue removed as a part of their surgery not required for diagnostic or clinical purposes to be used for research. Baseline demographics including age, sex, tumour staging, smoking history and lung function were collected as part of the study.

3.2 SURGICAL AND ANAESTHETIC MANAGEMENT

Patients underwent a 2 stage transthoracic oesophagectomy which included a laparoscopic abdominal stage followed by an open thoracotomy or minimally invasive technique with thoracoscopy. All approaches required one-lung ventilation to facilitate surgical access to the oesophagus in the chest. This was done by collapsing the right lung and ventilating the left lung.

Induction of anaesthesia for surgery was performed intravenously and dependent on the anaesthetists preferred practice, however, predominantly involved the use of propofol and an opiate. Maintenance of anaesthesia was with gaseous isoflurane, sevoflurane or desflurane. Following endotracheal intubation a right internal jugular central venous catheter was placed by the

anaesthetist as part of routine care. A modified 5 French 20cm arterial PiCCO® thermodilution catheter (PULSION Medical Systems, Munich, Germany) was placed in the right femoral artery using a seldinger technique by the research fellow.

A thoracic epidural was placed when possible to facilitate postoperative analgesia. Intraoperative analgesia was provided with intravenous opiates. A conservative fluid management approach was adopted perioperatively. During the thoracic phase of surgery one-lung ventilation was achieved by the use of a double lumen endotracheal tube or bronchial blocker. The fraction of inspired oxygen delivered was titrated to maintain oxygen saturations above 95%. Patients were ventilated with either pressure or volume control ventilation with the application of positive end expiratory pressure (PEEP) as per anaesthetic preference. Postoperatively patients were either extubated in theatre recovery or after a short period of stabilisation on the intensive care unit according to local practice. Patients participating in the optional bronchoscopy study underwent bronchoalveolar lavage in theatre at the end of surgery. The research team did not interfere with the routine anaesthetic and surgical local practice at any point.

3.3 LUNG BIOMARKER PARAMETERS

Invasive cardiovascular and transpulmonary measurements were performed using the Pulse Contour Cardiac Output PiCCO₂® system (PULSION Medical Systems, Munich, Germany). The PiCCO₂® monitor was connected to the

femoral arterial catheter with the thermo-transducer at its tip sitting in the descending aorta. An injectable sensor was connected to the distal port of the right internal jugular central venous catheter that was inserted as routine care and to the PiCCO₂[®] monitor.

The PiCCO₂[®] monitor calculates cardiac output (CO), extravascular lung water (EVLW) and volumetric parameters by automated analysis of the thermodilution curve created by measuring the downstream temperature change by the arterial thermistor following injection of 15mls of ice-cold 0.9% saline (thermal indicator) into the injectable sensor on the central venous catheter. These readings were obtained by triplicate injections per study and the measures recorded from the monitor represented the mean of the 3 readings. Baseline studies were performed in theatre immediately prior to surgery, then immediately postoperative within 1 hour and at 9am on day 1 post-surgery.

The single transpulmonary thermodilution curve allows accurate measurement of the CO using the modified Stewart-Hamilton algorithm utilising the area under the curve (Figure 3.1).²⁰⁰ Further analysis of the curve using the Newman 'slope-volume' method²⁰¹ enables volume determination from point of injection (superior vena cava) to point of detection (descending aorta).

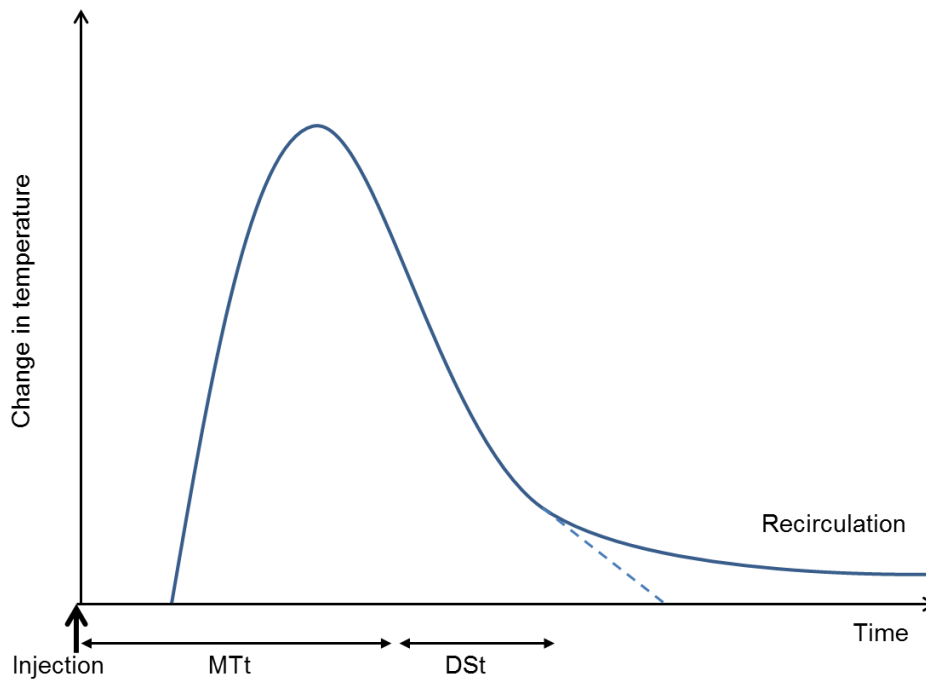


Figure 3.1: Diagram of a temperature-time curve of a thermodilution measurement.

MTt: mean transit time; DSt: downslope time of the curve

The volume of distribution of the thermal indicator represents the intrathoracic thermal volume (ITTV):

$$ITTV (ml) = CO \times \text{mean transit time of the thermal indicator (MTt)}$$

ITTV reflects both total intravascular and extravascular fluid volume within the chest (heart and lungs). The MTt reflects the transit time of the thermal indicator through the entire volume. However the largest volume of fluid and mixing chamber is the lungs (intra and extravascular) and reflected by the pulmonary thermal volume (PTV):

$$PTV (ml) = CO \times \text{exponential downslope time of thermodilution curve (DSt)}$$

Thus the difference between ITTV and PTV represents the volume of the non-pulmonary compartment consisting primarily of the heart and since this is greatest at end-diastole is called the global end-diastolic volume (GEDV)²⁰¹:

$$GEDV (ml) = ITTV - PTV$$

Intrathoracic blood volume (ITBV) is the volume of blood within the heart and the lungs and is a function of the GEDV and determined by the equation:

$$ITBV (ml) = 1.25 \times GEDV - 28.4ml$$

The relationship between GEDV and ITBV has been validated and shown to have a fixed linear correlation.^{202,203} EVLW is a measure of the fluid contained within the lung but outside the vasculature (interstitial, intracellular, alveolar and lymphatic)²⁰⁴ and is calculated as the difference between the thermal indicator distribution in the chest (ITTV) and the blood volume of the chest (ITBV):

$$EVLW (ml) = ITTV - ITBV$$

This thermodilution volumes are depicted in Figure 3.2.

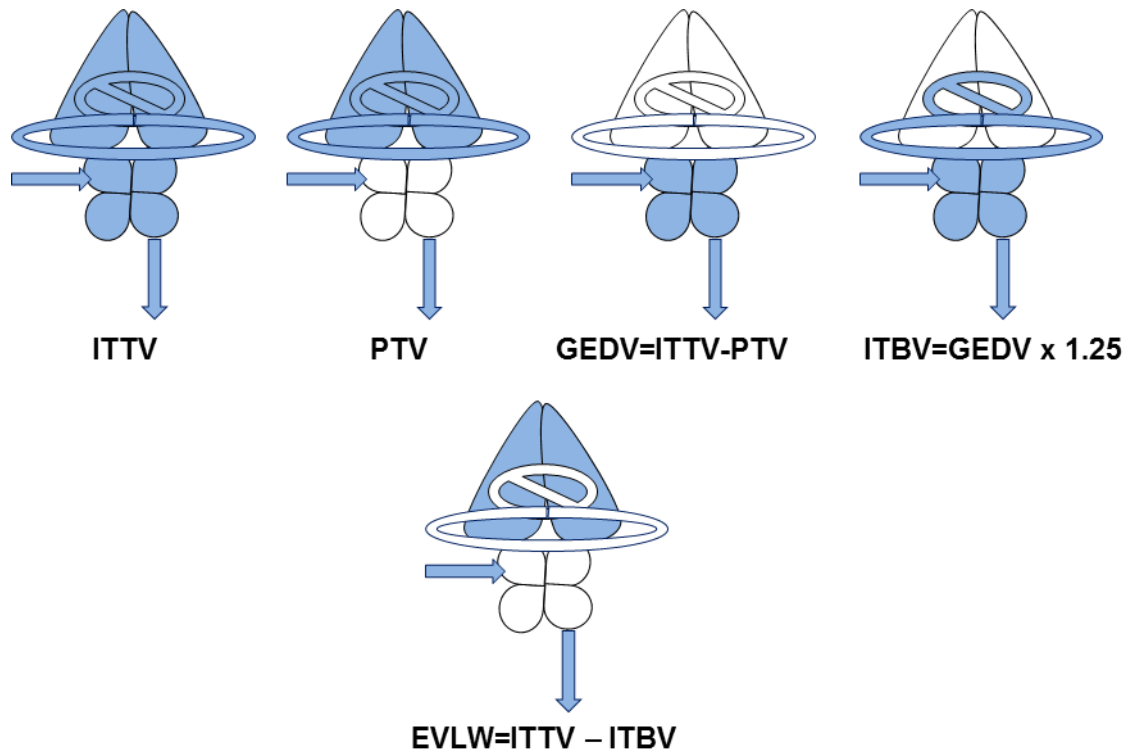


Figure 3.2: Diagrammatic representation of volume of distribution of the cold injector when measuring EVLW

ITTV: intrathoracic thermal volume; PTV: pulmonary thermal volume; GEDV: global end diastolic volume; ITBV: intrathoracic blood volume; EVLW: extravascular lung water. (adapted from Isakow *et al*)²⁰¹

The extravascular lung water index (EVLWI ml/kg) was calculated by dividing the EVLW by predicted body weight. EVLW indexed to predicted rather than actual body weight has been shown to have a greater correlation with mortality in the intensive care unit.⁸⁷ A recent study has shown that height was the only biometric parameter associated with EVLW.²⁰⁵ Throughout this thesis results are represented as EVLWI. Extravascular lung water measurements have been shown to correlate with gravimetric lung water at post mortem^{206,207} and been shown to be a predictor of adverse outcomes in the critically ill.^{87,208} Increases in EVLW are related to the

pathophysiological changes of pulmonary oedema and ARDS²⁰⁹ and an early reduction in EVLWI is a prognostic indicator of survival.²¹⁰

Transpulmonary thermodilution also allows for the measurement of pulmonary vascular permeability index (PVPI), which is thought to estimate the permeability of the alveolar capillary barrier. PVPI is the ratio between EVLWI and pulmonary blood volume (PBV), where PBV is the difference between ITBV and GEDV. PVPI has been validated in some animal²⁰⁶ and human studies which show it to be higher in patients with ARDS than hydrostatic pulmonary oedema.^{209,211} The thermodilution procedure has been utilised by our group for many years, however to test reproducibility, studies were performed six times in a single patient at 3 different time points to determine the coefficient of variation (CV). The CV was determined by dividing the standard deviation of the 6 measurements by their mean. The CV of CO, EVLW and PVPI at the 3 different time points is shown in Table 3.1.

Measurement	Preoperative	Postoperative	Day 1	Mean CV (%)
CO (L/min)	6.5	6.9	7.2	6.8
EVLW (mls)	5.1	6.3	3.8	5.1
PVPI	7.7	3.9	6.6	6.1

Table 3.1: Coefficient of variation for PiCCO measurements.

CO; cardiac output; EVLW; extravascular lung water; PVPI; pulmonary permeability index.

3.4 SAMPLE COLLECTION

3.4.1 Blood collection

Blood was taken from patients by peripheral venepuncture or via existing arterial or venous lines using a vacutainer® system (Becton Dickinson Ltd, Oxford, UK) into 4 Lithium heparin and 2 EDTA bottles (Total 36mls) at recruitment pre-drug administration, immediately pre-operatively, post-operatively, 9am on day 1 and day 3/4. Samples were then immediately transported on ice to the laboratory for processing.

Blood was taken from healthy volunteers by peripheral venepuncture into 5 Lithium heparin vacutainer® bottles (Becton Dickinson) at the University of Birmingham Research Laboratories after taking consent as per University guidelines and immediately processed in the laboratory.

3.4.2 Bronchoscopy and bronchoalveolar lavage

Bronchoscopy was performed immediately following oesophagectomy after completion of one-lung ventilation and prior to the patient being woken up and extubated. An Olympus LF-TP fibrescope (Olympus Keymed Medical, Southend-on-Sea, UK) was inserted into the endotracheal tube into the lung that had been deflated during one-lung ventilation, which was the right lung in all patients in this study. The scope is 5.2mm in diameter and has a sufficient sized suction channel. Bronchoalveolar lavage was performed using a standardised protocol that has previously been described.²¹² The tip of the bronchoscope was wedged into a sub-segmental branch of the middle lobe

and three 50 millilitre aliquots of sterile 0.9% saline at room temperature were instilled down the bronchoscope and the bronchoalveolar lavage fluid (BALF) aspirated back into a 100 millilitre collection chamber attached to the suction port. The sample was placed immediately on ice and shipped to the laboratory for processing and analysis.

3.4.3 Lung resection tissue

Lung resections samples collected as part of the MLTC were immediately examined in theatre by an experienced member of the surgical team. A section of the sample of at least 10grams in weight and distant from the tumour, without evidence of macroscopic malignancy that was not required for diagnostic or clinical purposes was immediately placed in a sterile sealed container with 0.9% saline and handed to the research team. Samples were transported on ice to the laboratory for processing and analysis.

3.4.4 ARDS bronchoalveolar lavage fluid

Stored ARDS BALF samples were obtained from 4 patients from the BALTI trial of salbutamol to treat ARDS¹¹⁴. This was a randomised study of patients with confirmed ALI/ARDS as per AECC criteria that were treated with placebo or IV salbutamol. Patients underwent a sub-study bronchoscopy and lavage on the day of recruitment as part of the study and unused BALF was stored as per ethical approval for use in future ARDS studies. Samples were pooled and mixed with 1:1 RPMI 1640 media and 10% FBS (Sigma-Aldrich) for further macrophage stimulation as described in section 6.2.1.1.

3.5 SAMPLE PROCESSING

3.5.1 Blood processing

Upon arrival in the laboratory samples were spun at 560g for 10 minutes in a centrifuge pre-chilled to 4°C (Rotina 46R centrifuge, Hettich AG, Bach, Switzerland). The supernatant (plasma) was then aspirated and aliquoted into 0.5ml cryovials and stored at -80°C until further analysis was performed. The blood cell pellet was then mixed in a ratio of 1:1 with phosphate buffered saline (PBS; Gibco Invitrogen, Paisley, UK) in a 50ml sterile Falcon™ tube (Becton Dickinson Ltd) in preparation for monocyte isolation.

3.5.2 Monocyte Isolation

The blood cell pellet and PBS (Gibco Invitrogen) mixed solution was gently overlay onto Lymphoprep™ (Axis-Shield, Oslo, Norway) in a ratio of 3:2 blood to Lymphoprep™ in a 50ml sterile Falcon™ tube (Becton Dickinson Ltd) and centrifuged (Universal 32 centrifuge, Hettich AG, Bach, Switzerland) at 800g with minimal acceleration and zero brake for 30 minutes at 20°C. Lymphoprep™ is a density gradient medium for the isolation of mononuclear cells from peripheral blood as granulocytes and red blood cells have a higher density than mononuclear cells and sediment through the Lymphoprep™ layer during centrifugation. The interphase created which contains mononuclear cells was then gently aspirated using a 2.5 ml Pasteur pipette (Alphalabs, Eastleigh, UK) and transferred into a clean Falcon™ tube (Becton Dickinson Ltd) and washed by the addition of 30mls of sterile PBS (Gibco Invitrogen) with 10% foetal bovine serum (FBS, Sigma-Aldrich, Poole, UK)

and centrifuged at 250g for 10mins (Universal 32 centrifuge, Hettich AG, Bach, Switzerland). This process enables removal of most of the platelet population. The supernatant was then discarded and cells re-suspended with culture medium [RPMI 1640 media; 10% FBS; penicillin 100U/mL; streptomycin 100ug/mL (Sigma-Aldrich)].

Total cell count, viability and differential cell count were performed as described in 3.5.6 and 3.5.7. Cells were then re-suspended in culture media dependent on monocyte cell percentage and total cell count to a volume such that the concentration was 1×10^6 monocytes/mL and plated at 0.5×10^6 per well in a 24 well flat bottom cell culture plate (Costar®, Corning®, Wiesbaden, Germany) and incubated at 37°C in a 5% CO₂ atmosphere in preparation for cellular experiments.

3.5.3 Bronchoalveolar fluid

The lavage fluid was initially filtered through sterile surgical gauze to remove mucous and particulate matter in a sterile 50ml Falcon™ tube (Becton Dickinson Ltd) and the total volume of lavage documented. A total cell count was performed using trypan blue and the haemocytometer and a differential cell count performed using Quick-diff (Gentaur Europe, Kampenhout, Belgium) as described in 3.5.6 and 3.5.7. The BALF was then centrifuged in a pre-chilled centrifuge (Rotina 46R centrifuge, Hettich AG) 4°C at 560g for 10 mins. BALF supernatant was aspirated and stored in aliquots of 0.5ml at -80°C for future analysis. The cell pellet was then re-suspended in 10mls of

RPMI 1640 media supplemented with 10% FBS (Sigma-Aldrich) for further processing.

3.5.4 Lung resection samples

Upon arrival in the laboratory lung resection samples were immediately washed through with 2000mLs of sterile 0.9% sodium chloride (Baxter, Newbury, UK) using a 14 gauge needle (Vasofix®, Braun, Melsungen, Germany). The needle was moved through the lung sample whilst continuous fluid was connected using an intravenous giving set and allowed to run freely. The washed through fluid or lavage was collected and decanted into 50ml Falcon™ tubes (Becton Dickinson Ltd) and centrifuged in a pre-chilled centrifuge (Rotina 46R centrifuge, Hettich AG) 4°C at 560g for 10 mins. The supernatants were discarded and the cell pellets pooled and re-suspended in 10mls of RPMI 1640 media supplemented with 10% FBS (Sigma-Aldrich) for further processing.

3.5.5 Alveolar macrophage isolation

Re-suspended cell pellets from BALF and lung resection washings were treated identically from this point onwards. The re-suspended cell pellets were overlay onto 10mls of Lymphoprep™ (Axis-Shield) and centrifuged at 800g for 30minutes at 20°C with minimal acceleration and no brake (Universal 32 centrifuge, Hettich AG). The interphase or buffy layer of mononuclear cells was aspirated carefully with a 2.5 ml Pasteur pipette (Alphalabs) into a fresh sterile 50mL Falcon™ tube (Becton Dickinson Ltd)

and washed with PBS (Gibco Invitrogen) supplemented with 10% FBS (Sigma-Aldrich) at 300g for 10 mins (Universal 32 centrifuge, Hettich AG) to facilitate removal of platelets. A further cell count and viability was performed on all samples as described in 3.5.6 and 3.5.7 (purity >95%) and cells re-suspended in culture medium [RPMI 1640 media; 10% FBS; penicillin 100U/mL; streptomycin 100ug/mL (Sigma-Aldrich)] to a concentration of 1×10^6 cells/mL. Cells were then plated into a 24 well flat bottom culture plate (Costar®, Corning®) at 0.5×10^6 cells/mL and cultured in an incubator at 37°C in a 5% CO₂ atmosphere in preparation for cellular studies. For the *in-vitro* studies, cells were treated with experimental conditions at this point.

3.5.6 Cell count and viability

Fifty microlitres of cell solution was mixed with equal volume of 0.4% (w/v) trypan blue solution and left to incubate for 5 minutes. Cell count and viability was performed by adding 10ul of the solution to a haemocytometer with improved Neubauer markings and cells counted in each chamber. Non-viable cells take up the dye and appear blue whilst viable cells exclude the dye. Total viable cell count was performed 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 (number of cells per millilitre from haemocytometer).

3.5.7 Differential cell count

A differential cell count was performed using a cytopsin preparation. Fifty microlitres of re-suspended cells were placed in the cytopsin apparatus and spun at 300 r.p.m (rotations per minute) for 5 minutes in a Shandon Mk II cyto-centrifuge (Thermo electron corporation, Basingstoke, UK). Slides were air dried and stained with Quick-Diff (Gentaur Europe, Kampenhout, Belgium). The differential cell count was then determined by counting 100 cells under 40 x magnification and noting the number of individual cell types.

3.6 EFFEROCYTOSIS ASSAY

An efferocytosis assay was undertaken to assess the phagocytic capacity of apoptotic neutrophils by alveolar macrophages and cultured peripheral blood monocytes. This assay has previously been validated by our group.

3.6.1 Isolation and preparation of neutrophils

Neutrophils were isolated from whole blood using a Percoll[®] (pH 8.5-9.5; Sigma-Aldrich) discontinuous density gradient as previously described.²¹³ Blood was drawn from healthy volunteers by peripheral venepuncture into lithium heparin vacutainer[®] bottles (Becton Dickinson Ltd) and transferred into a 50ml sterile Falcon[™] tube (Becton Dickinson Ltd). One millilitre of 2% dextran (Sigma-Aldrich) was added for every 6mls of blood and gently mixed prior to incubation for 30minutes at room temperature to facilitate sedimentation of the red blood cells.

A working 90% isotonic stock solution of Percoll® was made by mixing 45 ml of Percoll® (Sigma-Aldrich) with 5 ml of sterile 9% (w/v) sodium chloride (Sigma-Aldrich). The 80% Percoll® was prepared by diluting 40 ml of the isotonic Percoll® stock with 10 ml 0.9% (w/v) sodium chloride (Baxter, Newbury, UK) whilst 56% Percoll® comprised of 28 ml of the working isotonic Percoll® stock and 22 ml of 0.9% (v/v) sodium chloride (Baxter). Gradients were then prepared by carefully under-layering 2.5ml 80% Percoll® with a 3ml Pasteur pipette (Alphalabs) under 5 ml of 56% Percoll® in a 15ml sterile Falcon™ tube (Becton Dickinson, Ltd).

The white cell rich plasma top layer created by the dextran step above was then aspirated with a 3ml Pasteur pipette (Alpha labs) and carefully layered onto the Percoll® density gradient. The tubes were then centrifuged at 220g and 20°C for 20 minutes with minimal acceleration and no brake (Universal 32 centrifuge, Hettich AG). This process separates blood into distinct layers, with the uppermost being diluted plasma, followed by an interphase of mononuclear cells, a layer of 56% Percoll®, then a neutrophil cellular interphase followed by the 80% Percoll® layer and red cell pellet at the base. Neutrophils were carefully aspirated from the 80% and 56% gradient interface using a fine bore Pasteur pipette (Alphalabs) and re-suspended and washed twice in phosphate buffered saline (PBS; Gibco Invitrogen) at 440G for 10 minutes at room temperature. Post-centrifugation the supernatant was discarded and the cells re-suspended in RPMI 1640 (Sigma-Aldrich).

Cells were counted with trypan blue on a haemocytometer and a differential cell count was also performed as described above to ensure purity of >95%.

Cells were then re-suspended in a 1 micromolar (μM) solution of Celltracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) (Life Technologies Ltd, Paisley, UK) in warmed, serum free RPMI 1640 (Sigma-Aldrich) at a concentration of 2×10^6 cells/mL and incubated for 30 minutes at 37°C in a 5% CO_2 atmosphere to allow uptake of the marker. This fluorescent stain passes through cell membranes; however, once inside the cell is transformed by intra-cellular esterase enzymes into cell-impermeable fluorescent cytoplasmic reaction products with a green excitation/emission spectra (492/517 nm maxima). This product has both good fluorescent retention of over 72 hours and low cytotoxicity. This was followed by 2 washes with warm serum free RPMI 1640 (Sigma-Aldrich) by centrifuging for 5 minutes at 1500g at room temperature (Universal 32 centrifuge, Hettich AG). Finally the cells were re-suspended at a final concentration of 2×10^6 in warm serum free RPMI with penicillin 100U/mL; streptomycin 100 μg /mL (Sigma-Aldrich) and left for 24 hours at 37°C in a 5% CO_2 atmosphere.

Neutrophils are known to undergo apoptosis in a time-dependant manner. It has previously been shown by our group (Dr Christopher Bassford, University of Warwick, 2011) that features of apoptosis as evidenced by cellular vacuolation and maximal trypan blue uptake occurred at 20 hours. To confirm this, a flow cytometry quantitative assay of the time course of neutrophil apoptosis was performed of 4 healthy control neutrophils using

Fluorescein Isothiocyanate (FITC) conjugated Annexin V (BD Biosciences, Oxford, UK) and violet conjugated Sytox blue (Invitrogen, Paisley, UK). Cells that were annexin only positive (early apoptosis) and double positive (late apoptosis) were counted to calculate the percentage of total apoptosis over 24 hours. Figure 3.3 demonstrates that by 20 hours 78.2% and by 24 hours 93.8% of neutrophils were apoptotic. Very few cells were necrotic <2% (Sytox only positive) at any time point. Therefore in this assay we incubated neutrophils for 24 hours to achieve maximal apoptosis.

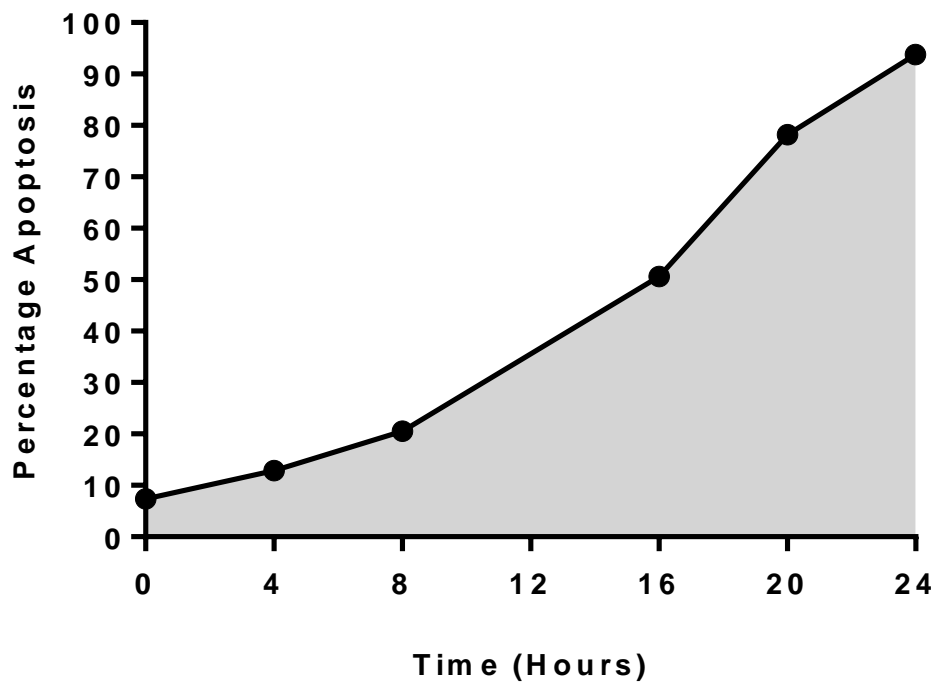


Figure 3.3: Time course of neutrophil apoptosis.

Apoptosis was assessed at 0, 4, 8, 16, 20 and 24 hours. Values represent means of 4 duplicate experiments.

3.6.2 Quantification of efferocytosis

After 24 hours incubation media was removed from the cultured alveolar macrophages or peripheral blood monocytes and the cells washed gently with serum free media. Cells as expected were adhered to the flat bottom wells of the culture plates. The stained apoptotic neutrophils were washed as described above and re-suspended in a concentration of 2×10^6 cells/mL in RPMI 1640 (Sigma-Aldrich). The suspension was then added to the mononuclear (MNCs) at 1mL per well thereby flooding the cultured mononuclear cells with four fold excess of apoptotic neutrophils. Cells were incubated for 90 minutes in a 5% CO₂, 37°C atmosphere to allow the phagocytosis of the neutrophils. The media was then removed and wells washed twice with room temperature PBS (Gibco Invitrogen) to remove any non-adherent or non-engulfed neutrophils. Five hundred microliters of trypsin solution (TripLE™ express, Gibco Invitrogen, Paisley, UK) was added to each well and incubated for 15 minutes at 37°C, 5% CO₂ atmosphere. Cells were visualised under a microscope to ensure that they had detached from the culture well floor. If not, they were returned to the incubator and re-inspected every 5 minutes. The cell suspensions were then pipetted and transferred into 5ml polypropylene flow cytometry tubes (Falcon™, Becton and Dickinson) and spun at 400g for 4minutes at 4°C and re-suspended in 100ml of PBS (Gibco Invitrogen) and immediately placed on ice and examined on a flow cytometer (CyAN_{ADP}, Beckman Coulter, Brea, California, USA).

Cells were agitated on a vortex (Varimix, Sciquip, Newtown, UK) immediately prior to running on the flow cytometer. Pure alveolar macrophage or cultured monocytes and pure stained apoptotic neutrophils were used to set forward and side-scatter gating on the flow cytometer. The FL-1 channel that detects the FiTC fluorescent spectrum was used to identify CMFDA labelled apoptotic neutrophils that had been engulfed by macrophages. An example of the flow plots and histograms obtained are shown in Figure 3.4. A minimum of 5000 events (cells gated as MNCs) were counted for each experimental condition and the proportion of CMFDA positive cells and hence engulfed apoptotic neutrophils presented as and efferocytosis percentage.

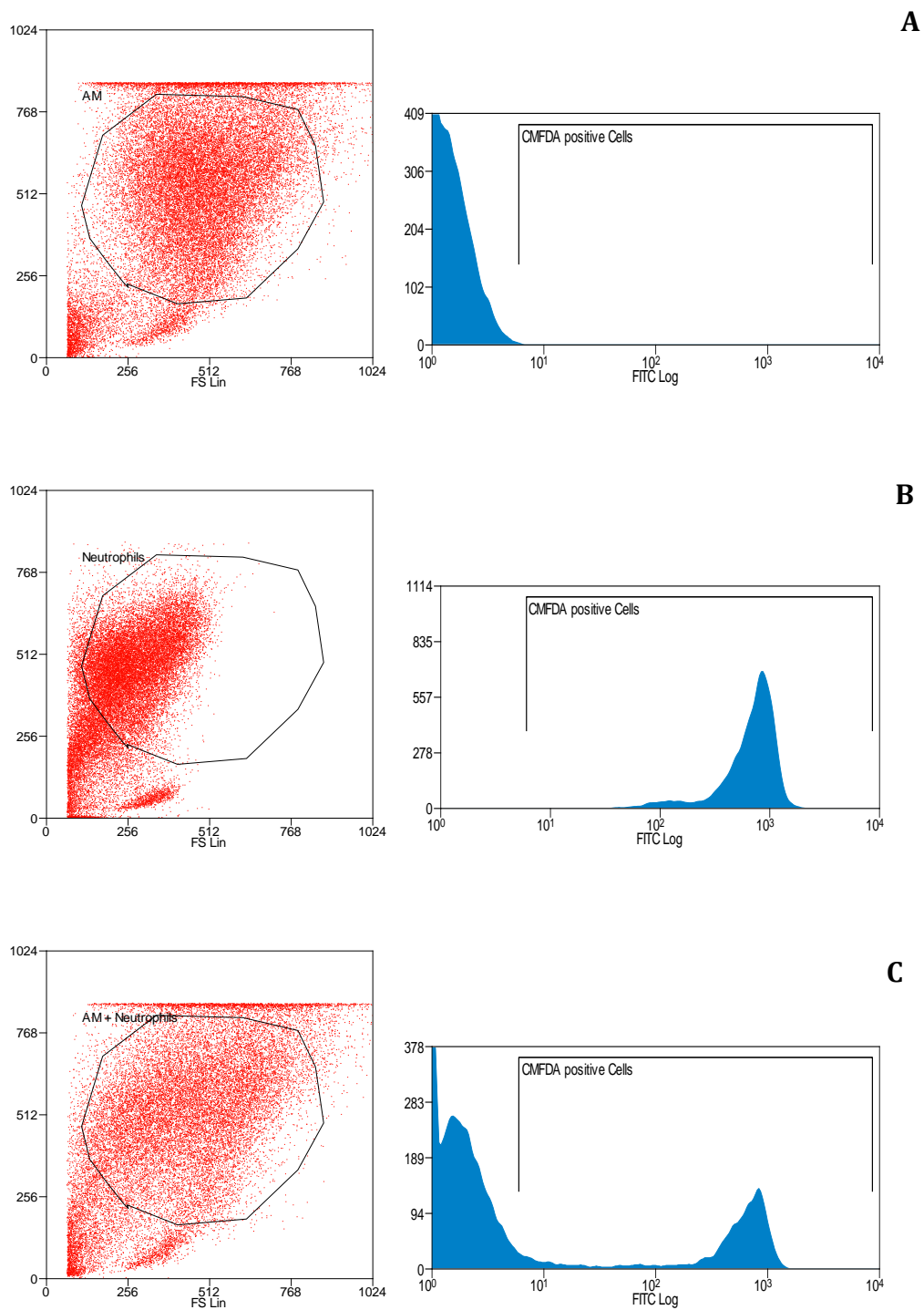


Figure 3.4: Example flow cytometry plots and histograms of efferocytosis.

Forward-side scatter plot and corresponding histogram of **A**: Negative control alveolar macrophages (AM). **B**: Positive control CMFDA stained apoptotic neutrophils and **C**: Proportion of AMs that had engulfed CMFDA stained apoptotic neutrophils.

This flow cytometric assay has been previously validated by our group by comparing it with direct fluorescent microscopy and been found to have differences in results of less than 10% (Dr Chris Bassford, PhD thesis, University of Warwick, 2011). However to ensure validity, intra-assay CV was re-assessed by repeating one condition 6 times within the same subject and the standard deviation of the measurements being divided by their mean. The intra-assay CV was 7.2%.

3.7 MONOCYTE DIFFERENTIATION AND PHENOTYPE

Isolated peripheral blood monocytes were incubated for 24 hours as described above. After which the media was changed and the cells treated with the relevant conditions [untreated, 50nmol/L 25(OH)D₃ (Merck Millipore, Watford, UK), 100ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF, PeproTech, Rocky Hill, USA), 50ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech) with 50ng/mL IL-10 (PeproTech)] in culture media for a further 72 hours. After 72 hours the supernatant was aspirated and the cells detached with 500mL trypsin solution (TripLE™ express, Gibco Invitrogen, Paisley, UK) and incubated for 15 minutes at 37°C, 5% CO₂ atmosphere. Cells were visualised under a microscope to ensure that they had detached from the culture well floor. If not, they were returned to the incubator and re-inspected every 5 minutes. The cells were then aspirated into a 96 well flexi-plate at 2 x 10⁵ cells per well (a well for cells only, each single immunofluorescent antibody stain, control,

treatment conditions in duplicate and isotype controls) and washed twice with 100mL PBS (Gibco, Invitrogen) with 1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, UK) at 4⁰ C, 400g for 4 minutes (Rotina 46R centrifuge, Hettich AG, Bach, Switzerland). Cells were then gently agitated and placed on ice and stained with the immunofluorescent antibody panel as shown in Table 3.2 for 30 minutes on ice in the dark. After incubation the plate was washed twice with 100mL of 1%BSA/PBS as above and cells re-suspended in 300mL of PBS (Gibco, Invitrogen) and immediately run through a flow cytometer (CyAN_{ADP}, Beckman Coulter, Brea, California, USA). Colour compensation and analysis were performed using Summit 4.3 software (Dako, Beckman Coulter). The mean fluorescent intensity (MFI) was analysed for treated and untreated cells for each antibody stain and the results expressed as a fold change in MFI compared with untreated cells.

Antibody	Clone	Isotype	Fluorochrome	Concentration	M1*	M2*
Anti-hCD14	TUK4	Mouse, IgG2a	PE Vio770™	1:20	++	+
Anti-hCD80	2D10	Mouse, IgG1	APC	1:11	++	-
Anti-hCD206	DCN228	Mouse, IgG1	APC Vio770™	1:11	++	+
Anti-hCD163	GHI/61.1	Mouse, IgG1	VioBlue®	1:11	-	++
Anti-hCD16	VEP13	Mouse, IgM	VioGreen™	1:11	-	++
Anti-hCD200R	OX108	Mouse, IgG1	Alexa Fluor®488	1:20	-	++
Anti-hMer TK	125518	Mouse, IgG1	PE	1:10	-	++

Table 3.2: Human antibody staining panel used for flow cytometry of cultured monocyte cell surface expression.

h: human; MerTK: Mer tyrosine kinase receptor; PE: phycoerythrin; APC; allophycocyanin.

*: The expression of each antigen by M1 (classically differentiated) and M2 (alternatively differentiated) mononuclear cells is presented as high (++) , intermediate (+) and low/negative (-).

CD14, CD80, CD206, CD163 and CD16 (Macs, Milteyni Biotech, Auburn, USA); CD200R (AbD Serotec, Bio-Rad, Kidlington, UK);

Anti-Mer TK (R&D systems, Abingdon, UK)

3.8 BIOCHEMICAL ASSAYS

3.8.1 Vitamin D Levels

Vitamin D levels (25(OH)D₃ and 1,25(OH)₂D₃) were batch transported on dry ice and measured by the Supra-regional Assay Service for Metabolic Bone Assays, Biomedical Research Centre, University of East Anglia, Norwich Medical School, Norfolk, Norwich, UK. This laboratory has appropriate Clinical Pathology Accreditation (CPA UK Ltd) and all assays have been validated to national standards. 25(OH)D₃ was measured by tandem mass spectrometry using the appropriate vitamin D External Quality Assessment Scheme (DEQAS) control. 1,25(OH)₂D₃ levels were measured by enzyme immunoassay (EIA) (immunodiagnostic systems Ltd, UK). A summary of the validation data provided for these assays is provided in Table 3.3 and 3.4.

25 (OH)D ₃ (nmol/L)			
Lower limit of detection	Intra-assay CV (%)	Inter-assay CV (%)	Spike recovery (%)
5nmol/L	9.6	9.2	96.1

Table 3.3: Validation of 25(OH)D₃ mass spectrometry assay
Data courtesy the Professor William Fraser, Biomedical Research Unit, University of East Anglia, Norwich, UK. CV: coefficient of variation.

1,25(OH) ₂ D ₃ (pmol/L)		
Lower limit of detection	Intra-assay CV (%)	Inter-assay CV (%)
20pmol/L	10.5 – 15.9	17.6 – 16.3

Table 3.4: Validation of 1,25(OH)₂D₃ enzyme immunoassay (EIA)
Data courtesy of Professor William Fraser, Biomedical Research Unit, University of East Anglia, Norwich, UK. CV: coefficient of variation.

3.8.2 Vitamin D binding protein

Vitamin D binding protein (DBP) was measured by using a commercially available ELISA kit (K2314KO1, Immundiagnostik, Bensheim, Germany). The pre-coated 96 well microplate (polyclonal anti-DBP antibodies) was manually washed 5 times with 250mL of wash buffer using a multichannel pipette (Starlabs, Milton Keynes, UK). Pre-prepared lyophilized 5 standards and 2 controls were reconstituted in 500mL of standard dilution buffer and 1 in 40,000 dilutions of plasma samples prepared by 3 serial dilutions in the sample dilution buffer. The enzyme linked antibody (rabbit –anti-DBP, peroxidase labelled) was diluted 1:100 in wash buffer. One-hundred microliters of standards, controls and pre-diluted plasma samples were added to the pre-coated 96 well plate, covered and incubated for 1 hour on a horizontal plate mixer at room temperature. At the end of the incubation period wells were manually washed 5 times with wash buffer as described above. One hundred microlitres of the peroxidase labelled antibody were added to each well and the plate covered with an adhesive strip and incubated for a further hour on a horizontal plate mixer. Following this the contents were decanted and plates washed 5 times as described above. One hundred microliters of substrate tetramethylbenzidine (TNB) were added to each well and incubated in the dark for 20 minutes prior to adding 100mL of stop solution. The plate was immediately read at 450nm (against 690nm) using a Synergy HT fluorometric plate reader (BioTek, Swindon, UK). The DBP concentration was calculated by interpolation of the standard curve and correction for sample dilution factor.

Spiking experiments were performed in which pooled healthy control plasma were measured with and without supplementation with a known concentration of DBP. Observed values were compared against expected values and the mean spike recovery calculated. Validation experiments were performed in a 1 in 40,000 sample dilution. Experiments were performed in replicate 12 times intra-assay and known spiked sera compared between 10 plates. Adding 2 standard deviations of the mean optical density value of 10 zero standard replicates and calculating the corresponding concentration calculated the lower limit of detection. The lower limit of detection, intra and inter assay CV and percentage recovery are shown in Table 3.5 and standard curve in Figure 3.5. Limitations of this assay include the high sample dilution required, reducing its sensitivity and its lack of detection of DBP-Actin complexes that may result in lower levels of DBP.

DBP ng/mL			
Lower limit of detection	Intra-assay CV (%) n=12	Inter-assay CV (%) n=10	Spike recovery mean (%) n=12
2.96ng/mL	2.4	27.8	104.8

Table 3.5: Validation of vitamin D binding protein (DBP) enzyme-linked immunosorbent assay. CV: coefficient of variation.

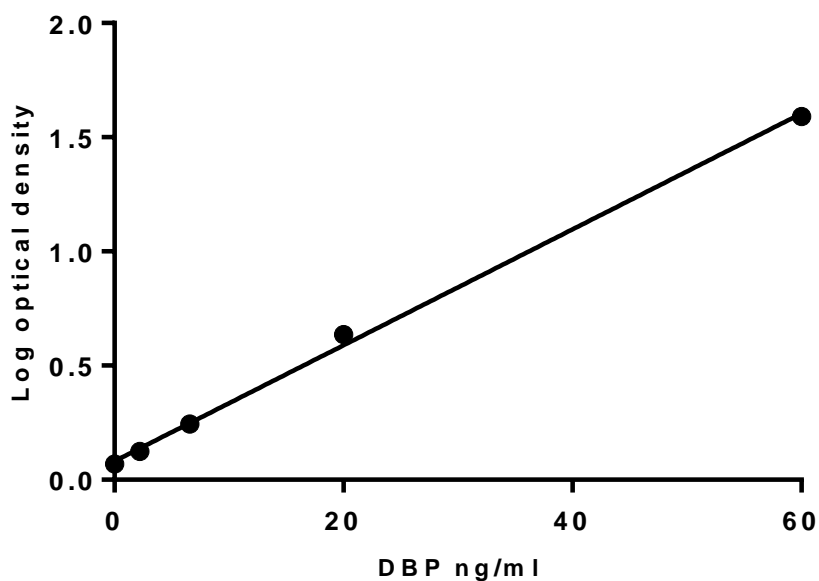


Figure 3.5: Standard curve for vitamin D binding protein (DBP)

3.8.3 Cytokines

Plasma inflammatory cytokines (IL-6, IL-8, TNF- α , IL1- β and IL-10) were measured by a commercially available Magnetic Luminex[®] Performance Assay (LUHM000, R&D Systems, Abingdon, UK) and soluble cytokine receptors (sRAGE, TNFR-1 and TNF-2I) by a MILLIPLEX[®] MAP assay (HSCRMAG-32K, Millipore, Billerica, USA). This method uses precise concentrations of color-coded magnetic microparticles which have been pre-coated with analyte specific capture antibodies that allow multiple distinctly coloured bead sets to be created. After capture of the analyte by the beads, a biotinylated antibody cocktail specific to the analytes is added. Finally a Streptavidin-Phycoerythrin conjugate (Streptavidin-PE) that binds to the biotinylated antibody is added. A dual laser analyser allows one laser to identify the analyte in question and the other quantifies the magnitude of the

PE-derived signal. This technique allows measuring of multiple cytokines simultaneously in a limited sample volume.

The two kits used above follow similar procedures albeit with slight variations. For the inflammatory cytokine kit (LUHM000, R&D Systems), standards were created by seven doubling dilutions of the standard cocktails provided with calibrator diluent. Plasma samples were diluted 4 fold in calibrator diluent. Microparticle beads were gently vortexed and 50mL made up to 5mls with microparticle diluent. Fifty microliters of microparticles were added to each well of a 96 well microplate followed by 50 μ L of standards or sample, covered with a foil plate sealer and left to incubate for 3 hours on a horizontal microplate shaker (800rpm). Following incubation the plate was washed 3 times with 100 μ L of wash buffer using an automated magnetic plate washer (Bioplex Pro™, Bio-rad, Hercules, USA) to ensure beads are not washed away. Fifty microliters of diluted biotin antibody cocktail was added to the wells and incubated for a further hour at room temperature on a shaker. After the wash step was repeated 50 μ L of Streptavidin-PE was added to each well and left to incubate in the dark as above for 30mintues. A further wash step was repeated followed by the addition of 100 μ L of wash buffer and incubation for 2mins on the shaker as above. The plate was then read on a Bioplex® 200 analyser (Bio-rad, Hercules, USA). Each bead is assigned a region on the analyser and standard concentrations and dilution factor for each analyte is input from which the BioRad software package creates a standard curve and using 5 parameter

logistic regression determines the concentrations of analytes within the samples determined by interpolation, and multiplication by the appropriate dilution factor.

In brief the cytokine soluble receptor kit (Millipore, Billerica, USA) uses the same method. Microparticle beads were sonicated and 60 μ L were made up to 3mls with bead diluent. The supplied 96 well microplate was washed 3 times with 200 μ L of wash buffer using an automated plate washer (Bioplex Pro™, Bio-rad, Hercules, USA). Twenty-five microliters of standards (seven serial dilutions), quality controls, samples (1 in 5 dilutions), assay buffer and serum matrix were added to the wells followed by 25 μ L of magnetic beads. These were sealed and incubated for 2 hours at room temperature as described above. The plate was then washed twice with 200 μ L of wash buffer on a magnetic automated plate washer (Bioplex Pro™, Bio-rad). Twenty-five microliters of detection antibodies were added and incubated for a further hour followed by the addition of 25 μ L of Streptavidin-PE without performing a wash step. The plate was then incubated for a further 30minutes and washed twice followed by the addition of 150 μ L of sheath fluid. The plate was analysed on the Bioplex® (Bio-Rad) as described above. These assays are calibrated against highly purified recombinant human cytokines produced at R&D Systems and Millipore.

3.9 ANIMAL STUDIES

3.9.1 Ethical statement

All procedures were performed in accordance to UK laws under the Animal [Scientific Procedures] Act 1986 (project licence code 30/2836; personal licence number I77F587B4) and with approval and oversight from the local animal ethics committee, Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NAQWO). The principles of the 3Rs (Reduction, Replacement and Refinement) provide the foundations for how the animals were cared for and the research was designed.

3.9.2 Experimental animals and study design

Male wild-type (WT) C57Bl/6 mice (aged 3-4 week old) were obtained from Harlan UK Limited, Oxford, UK and maintained at the Biomedical Services Unit (BMSU), Birmingham University, UK. A maximum of 16 mice were obtained at any one time to account for 8 mice per group allocation. Once housed the mice were randomly numbered and tagged 1 to 4 per cage in 4 cages. All experiments were carried out on WT mice that had undergone dietary manipulation. Vitamin D deficiency was induced in WT pups by feeding them a vitamin D deficient chow (TD 89123, Harlan, USA) or normal chow for 6 weeks thus producing 2 experimental arms (n=8 per arm), vitamin D deficient mice (VDD) and vitamin D sufficient (VDS) mice. Caecal ligation and puncture surgery was carried out on individual mice in batches of 2-4 mice at any one time. This was to ensure mice could be monitored adequately during and after the procedure. The experimental protocols have

been designed to minimise the numbers of animals used but to maximise scientific outcome (e.g. the consistent use of C57B6 strain, sex and consistent age of animal and interventions to allow comparisons across wild type animals and measurements of multiple outcomes in the same animal).

3.9.3 Housing and husbandry

All animals were housed in cages of 4 in a 12-hour light and 12-hour dark cycle with food and water provided *ad libitum*. All mice were weighed before and 16 hours after surgery. The health of each individual mouse was assessed and documented before, during and after surgery on in-house standardised mouse assessment scoring sheets to ensure welfare. If at any time there was concern that the mouse was becoming too unwell, in pain despite increased analgesia or distressed a decision was made to stop the experiment and euthanise the animal humanly and immediately according to Schedule 1 of the Animal (Scientific Procedures) Act 1986.

3.9.4 Caecal ligation and puncture (CLP) surgery

Pre-medication was given in the form of subcutaneous buprenorphine 0.1mg/kg body weight (Temgesic®, Reckitt Benckiser, Wallisellen, Switzerland) 15-30 minutes prior to the procedure. Animals were anaesthetised with isoflurane gas (5% with 1.5L/min of oxygen) for induction and maintenance (1-3% with 1.5L/min of oxygen) anaesthesia. All surgery was performed with aseptic techniques (sterile gown, gloves, drapes, and surgical equipment) and in dedicated animal surgical theatres in the

BMSU, University of Birmingham. The CLP experimental design was adapted from previously published protocols.²¹⁴ After shaving the fur off the abdomen and application of 2% chlorhexidine (Avagard™, Pymble, Australia) a midline laparotomy was performed followed by exposure of the caecum, ligation of the lower 30% with 2.0 Ethilon™ nylon suture (Ethicon inc) and single through-and-through puncture of ligated caecum with a 19G microlance needle (Becton Dickinson Ltd). A small amount of faeces was then gently expressed by compressing the ligated caecum with forceps prior to being placed back into the abdomen and closed with 6.0 Vicryl® (Ethicon inc) followed by skin closure with 4.0 Prolene® (Ethicon inc). Surgery was performed on heated tables set at 37.5°C. All animals were recovered in heat boxes and recovery incubators until sacrificed. Postoperative resuscitation was with 0.5mls of fluid (Hartman's solution, Aquapharm 11®, Animalcare Ltd, York, UK) and a further dose of buprenorphine. Animals were reassessed 6 hours post-operatively and given a further 0.5mls of fluid and sacrificed at 16 hours post surgery. Sham surgery was identical except for the lack of ligation and puncture of the caecum after exteriorisation from the abdomen.

3.9.5 Sample collection and processing

At 16 hours post CLP animals were deeply anaesthetised with 5% isoflurane. Cardiac puncture was performed and death confirmed. Immediate post-mortem peritoneal lavage fluid (PLF) was collected by instilling 1.0ml of PBS/EDTA (200nM) (Gibco, Invitrogen) into the right and left upper quadrant of the abdomen and aspirating back in both lower quadrants.

Bronchoalveolar fluid (BALF) was collected by opening the chest cavity and neck, dissecting down, manipulating the trachea and inserting a fine bore polythene tubing to the level of just above the carina. Lungs were then lavaged twice with 0.6mls of PBS/EDTA (200nM) (Gibco, Invitrogen). Examination of the caecum was carried out to confirm necrosis and colour of liver recorded to ensure adequate sepsis was induced. All samples were immediately transferred on ice to the laboratory for processing and analysis.

Cardiac blood was centrifuged at 13,500RPM in a micro-centrifuge (Stuart SCF2, Bibby Scientific Ltd, Stone, UK) at for 10 minutes. Prior to centrifugation 15ul of whole blood was taken for bacterial culture. The sera layer was aspirated and stored at -20°C. PLF and BALF were centrifuged at 400g for 10minutes. The supernatant was aspirated and stored at -20°C. The cell pellet was then re-suspended in PBS/EDTA (200nM) (Gibco, Invitrogen) for further analysis.

3.9.6 Bacterial culture

One hundred microliters of whole blood, PLF and BALF were diluted in 1ml of sterile RPMI 1640 in the first well of corresponding 24 well cell culture plates (CoStar®, Corning®). Eight serial dilutions of 1:10 (100mL of preceding well into the next well of 1640 RPMI) were sequentially carried out to a factor of 10⁸. Twenty microliters of each dilution and neat BALF were plated in corresponding pre-prepared Lysogeny broth (LB-Lennox, Sigma-Aldrich) agar plates split into eighths and labelled with dilution factors and incubated

overnight at 37°C. Culture plates were made in advance by adding 35g of LB-Lennox powder to 1L of warm distilled water and stirring to dissolve the powder. The broth was then autoclaved for 15 minutes at 121°C to sterilise. It was then allowed to cool slightly and poured into petri dishes. After 24 hours of incubating Colony Forming Units (CFU) were counted and CFU/ml calculated from the original dilutions and initial total volumes of blood, BALF and PLF. An example of a culture plate is given in Figure 3.6.

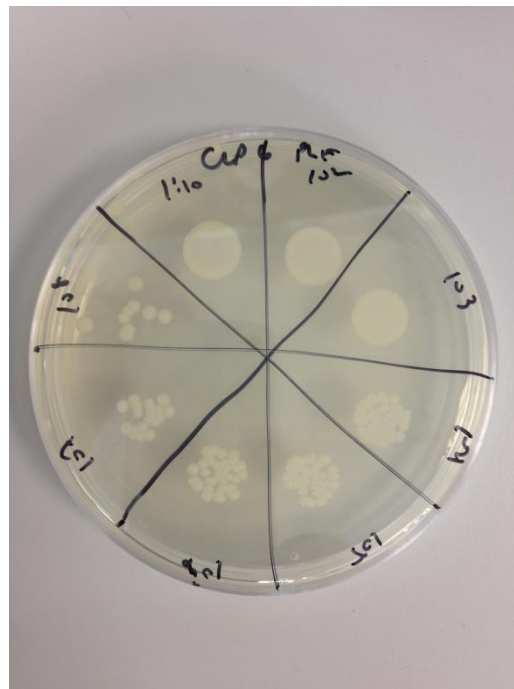


Figure 3.6: Example of bacterial culture plate for peritoneal lavage fluid

In this particular plate the colony forming units (CFU) would be read as 7×10^8 (since this is where the clearest CFUs can be read). The final calculation of CFU/mL was determined by the total volume of PLF and the initial dilution (1 in 50) prior to serial dilutions being performed: e.g. $\text{CFU/mL} = (7 \times 10^8 \text{ CFU}/1.25\text{mL PLF}) \times 50 \text{ dilution factor} = 2.54 \times 10^{10} \text{ CFU/mL}$

3.9.7 Cell identification and enumeration

The re-suspended BALF and PLF cells were counted on a haemocytometer as described in section 3.5.6 and the total number of cells per ml of BALF or PLF recorded. Cells were then resuspended to a concentration of $1-2 \times 10^6$ cells per ml in 2%EDTA/PBS (Gibco, Invitrogen) and 200 μ L plated onto a pre-labelled 96 well flexiplate (CoStar®). The plate was then centrifuged at 400g 4°C for 4minutes (Rotina 46R centrifuge, Hettich AG) and the cells gently agitated to release the cells from the bottom of the plate. Ten microliters of mouse serum (Sigma-Aldrich) was diluted in 1000mL of 2%EDTA/PBS and 50 μ L added to each well and the plate incubated on ice for 15minutes to block non-specific Fc receptor binding. The plate was then spun as previously and anti-mouse antibodies made up in 2%EDTA/PBS (Gibco, Invitrogen) and added to the appropriate wells as described in Table 3.6 (control, single antibodies and samples). This method has previously been validated by our group and therefore was not repeated. The cells were then incubated in the dark on ice for a 20minutes after which the plate was spun as described above.

Apoptosis of cells was assessed by adding one hundred microliters of Annexin V buffer (BD Biosciences, Oxford, UK) to the wells spinning the plate as described above followed by the adding Annexin V antibody (BD Biosciences at a concentration of 1:100 (in Annexin V buffer) and the plate left to incubate for a further 10 minutes in the dark on ice. Cells were then washed as above, transferred to polypropylene flow cytometry tubes

(Falcon™, Becton and Dickinson Ltd) and were analysed by flow cytometry (CyAn™ ADP, Beckman Coulter). SYTOX Blue™ (Molecular Probes™, Invitrogen) was used to assess cellular necrosis and 30µL (1:1500) was added prior to running the cells on the flow cytometer. Colour compensation and analysis were performed using Summit 4.3 software (Dako, Beckman Coulter). Relevant cell identification by markers are summarised in Table 3.7 and typical flow plots and gating strategy are shown in Figure 3.7.

Antibody	Clone	Isotype	Fluorochrome	Concentration
Anti-mF4/80	BM8	Rat, IgG2a kappa	PE	1:100
Anti-mCD11b	M1/70	Rat, IgG2b kappa	APC	1:1000
Anti-mCD11c	N418	Hamster, IgG	PE Cy7	1:150
Anti-mGr1/Ly6G	RB6-8C5	Rat, IgG2b kappa	APC Cy7	1:200

Table 3.6: Mouse antibody staining panel used for flow cytometry to identify cells

m: mouse; PE: phycoerythrin; APC; allophycocyanin; Cy7 cyanine 7. All markers acquired from eBiosciene, Hatfield, UK.

Cell	F4/80	CD11b	CD11c	Gr1
Alveolar macrophages	++	-	++	-
Peritoneal macrophages	++	+	-	-
Neutrophils	-	++	-	++

Table 3.7: Cell surface marker expression strategy for the identification and quantification of murine cells

The expression of each antigen by peritoneal and bronchoalveolar fluid cells is presented as high (++), intermediate (+) and low/negative (-).

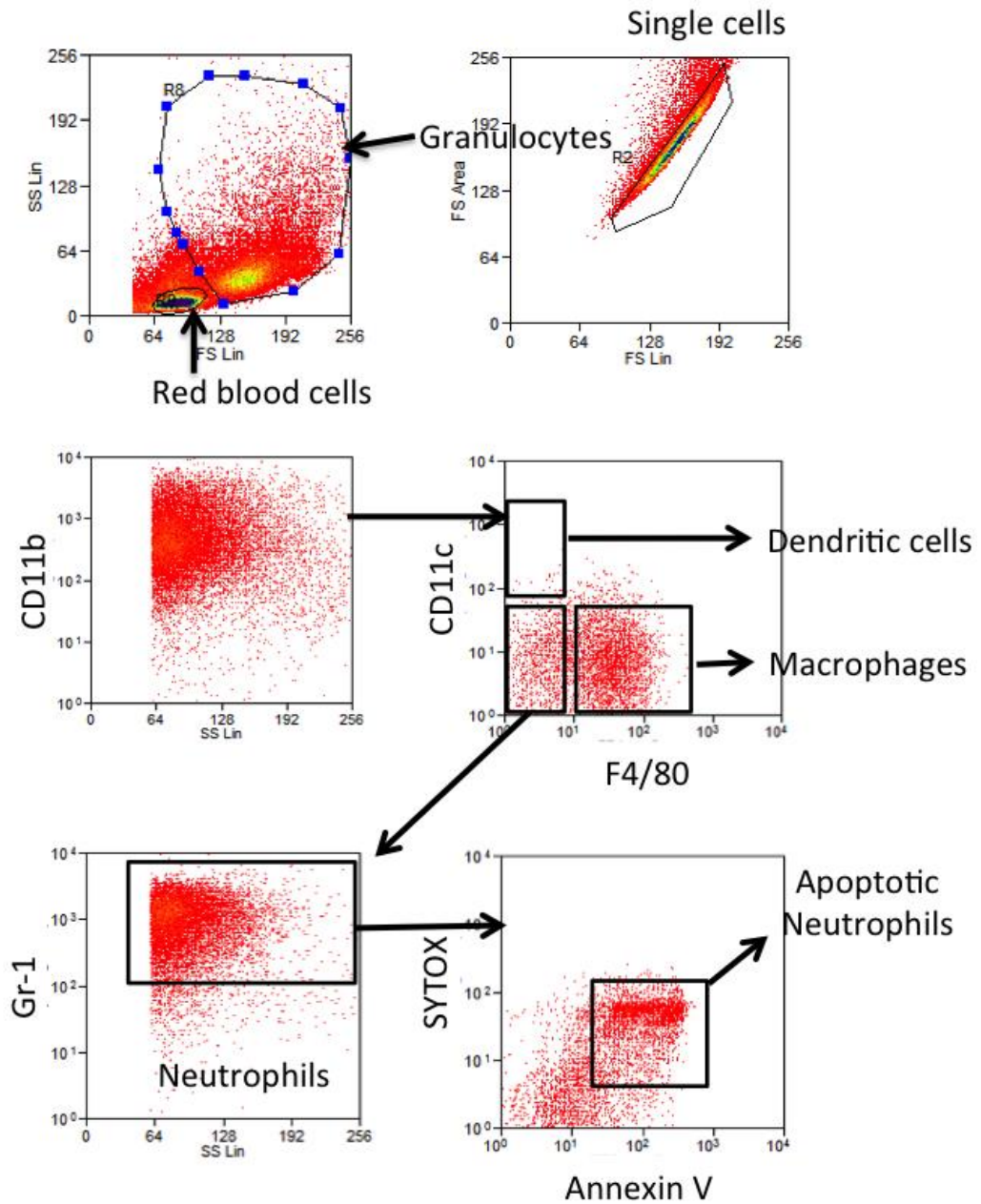


Figure 3.7: Gating strategy and flow plot for the identification of cells in
 Macrophages $CD11b^+F4/80^+ CD11c^-$; Neutrophils $CD11b^+F4/80^-CD11c^-Gr1^+$;
 Apoptosis: $Annexin V^+$ and $SYTOX^+$.

3.9.8 Ex-vivo macrophage phagocytosis assay

In brief extracted peritoneal cells underwent red cell lysis using Gibco ACK (Gibco, Invitrogen) lysing buffer 100 μ L per sample and incubated at room temperature for 5 minutes. The reaction was stopped with 1mL of cold PBS (Gibco, Invitrogen) and cells washed with PBS/BSA 2% (Gibco, Invitrogen) at 400g 4 $^{\circ}$ C for 10 minutes, resuspended in PBS/BSA 2% (Gibco, Invitrogen) to a concentration of 1×10^6 cells per mL 100 μ L added to 3x 5mL polypropylene tubes (FalconTM, Becton and Dickinson Ltd). pHrodo[®] Green labelled *Escherichia coliform* (E.Coli) bioparticles (Molecular ProbesTM, Invitrogen, Paisley, UK) were re-suspended in 2mL of Hanks' Balanced Salt Solution with 20mM HEPES pH 7.4 (Gibco, Invitrogen) and sonicated for 10minutes. Fifty microliters of the bioparticles were then added to the cells and incubated for 30 mins at 37 $^{\circ}$ C with one tube incubated on ice (to act as a negative control). Following this incubation cells were washed with PBS/BSA 2% as above. The supernatant was removed and the cell pellet re-suspended in 50mL of mouse serum, transferred to a flexiplate to Fc receptor block and stained as per cell analysis protocol above except the F4/80 antibody was now conjugated to FiTC (Clone BM8, eBiosciences, Oxford, UK) as pHrodo[®] green particles where excitation/emission is similar to that of phycoerythrin (PE). Macrophage phagocytosis of the particle results in acidic pH change resulting in fluorescence (excitation/emission 509/533nm) that was measured by flow cytometry (CyAnTM ADP, Beckman Coulter) as described above. The proportion of F4/80⁺CD11b⁺ CD11c⁻ gated cells that were also pHrodo[®] positive were presented as a phagocytosis percentage.

3.9.9 Biochemical Assays

3.9.9.1 Murine vitamin D analysis

25(OH)D₃ was measured by a commercially available direct ELISA (K2109, ImmunDiagnostik, Bensheim, Germany). 1,25(OH)₂D₃ was measured by a commercially available EIA (AC-62F1, Immunodiagnostic Systems, Tyne and Wear, UK). Calcium was measured by a commercially available colorimetric assay (ab102505, Abcam, Cambridge, UK) Dr Sian Lax (post-doctoral fellow) kindly performed all these assays as per manufacturer's protocols without deviation. All inter and intra assay CVs were reported to be below 10%. The 25(OH)D₃ direct ELISA was not validated for murine plasma and therefore underwent internal validation and was found to have an intra assay CV of 12.8% and inter assay CV of 15.6% and a mean spike recovery of 82%.

3.9.9.2 Cathelicidin related antimicrobial peptide (CRAMP) assay

Total CRAMP levels were measured in sera, BALF and PLF by an indirect ELISA derived from a method previously used for LL-37 quantification.²¹⁵ CRAMP standards of known concentrations and samples were made up to 50 µl in serum free RPMI 1640 (Sigma-Aldrich) and incubated in Greiner high binding plates (Sigma-Aldrich) overnight at 37 °C in an unsealed plate to allow adherence. Plates were washed 3 times with PBS (Gibco, Invitrogen) containing 0.05% tween-20 detergent (PBST, 0.05%) and blocked in PBST containing 1% BSA (Sigma-Aldrich) for 1 hour at room temperature with gentle agitation. Rabbit anti-CRAMP antibody (PA-CRPL-100, Innovagen, Lund, Sweden) was diluted 1:2500 in PBST (0.05%) and 100 µl added to each

well for 2 hours at room temperature. Plates were again washed 3 times with BST (0.05%) and 100µl of goat anti-rabbit horse radish peroxidase (HRP) conjugated antibody (7074, Cell Signalling technologies, Denver, USA) diluted 1:2500 in PBST (0.05%) added per well for 1 hour at room temperature. Plates were washed with PBST (0.05%) and HRP activity was measured by the addition of TMB (100µL per well). Plates were incubated with substrate for 10 minutes in the dark before the reaction was stopped by addition of 100 µl of stop solution (1M HCl). Absorbance values were obtained at 450 nm within 10 minutes, (Synergy 2 Gen 5™, Biotek). Sample concentrations were then extrapolated from the appropriate standard curve.

CRAMP levels were corrected per mg of protein and represented as ng/mg. This assay had an intra assay CV of 4.7% and inter assay CV of 14.2% with a mean spike recovery of 94.6%. A limitation of this assay is that it was only validated in sera within a range of 0.934ng/ml to 75ng/ml with no experiments performed to exclude cross-reactivity. If the upper limit is extended to 200ng/ml the intra-assay CV increased to 18% and lower limit decreased to 0.235ng/ml the CV increased to 20.34%.

3.9.9.3 Protein assay

A Bio-rad® (Hertfordshire, UK) simple colorimetric assay was used to measure total protein in sera, BALF and PLF. Bovine serum albumin (BSA, Sigma-Aldrich) standards of known concentration were made up to 10ul and plated along with 10ul of sample. Bio-Rad® solution was diluted 1 in 5 with

distilled water and 200ul added to each well which turns blue on contact with protein. Absorbance was measured immediately at 595nm (Synergy 2 Gen 5™, Biotek). Protein permeability index was calculated as a ratio fluid protein to plasma protein. This assay has previously been validated by our group and was not repeated but intra assay CV was 7.8% and inter assay CV 9.1%.

3.9.9.4 Cytokine assays

Murine serum inflammatory cytokines (IL-6, KC/CXCL-1, TNF α , MIP-2, VEGF and GM-CSF) were measured by a Luminex® screening assay (LXSAMS, R&D systems) and BAL sRAGE by a commercially purchased ELISA (MRG00, R&D systems). These assays were kindly performed with the assistance of Dr Sian Lax (Post-doctoral fellow) as per the manufacturer's protocols with no deviations and have previously been validated by our group and therefore not repeated.

CHAPTER 4

EFFECTS OF VITAMIN D DEFICIENCY ON A MURINE MODEL OF SEPSIS INDUCED LUNG INJURY

Preliminary data from this chapter have been presented and published in
abstract form:

Parekh D, Lax S, Dancer RCA, Perkins GD, Thickett D

*Vitamin D deficiency and bacterial load in a murine model of sepsis-induced
lung injury*

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4.1 INTRODUCTION

The acute respiratory distress syndrome (ARDS) is a heterogeneous syndrome that occurs in response to a variety of direct pulmonary (e.g. pneumonia and aspiration) and non-pulmonary (e.g. sepsis, trauma and blood transfusion) injurious events.^{6,216} Sepsis remains the commonest indirect cause of ARDS,¹⁷ however only 18-38% of patients with sepsis develop ARDS.²¹⁷ Patient demographics, comorbidities, smoking status and genetic factors may determine those at risk of developing ARDS.¹⁶ A large prospective study has shown patients with sepsis related ARDS were more likely to be women and to have diabetes as well as a higher disease severity, poorer recovery from lung injury, and higher mortality than non-sepsis related ARDS.²¹⁸ The same study has found age, acute physiology and chronic health evaluation (APACHE III) scores, liver cirrhosis, metastatic cancer, serum bilirubin and serum glucose to be independent predictors of ARDS mortality. Therefore identifying potential modifiable risk factors may play an important role in preventing ARDS and improving outcomes in those with established ARDS.

Worldwide vitamin D deficiency is common.²¹⁹ Epidemiological studies have demonstrated that levels of 25-hydroxyvitamin D₃ (25(OH)D₃) are related to geography and season.^{220,221} In a study of middle-aged adults in the United Kingdom, 40% had serum 25(OH)D₃ concentrations above 30ng/L (75nmol/L) in the summer months but this fell to less than 17% in the winter.¹²⁹ The incidence and mortality of sepsis is higher during the winter

months when vitamin D levels are lower.²²² Vitamin D has important pleiotropic effects on human immunity, acting as a modulator of cells of the innate and adaptive system.⁸⁵ Biologically active 1,25(OH)₂D₃ directly enhances signalling to increase the induction of antimicrobial peptides, cathelicidin (LL-37, its active form) and β-defensin by the innate immune system.²²³ CRAMP (cathelicidin related antimicrobial peptide) has been widely identified as a vitamin D dependent anti-microbial peptide that binds bacteria. Cathelicidin rapidly destroys the lipoprotein membranes of microbes enveloped in phagosomes after fusion with lysosomes in macrophages.²²⁴ Gram-positive bacteria, invasive pneumococcal disease, meningococcal disease and group A streptococcal disease are more common when 25(OH)D₃ levels are low.²²⁵ In addition to its direct antimicrobial capability, *in-vitro* and *in-vivo* studies of LL-37 suggest a broad range of activities that could modify innate inflammatory processes and adaptive immune responses by neutralising lipopolysaccharide (LPS), and acting as a chemoattractant for neutrophils, monocytes and T cells through a formyl peptide receptor.^{85,145}

Recent observational studies suggest vitamin D deficiency is common in critically ill patients, is associated with adverse outcome ²²⁶ and pre-hospital vitamin D deficiency is a predictor of sepsis and increases the risk of mortality following critical care initiation.²²⁷ Additionally, vitamin D deficiency has been associated with an increased risk of intensive care admission and mortality in patients with pneumonia.²²⁸ Severe deficiency

(<10ng/ml) before hospitalisation has also been shown to be associated with increased risk of hospital acquired infections.²²⁹ Furthermore 2 recent meta-analyses support the association of vitamin D deficiency with increased susceptibility for severe infection, sepsis and mortality in the critically ill.^{230,231}

Animal studies to date investigating the role of vitamin D deficiency and its supplementation in ARDS and sepsis have conflicting results. In a rat model of abdominal sepsis pre-treatment with 1,25(OH)₂D₃ reduced thrombocytopenia²³² and disseminated intravascular coagulation in a LPS model of sepsis.²³³ 1,25(OH)₂D₃ inhibited neutrophil but not monocyte recruitment by 40% in a hamster model of ARDS, thought to be in part due to its inhibitory effects on IL-8.¹⁷⁵ It also ameliorated lung damage secondary to ischaemia reperfusion injury after femoral artery ligation in rats.¹⁷⁶ A further study has shown reduced interstitial inflammation and collagen deposition post radiotherapy in rats administered vitamin D.¹⁷⁷ We have shown in a murine intra-tracheal LPS model of lung injury that vitamin D deficiency leads to exaggerated lung cellular inflammation and epithelial damage resulting in reduced oxygenation.¹⁹⁸ Conversely, a recent study of LPS induced lung injury in vitamin D deficient mice found no difference in the degree of lung injury.¹⁸⁰ Issues centred on differing aetiologies of lung injury as well as methodology of when lung injury is measured or defined may go some way to explaining this apparent contradiction.

Murine models of lung injury albeit imperfect are invaluable in investigating the mechanism of disease and their potential therapies. In conjunction with translational studies and human disease models they can form the basis of strong evidence to pursue novel therapies and targets. Unfortunately, no single murine model of lung injury represents the complex heterogeneous pathophysiology of human ARDS.¹⁰⁰ An ideal model would feature a triad of 1] evidence of neutrophilic alveolitis and alveolar inflammatory response; 2] deposition of hyaline membranes or evidence of disruption of the alveolar/capillary membrane and 3] formation of microthrombi, indicating the presence of endothelial injury.²³⁴ We aimed to investigate the role of vitamin D deficiency on sepsis and sepsis induced lung injury. For this reason we chose the caecal ligation and puncture (CLP) model of sepsis. This is recognised as a good murine polymicrobial model of human sepsis²³⁵ that in the acute phase increases epithelial permeability, with increased neutrophil accumulation in the interstitium and alveolar spaces.¹⁰⁰ Although it does not represent all the features of ARDS it provided us with a model to investigate the effects of vitamin D deficiency in both conditions at the same time. Furthermore, since murine cathelicidin related antimicrobial peptide (CRAMP) is homologous to its human counterpart would provide evidence of the functional importance of vitamin D deficiency if these CRAMP levels are suppressed.

The observational and experimental studies presented suggest that vitamin D deficiency is a determinant of sepsis and ARDS. The aim of this study was to

establish if vitamin D deficiency influences innate immune responses and exaggerates sepsis induced lung injury in a murine model.

4.2 MATERIALS AND METHODS

Detailed methods are provided in section 3.9

4.2.1 Ethical statement and study design

All procedures were performed in accordance with UK laws under the Animal [Scientific Procedures] Act 1986 (project licence code 30/2836; personal licence number I77F587B4). Local policies and procedures were followed as described in section 3.9.1 and study design as per section 3.9.2. The priori study design was to assess if inducing vitamin D deficiency exaggerates sepsis and sepsis- induced lung injury secondary to CLP. Pre-defined measurements were lung cellular recruitment and protein permeability index, bacterial numbers and cytokine measurements. Secondary measurements of macrophage phagocytosis and antimicrobial peptide were performed to subsequently investigate the mechanism of exaggerated inflammation and sepsis.

4.2.2 Induction of vitamin D deficiency

Male wild-type mice (WT) C57Bl/6 (aged 3-4 weeks) were obtained from Harlan UK Limited, Oxford, UK and maintained at the Biomedical Servicing Unit (BMSU), Birmingham University, UK. Vitamin D deficiency was induced in WT pups by feeding them a vitamin D deficient chow (TD 89123, Harlan, USA) or sufficient chow for 6 weeks. All animals were housed in cages of 6 in a 12-hour light and 12-hour dark cycle with food and water provided *ad libitum*.

4.2.3 Surgical procedure

Detailed methods are described in section 3.9.4. In brief, pre medication was given in the form of subcutaneous buprenorphine (Temgesic®, Reckitt Benckiser, Wallisellen, Switzerland at, Animalcare Ltd, 0.1mg/kg body weight) prior to the procedure. Animals were anaesthetised and maintained with isoflurane gas and oxygen. All surgery was performed with aseptic techniques. Midline laparotomy was performed followed by exposure of the caecum, ligation of the lower 30% and single through-and-through puncture of ligated caecum. A small amount of faeces was gently expressed by compressing the ligated caecum prior to being placed back into the abdomen and wound closed with non-absorbable sutures. All animals were recovered in heat boxes and recovery incubators until sacrificed. Postoperative resuscitation was with 0.5mls of fluid (Hartman's solution, Aquapharm 11®) and a further dose of buprenorphine. Animals were reassessed 6 hours post-operatively and given a further 0.5mls of fluid and sacrificed at 16 hours post surgery. Sham surgeries were identical except for the lack of ligation and puncture of the caecum after exteriorisation from the abdomen and therefore deemed sterile.

4.2.4 Sample collection and processing

At 16 hours post CLP animals were deeply anaesthetised with 5% isoflurane. Cardiac puncture was performed and death confirmed. Immediate post-mortem peritoneal and bronchoalveolar lavage was performed (as described in section 3.9.5) and the retrieved lavage fluid (PLF and BALF) transferred on

ice to the laboratory for further processing and analysis as described in section 3.9.5. Post mortem examination of the caecum was carried out to confirm necrosis and colour of liver recorded to ensure adequate sepsis was induced.

4.2.5 Bacterial culture

Whole blood, PLF and BALF were diluted accordingly and incubated at 37°C in pre-prepared agar plates as described in section 3.9.6. Colony forming units (CFU) were counted at 24 hours and CFU/ml calculated from the original dilutions and total starting volumes of samples retrieved.

4.2.6 Cell analysis

BALF and PLF was analysed for cellular inflammation by differential cell count, neutrophil count and the proportion of neutrophil apoptosis by cell surface staining and flow cytometry as described in section 3.9.7

4.2.7 Protein assay

A simple colorimetric assay (Bio-rad®, Hertfordshire, UK) to measure total protein in sera, BALF and PLF was performed as described in section 3.9.9.3. Protein permeability index was calculated as a ratio of fluid protein to plasma protein.

4.2.8 Cytokine assays

Inflammatory cytokines were measured by luminex array (R&D systems, Abingdon, UK) as per manufacturer's protocols. BAL RAGE was measured by a commercially purchased ELISA (R&D systems, Abingdon, UK). Detailed methods are described in section 3.9.9.4.

4.2.9 Ex-vivo phagocytosis assay

Detailed methods can be found in section 3.9.8. In brief after undergoing red cell lysis extracted peritoneal cells were plated into a microplate and pHrodo® labelled *Escherichia coliform* (*E.Coli*) bioparticles added and incubated for 30 mins at 37°C with a 4°C negative control. Phagocytosis of the particle results in acidic pH change resulting in fluorescence that was measured by flow cytometry.

4.2.10 Cathelicidin related antimicrobial protein (CRAMP) assay

Total CRAMP levels were measured in sera, BALF and PLF by an indirect ELISA as described in section 3.9.9.2. CRAMP levels were corrected to per mg of protein and represented as ng/mg.

4.2.11 Animal numbers

Animal numbers were minimised by the consistent use of the C57Bl/6 strain, age and sex of animals, lung injury interventions to allow comparisons across wild type (WT) mice and measurements of multiple outcomes in the same animal. Precise power calculations were not available for the 16 hours CLP

time point and therefore initial power calculations for 8 animals in each arm (control; deficient; sufficient; sham) were based upon preliminary data (verbal communication with Professor David Thickett) of increased lung permeability index 24 hours after CLP of 0.46mg/ml allowing the detection of a change of 0.09 (20%) between vitamin D sufficient and deficient mice. For BALF % neutrophils (mean 68% (SD10.1)) treatment effect would be 15.2% at a power of 0.8 $p=0.05$. To account for a failure rate and procedure optimisation a total number of 50 mice were accounted for this study. A failure rate of 10% was anticipated and accounted for the severity of the model and requirement to cull the animal perioperatively due to animal welfare as well as technical failure in retrieving lavage samples. Experiments were performed in batches of 4 mice per arm in view of lack of precise power calculations to minimise and justify animal use. This was un-blinded. Surgery was not repeated if a result was clearly significant or not to minimise animal numbers. Results that have $n=12$ account for $n=4$ CLP performed for phagocytosis experiments which allowed for cumulative analysis of bacterial, CRAMP and cell quantification in mice that had already undergone surgery maximising experiment sample utilisation. BALF cell analysis was not repeated after $n=8$ as no lung injury was demonstrated in the first experiments. Uneven numbers account for a failure in the experiment for reasons described above.

4.2.12 Statistical analysis

Statistical analysis was performed on GraphPad Prism 6.0[©] software (GraphPad Software, La Jolla, California, USA) Normality was assessed with the D'Agostino-Pearson omnibus test. Parametric data were analysed using t-tests. Non-parametric data were analysed using Mann-Whitney tests and Kruskal Wallis ANOVA and Dunn's test for multiple comparisons. A two-tailed p-value < 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Murine vitamin D status

Vitamin D deficiency (VDD) was successfully established in mice fed a deficient diet compared to a vitamin D sufficient (VDS) diet (Table 4.1). The magnitude of deficiency is similar to that previously reported by our group in patients with ARDS.¹⁹⁸ Deficiency did not result in a significant effect on serum calcium but was associated with reduced circulating bioactive 1,25(OH)₂D₃ concentration.

	VDD (n=8)	VDS (n=7)	p-value
25(OH)D₃ (nmol/L)	7.9 (4.5-9.4)	50.4 (48.1-51.9)	0.0003
1,25(OH)₂D₃ (pg/ml)	13.4 (7.3-18.9)	150.5 (125.0-175.3)	0.002
Calcium (mM)	3.4 (3.1-4.1)	3.3 (3.1-3.4)	0.69

Table 4.1: Effects of vitamin D deficient diet on circulating vitamin D levels.

Data presented as medians (interquartile range); VDD (vitamin D deficient); VDS (vitamin D sufficient); p-value Mann-Whitney test

4.3.2 Bacterial load

VDD mice had a significantly higher bacterial load compared to VDS in all three compartments: PLF, blood, and (BALF) as measured by CFU/mL 16 hours after CLP. PLF median [IQR] 856×10^3 [136×10^3 – 237×10^7] vs. 27.1×10^3 [3.15×10^3 – 1.27×10^7], $p=0.037$; blood median [IQR] 132×10^3 [5.96×10^3 – 758×10^3] vs. 0.55×10^3 [0.0 – 6.67×10^3], $p=0.019$ and BALF median [IQR] 2.51×10^3 CFU per mL [0.93×10^3 – 19.2×10^3] vs. 0.48×10^3 [0.0 – 1.71×10^3], $p=0.011$ (Figure 4.1). In sham experiments there was an absence of bacteria as measured by CFU/ml in all 3 compartments confirming a sterile procedure and surgery (data not shown).

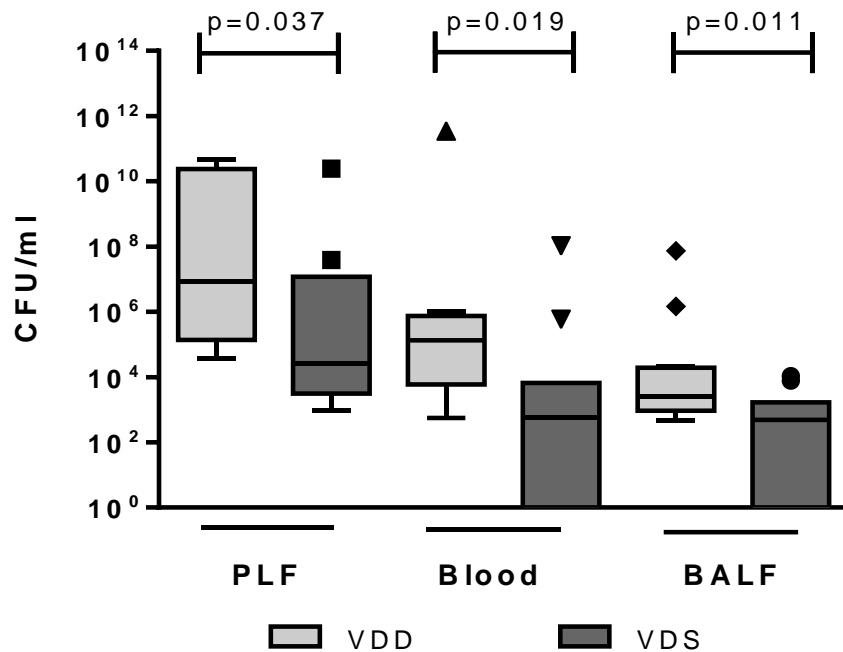


Figure 4.1: Effect of VDD on bacterial load in PLF, blood and BALF

Data presented as box and whisker plots with median and Tukey's distribution, logarithmic scale to allow graphical representation. VDD (vitamin D deficient) n=12; VDS (vitamin D sufficient) n=11; p-values: Mann Whitney tests.

4.3.3 Cathelicidin related antimicrobial peptide (CRAMP)

The CLP procedure increases CRAMP levels significantly in PLF, serum and BALF in VDS mice. However, significantly lower levels were observed in VDD mice supporting the observation that VDD mice have reduced anti-microbial capacity (Figure 4.2). Levels corrected for protein were higher in vitamin D sufficient mice in BALF and serum but this did not reach significance in PLF. PLF VDD median [IQR] 26.7ng/mg [22.1 – 36.9] vs. VDS 30.6ng/mg [16.5 – 48.9], p=0.684; BALF VDD median [IQR] 2.74ng/mg [0 – 11.47] vs. VDS 12.98ng/mg [7.36 – 20.9], p=0.007; Serum VDD median [IQR] 0.01ng/mg [0 – 0.05] vs. VDS 0.17ng/mg [0.03 – 0.47], p=0.02.

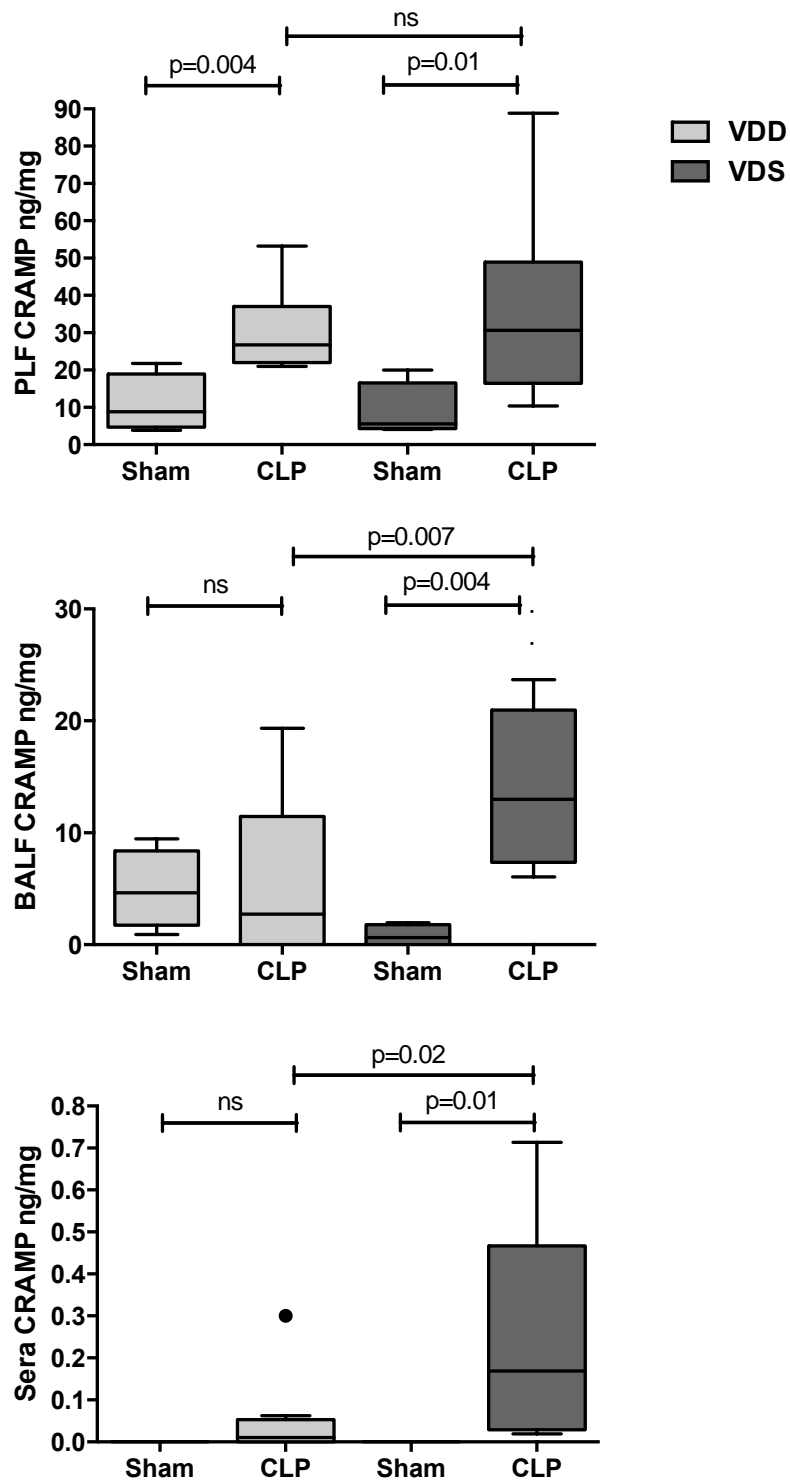


Figure 4.2: CRAMP (murine cathelicidin) expression in PLF, BALF and serum.

Box and whisker plots with medians and Tukey's distribution. VDD (vitamin D deficient) n=12; VDS (vitamin D sufficient) n=11. Sham n=4 per group. CRAMP was undetectable in sham treated sera.

4.3.4 Alveolar cellular inflammation

Little-to-no cellular recruitment into the alveolar compartment was observed at this time point (Figure 4.3A-C) however, there was evidence of a mild increase in BALF protein permeability index (PPI), suggesting early alveolar epithelial leak. This was significantly higher in VDD mice as compared to VDS mice (median [IQR] 3.30 [2.69-4.64] vs. 2.09 [1.82-2.90], $p=0.014$) (figure 4.3D).

CLP increased the number of alveolar macrophages in proportion to other cells but there was no difference between VDD and VDS groups. Neutrophil cellular recruitment in the BALF was low and therefore quantification of apoptosis and necrosis of neutrophils was not possible.

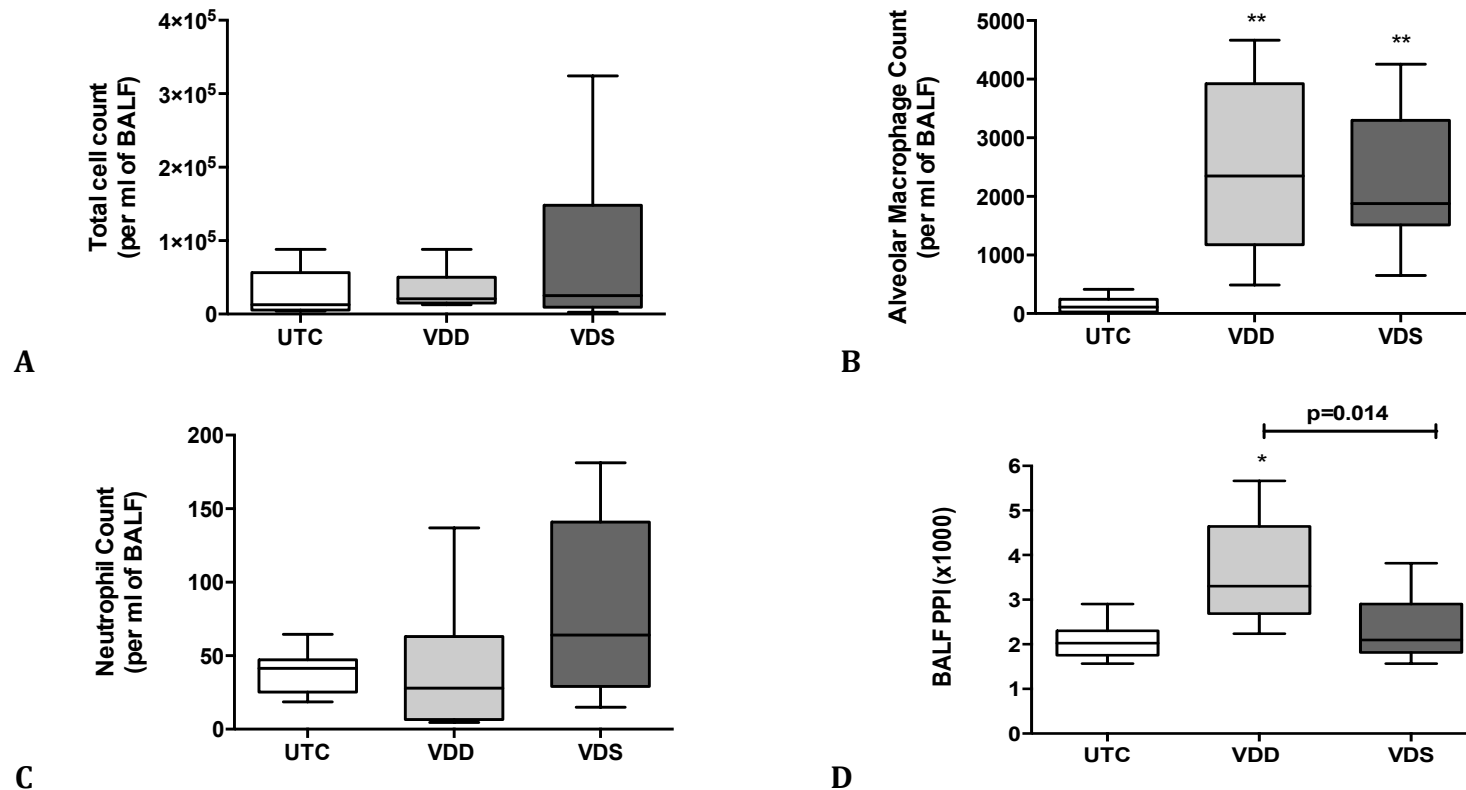


Figure 4.3: BALF cellular recruitment and protein permeability index at 16 hours post CLP

Box and whisker plots with median and Tukey's distribution. Kruskal-Wallis ANOVA and Dunn's multiple comparison test. **A]** Total cell count, $p=0.57$; **B]** neutrophil count, $p=0.33$ and **C]** alveolar macrophage count, $p=0.001$. **D]** BALF protein permeability index (PPI). VDD (vitamin D deficient) $n=8$; VDS (vitamin D sufficient) $n=7$; UTC (untreated WT control) $n=10$ in each group. * $p<0.05$; ** $p<0.005$ compared to UTC

4.3.5 Peritoneal cellular inflammation

Post CLP there was significant cellular recruitment in PLF (Figure 4.4A). As major players of the acute inflammatory response, neutrophils and F4/80+ macrophages were enumerated within the peritoneal cavity. Sham experiments did not significantly increase cellular infiltration. Significantly more neutrophils (Figure 4.4B) and F4/80+ macrophages (Figure 4.4C) were observed in VDD compared to VDS mice post CLP, with the neutrophil to macrophage ratio similar between both groups indicating a global increase in inflammatory mediators (Figure 4.4D). PLF PPI was also increased in VDD mice this did not reach statistical significance (median [IQR] 46.86 [28.17-58.33] vs. 29.81 [14.81-54.52], $p=0.06$) that may be suggestive of more vascular damage in VDD mice post CLP (Figure 4.4E). However this increase in protein in the peritoneal compartment may also be due to the CLP procedure itself as our protein assay did not specifically measure a plasma protein such as albumin or IgM.

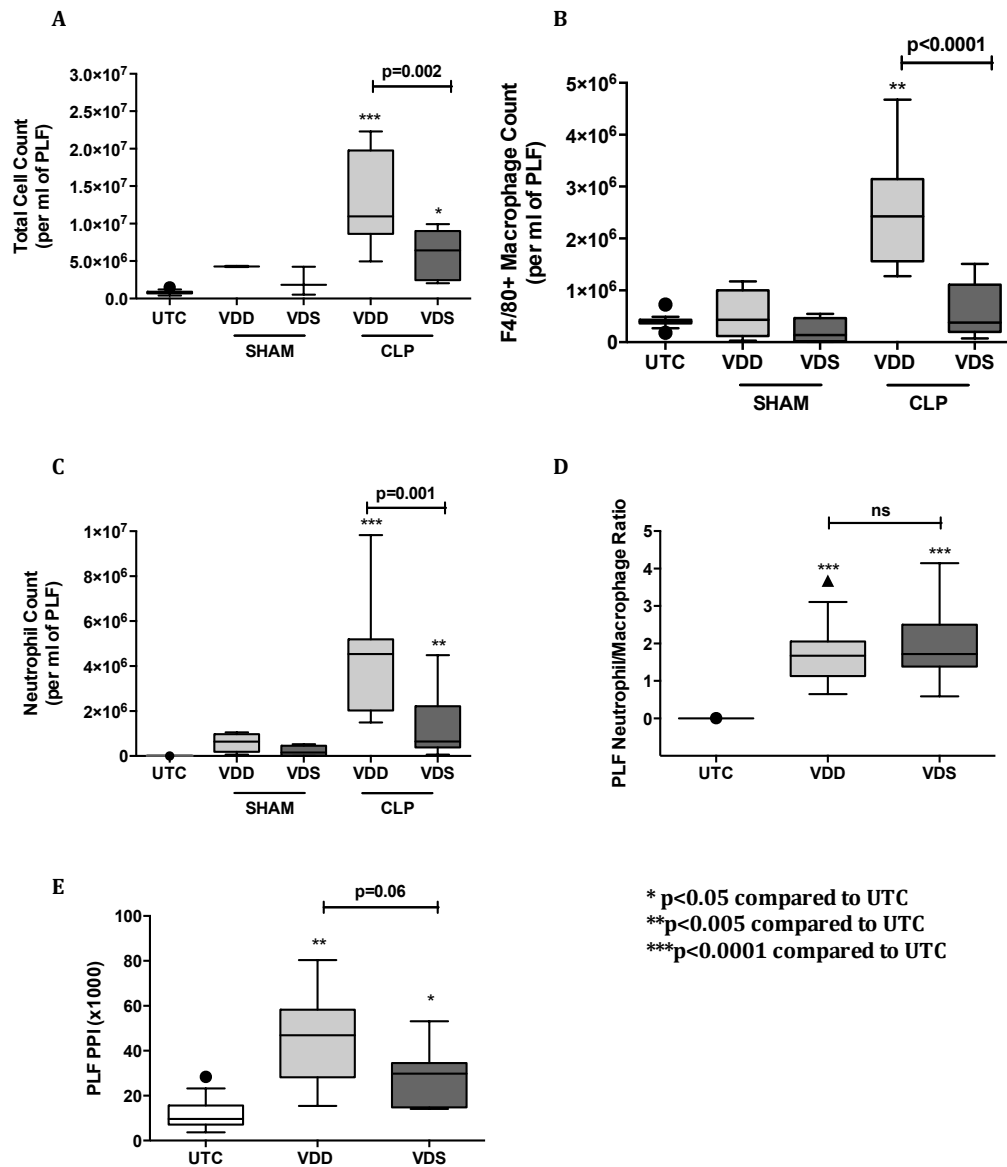


Figure 4.4: PLF cellular recruitment and protein permeability 16 hours post CLP.

Box and whisker plots with median and Tukey's distribution. Kruskal-Wallis ANOVA with Dunn's multiple comparisons. **A]** total cell count $p<0.0001$; **B]** neutrophil count $p<0.0001$ and **C]** macrophage count $p<0.0001$). **D]** PLF neutrophil/macrophage ratio. **E]** PLF protein permeability index (PPI). VDD (vitamin D deficient) $n=12$; VDS (vitamin D sufficient) $n=11$; UTC (untreated WT control) $n=12$

4.3.5.1 Neutrophil Apoptosis

There was a significant increase in the number of apoptotic neutrophils in VDD compared to VDS peritoneal lavage fluid (median [IQR] 1.87×10^5 [$0.89 - 4.19 \times 10^5$] vs. 0.51×10^5 [$0.20 - 0.54 \times 10^5$], $p=0.007$) (Figure 4.5), suggesting dysregulated neutrophil apoptosis and clearance of dying cells.

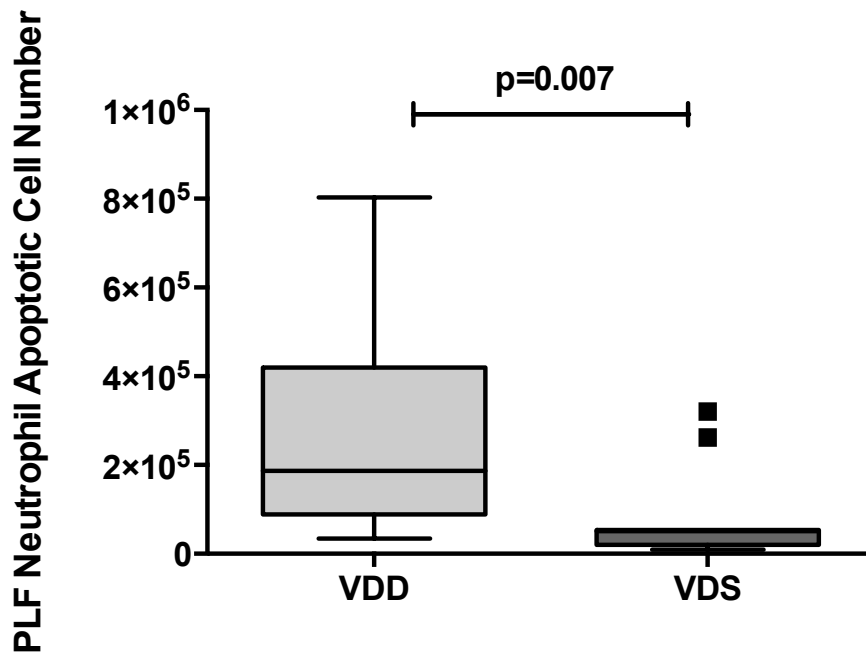


Figure 4.5: Peritoneal lavage fluid (PLF) neutrophil apoptosis.

Box and whisker plot with median and Tukey's distribution, VDD (vitamin D deficient) $n=12$; VDS (vitamin D sufficient) $n=11$. p -value; Mann Whitney test.

4.3.6 Macrophage phagocytosis

To determine whether the increased bacteraemia and/or accumulation of apoptotic neutrophils observed in VDD mice was due to impaired clearance by peritoneal macrophages, we assessed bacterial phagocytosis following CLP. Ex vivo phagocytosis of pHrodo labelled *Escherichia coliform* bacteria (E.Coli.) was significantly reduced in F4/80+ macrophages isolated from PLF of VDD compared to VDS mice following CLP (median [IQR] 6.89% [3.12 – 9.87] vs. 21.12% [17.56 – 24.29], $p=0.029$) (Figure 4.6).

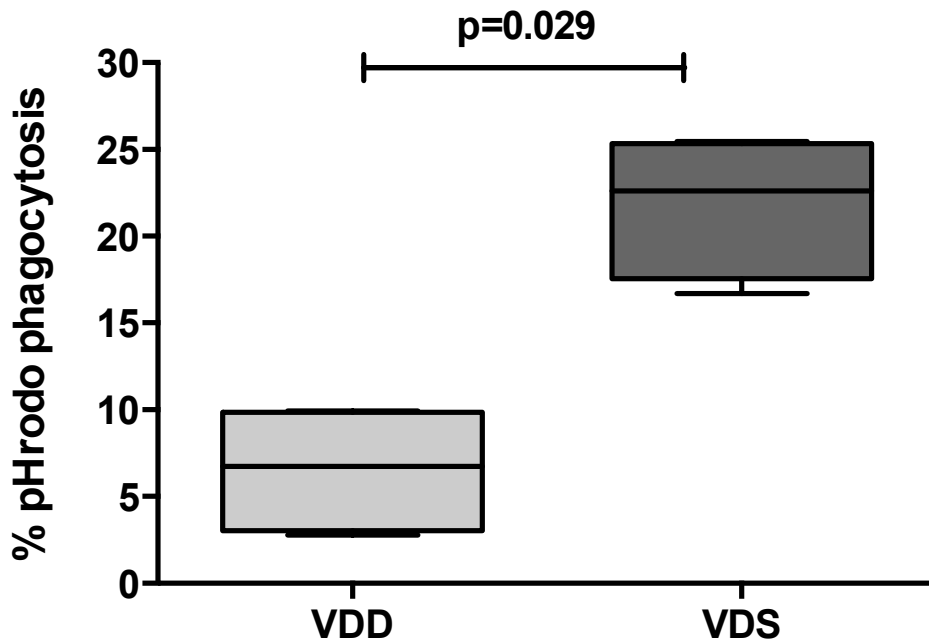


Figure 4.6: Peritoneal lavage fluid (PLF) phagocytosis of pHrodo[®] labelled *E.Coli* bacteria.

Box and whisker plot with median and Tukey's distribution. VDD (vitamin D deficient) $n=4$; VDS (vitamin D sufficient) $n=4$. p -value: Mann Whitney test.

4.3.7 Biomarkers of inflammation

There was no difference seen in inflammatory cytokines in PLF (Table 4.2), BALF (Table 4.3) or sera (Table 4.4) between VDD and VDS groups. BAL soluble receptor for advanced glycation products (sRAGE) concentrations were not significantly raised compared to untreated controls (UTC median [IQR] 1854pg/ml [1365 - 2219] vs. VDD median [IQR] 686.3pg/ml [245.8 - 952] vs. VDS median [IQR] 1301pg/ml [662.8 - 3066], p=0.09).

	VDD (n=8)	VDS (n=7)	p-value
IL-6 (pg/ml)	4054 (1872 – 8000)	4181 (521.5 – 7107)	0.61
KC/CXCL-1 (pg/ml)	1056 (597.8 – 1793)	1603 (81.8 – 4146)	0.90
TNF- α (pg/ml)	16.2 (4.64 – 55.7)	6.61 (2.12 – 9.82)	0.29
IL1 β (pg/ml)	181.4 (86.8 – 309.7)	74.4 (52.6 – 150)	0.12
VEGF (pg/ml)	120.1 (58.5 – 149.5)	107.1 (59.3 – 139.7)	0.60

Table 4.2: PLF biomarkers of inflammation

Data presented as median (interquartile range), Mann-Whitney U tests.

	VDD (n=8)	VDS (n=7)	p-value
IL-6 (pg/ml)	12.9 (5.7 – 14.0)	19.1 (5.8 – 23.4)	0.52
KC/CXCL-1 (pg/ml)	52.1 (15.15 – 142)	155.0 (62.3 – 256.6)	0.23
TNF- α (pg/ml)	2.34 (0.74 – 3.28)	1.25 (0.81 – 4.20)	0.75
MIP-2 (pg/ml)	6.95 (0 – 17.9)	15.1 (2.33 – 40.2)	0.41
VEGF (pg/ml)	167.6 (115.1 – 201.7)	185 (102.0 – 227.4)	0.91
GM-CSF (pg/ml)	1.19 (1.02 – 1.27)	1.11 (1.02 – 227.4)	0.54

Table 4.3: BALF biomarkers of inflammation

Data presented as median (interquartile range), Mann-Whitney U tests.

	VDD (n=8)	VDS (n=7)	p-value
IL-6 (pg/ml)	1561 (212.8 – 9894)	3284 (1171 – 21,089)	0.39
KC/CXCL-1 (pg/ml)	12,476 (2322 – 45,200)	13,164 (10,457 – 45,200)	0.86
TNF- α (pg/ml)	17.1 (7.18 – 53.9)	19.2 (9.32 – 52.5)	0.44
MIP-2 (pg/ml)	13.7 (10.9 – 16.2)	14.1 (13.6 – 16.2)	0.84
VEGF (pg/ml)	700 (162.1 – 2322)	2291 (530 – 4244)	0.33
GM-CSF (pg/ml)	3.94 (2.80 – 18.4)	4.92 (4.08 – 7.52)	0.44

Table 4.4: Serum biomarkers of inflammation

Data presented as median (interquartile range), Mann-Whitney U tests.

4.4 DISCUSSION

In this study we sought to establish whether inducing vitamin D deficiency by diet prior to injury in mice leads to exaggerated sepsis, lung injury and enhanced cellular inflammation/dysfunction. In the clinically relevant CLP model of early sepsis, VDD was associated with exaggerated bacterial growth in the peritoneal cavity, elevated systemic bacteraemia as well as increased bacterial translocation to the alveolar compartment. This was associated with abnormal protein permeability of the peritoneal and alveolar capillary barrier. In the PLF, there was exaggerated cellular inflammation in VDD mice with evidence of impaired antibacterial responses in terms of CRAMP release and the ability of peritoneal macrophages phagocytosis of *E.coli*. These cellular changes resulted in increased accumulation of apoptotic neutrophils in the PLF. These data support our hypothesis that VDD is mechanistically important in driving sepsis.

We successfully established severe deficiency in the mice, with levels of 25(OH) D₃ similar to those who have ARDS.¹⁹⁸ This deficiency was reflected also in reduced circulating 1,25(OH)₂D₃, the bioactive form of vitamin D.

Previous animal studies have utilised different experimental models of lung injury to investigate the role of vitamin D replacement with varying results.^{175,176} However, there is only one study to our knowledge that has shown a benefit of 1,25(OH)₂D₃ treatment on sepsis induced coagulopathy in a CLP model in rats.²³² Our group has recently shown a detrimental effect of

vitamin D deficiency with exaggerated lung injury and reduced oxygenation in a intra-tracheal LPS direct murine lung injury model.¹⁹⁸

Vitamin D is known to be a stimulator of antimicrobial peptides¹⁴³ with human LL-37 (cathelicidin) and β -defensin-2 expression dependent on circulating levels of 25(OH)D₃.¹⁴⁴ This study confirms that deficiency in vitamin D results in lower systemic and local circulating levels of CRAMP in the lung and blood. This confirms the findings of Jeng *et al.* of lower levels of LL-37 in vitamin D deficient patients on the intensive care unit with sepsis.²³⁶ This has been recently confirmed in a further prospective study of critically ill patients in which lower 25(OH)D₃ levels were associated with decreased cathelicidin peptide and 90-day mortality.²³⁷ In our study this difference was not as pronounced in the PLF and may be accounted for by overwhelming bacteraemia in the peritoneum. However an increased concentration of CRAMP may be one explanation for overall reduced bacteria in VDS mice.

Vitamin D₃ has been shown to have the ability to stimulate the differentiation of premature monocytes to mature phagocytic macrophages.¹⁵⁰ Macrophages are the 'first responder' cells of the innate immune system and are recruited to the site of infection forming the first line of defence towards pathogens. Vitamin D deficiency is known to rescue the phagocytic potential of macrophages to mycobacteria²³⁸. We have shown that macrophage phagocytosis of labelled *E.Coli* is reduced in vitamin D deficient mice post

CLP. This may be a second reason to account for the increased bacterial numbers seen in the deficient mice.

Protein permeability index was increased in the lung 16 hours post CLP in VDD mice suggesting a breakdown in epithelial-endothelial integrity and protein leak that is consistent with the onset of early lung injury. However unfortunately at 16 hours post injury there was no evidence of neutrophil alveolitis, a hallmark of ARDS. There was also no rise in BALF sRAGE, a marker of Type 1 epithelial cell damage compared to untreated control mice. Taken together it can be concluded that 16 hours post CLP does not replicate all the features of human ARDS and is not an ideal model to study the disease.

Although BALF cellular recruitment did not occur at this time-point there was a significant influx of cells to the peritoneum. Despite a larger number of macrophages and neutrophils recruited to the PLF in VDD mice, the overall neutrophil to macrophage ratio remained the same between both groups. Interestingly the number of apoptotic neutrophils in the VDD PLF was significantly higher, suggesting dysregulated neutrophil apoptosis or delayed clearance. Both of which could be important mechanistically in both ARDS and sepsis. The resolution of inflammation is dependent on the action of macrophages to ingest apoptotic neutrophils, a process termed efferocytosis, and prevent the release of their pro-inflammatory intra-cellular contents during secondary necrosis.⁹⁵ Furthermore, uptake of apoptotic cells is part of

an anti-inflammatory process⁹⁶ and there is evidence that a defect in the process of efferocytosis is a cause of on-going inflammation in ARDS⁹⁷⁻⁹⁹.

Vitamin D₃ is believed to be anti-inflammatory and 1,25(OH)₂D₃ has been shown to reduce the production of inflammatory cytokines and chemokines (IL-8 and CXCL-10) from stimulated epithelial cells by modulating NF-kb signalling.^{155,156} We did not find a difference in the inflammatory cytokine milieu between vitamin D sufficiency and deficiency in this study. This could be partly explained by low n numbers.

Recent studies have shown an association between vitamin D deficiency and incidence of sepsis^{230,231} with a suggestion that vitamin D deficiency may be a predictor of sepsis and increases mortality.²²⁷ Bacteraemic sepsis is more common in winter months when vitamin D levels are lowest.²²² Sepsis remains the most common cause of indirect ARDS and vitamin D deficiency is common in the critically ill.²²⁶ To date, human studies have indicated an association however direct causality is yet to be proven, as vitamin D levels may also be influenced by being critically ill and may itself, be a marker of severity of illness. This study confirms that vitamin D deficiency is a predeterminant of sepsis and mechanistically provides evidence that the deficiency is of functional importance.

The major limitation of this study was the CLP time point. In the UK, the regulatory framework for animal experiments dictated that we could not keep VDD animals alive post-CLP for more than 16 hours due to serious

adverse events (increased severity of illness and mortality seen at 24 hours in VDD mice in our preliminary experiments) so we were only able to model early sepsis using this technique and unable to assess if the effects seen could be abrogated by vitamin D₃ replacement therapy. However, a limitation of this study is the absence of pre-treatment, restoration of vitamin D sufficiency. Ideally, lung histology would have been extremely informative to assess interstitial neutrophil accumulation, alveolar epithelium and space. However this facility and expertise was not available. Finally the protein assay performed was not specific to a large plasma protein and measured total protein and therefore not specific for vascular leak and may in the peritoneum be confounded by the puncturing of the bowel in the CLP procedure that could be improved by measuring lavage albumin or IgM.

In conclusion, this study confirms that pre-existing vitamin D deficiency may be a modifiable risk factor of sepsis and identifies increased antimicrobial peptide, decreased macrophage phagocytosis of bacteria, dysregulated apoptosis and or clearance and decreased barrier integrity as potential cellular mechanisms that may account for the associations seen in clinical studies.

4.5 ACKNOWLEDGEMENT

Murine cytokine assays performed by Dr Sian Lax and CRAMP assay assisted by Dr Aaron Scott. Many thanks to Dr Sian Lax for teaching me all the techniques performed in this Chapter.

CHAPTER 5
THE VITAMIN D TO PREVENT ACUTE LUNG INJURY
FOLLOWING OESOPHAGECTOMY TRIAL

5.1 INTRODUCTION

The acute respiratory distress syndrome (ARDS) remains an important cause of morbidity and mortality in the critically ill patient.^{2,3} Preclinical trials of pharmacological therapies have shown promise but failed to demonstrate clinical efficacy in treating established ARDS.^{6,48} Early recognition of patients at risk of and preventing ARDS has become a focus of research in recent years,^{239,240} with the development of prediction scores^{43,241} prevention trials^{44,51} and a growing evidence of other potential biological therapies to prevent ARDS.²⁴⁰

ARDS is common after oesophagectomy,¹⁰⁵ which is used to treat oesophageal cancer. Access to the oesophagus during surgery is attained by deflation of one lung and maintenance of one-lung ventilation (OLV). This exposes the ventilated lung to oxidative stress and high inflation pressures risking the development of ventilator-induced lung injury (VILI).¹¹⁵ Ventilation with higher tidal volumes and peak pressures not only cause the alveoli to overstretch in a process called volutrauma but also cause biotrauma leading to recruitment of neutrophils and local production and release of inflammatory mediators,²⁴² which can spill over into the systemic circulation.^{243,244} In the perioperative setting, mechanical ventilation itself can induce an inflammatory response²⁴⁵ which can compound the local and systemic response induced by major surgery and contribute to the inflammatory cascade that leads to the development of lung injury and systemic organ failure.²⁴⁶ High inspired oxygen leads to increased reactive

oxygen species (ROS) production²⁴⁷ which causes alveolar cellular dysfunction and damage.^{248,249} There is also evidence to suggest a synergistic effect of hyperoxia and high tidal volume ventilation with increased alveolar-capillary permeability, neutrophil accumulation and pro-inflammatory mediator release.^{250,251} Concurrently the deflated lung is exposed to a period of ischaemia followed by reperfusion which causes endothelial damage, release of ROS and inflammatory mediators²⁵² and is associated with postoperative ARDS.¹¹⁵ Furthermore, evidence suggests that the pathophysiological changes of lung injury post oesophagectomy are similar to that of ARDS caused by other precipitants¹¹⁵ – with increased pulmonary capillary permeability¹¹², neutrophilic alveolitis,^{116,253} and release of plasma²⁵⁴ and pulmonary¹¹⁰ pro-inflammatory cytokines. To this effect, the model has already been utilised in a clinical trial to assess the effect of salmeterol on preventing lung injury post oesophagectomy.⁵¹

Studies report a high prevalence of vitamin D deficiency in the critically ill¹³⁴ with an association with increased rates of infection,^{227,229} acute respiratory failure,¹⁹⁷ acute kidney failure²⁵⁵ and mortality.²²⁶ Vitamin D is an activator of immune responses that may play a role in the pathogenesis of ARDS.⁸⁵ In humans, 25-hydroxyvitamin D₃ (25(OH)D₃) bound to its binding protein (DBP) is the most abundant circulating metabolite which requires hydroxylation to the active 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] by the mitochondrial enzyme 25(OH)D-1-alpha (CYP27B1).¹¹⁸ Its target, the vitamin D receptor (VDR) as well as CYP27B1 are now known to be

expressed by cells of the innate and adaptive immune system as well as by respiratory epithelial cells.¹⁷⁴ We have recently shown that patients with ARDS post-oesophagectomy have lower levels of 25(OH)D₃ (ARDS 16.97 nmol/L vs no ARDS 25.46 nmol/L, p=0.014) and severe deficiency of less than 20nmol/L was associated with an increased risk of ARDS (OR=4.2 (95% CI 1.13 to 15.9; p=0.032) after adjustment for gender, age, diagnosis, staging data, and smoking pack-years.¹⁹⁸ Severe deficiency was also associated with increased markers of alveolar epithelial damage. There are currently no human studies investigating the role of vitamin D replacement to treat or prevent ARDS.

The primary aim of this study was to determine if pre-treatment with a single high dose of vitamin D is safe and effective and investigate its effects on biomarkers of early acute lung injury and other clinical outcomes post oesophagectomy, in a group of patients at high risk of developing the condition.

5.2 MATERIALS AND METHODS

5.2.1 Trial Conduct

The vitamin D treatment to prevent lung injury post-oesophagectomy (VINDALOO) trial was a randomised double blind, placebo-controlled trial. Patients were recruited from October 3, 2012 until January 26, 2015 at three large tertiary hospitals in the United Kingdom. National research ethics was approved by South Birmingham Research Ethics Committee (REC 12/WM/0092). The trial is registered on the International Standard Randomised Controlled Trial Registry (ISRCTN27673620) and European Union database of randomised Controlled Trials (2012-000332-25). The study was conducted in accordance with the trial protocol which has previously been published¹¹⁶ (Appendix).

5.2.2 Patient recruitment

Patients were identified through the upper gastrointestinal multi-disciplinary meetings and pre-operative clinics. Patients were eligible if they were undergoing a planned thoracic oesophagectomy and aged 18 or older if male, aged 55 or more than 2 years since menopause if female and were able and willing to give written informed consent. Patients with a known intolerance of vitamin D, sarcoidosis, tuberculosis, lymphoma, hyperparathyroidism, or nephrolithiasis, known serum corrected calcium >2.65 mmol/L, undergoing haemodialysis, or had a known diagnosis of chronic obstructive pulmonary disease (COPD) with an FEV₁ less than 50% predicted or resting oxygen saturations of less 92% were ineligible. Patients taking the following

concomitant medication were also excluded – more than 1,000IU (25mcg)/day vitamin D supplementation a month preceding or more than 10mcg/day in the 2 months preceding enrolment; cardiac glycoside, carbamazepine, phenobarbital, phenytoin, primidone, or long-term immunosuppressant therapy; thiazide diuretic at a dose higher than recommended in the British National Formulary. If agreeable, written informed consent was gained following a face to face discussion.

5.2.3 Randomisation and blinding

Drug (oral cholecalciferol oily solution Vigantol, 300,000 IU [7.5mg]) and matching placebo (Miglyol 812 oil, the vehicle for cholecalciferol in Vigantol) were supplied and packaged by Novalabs (Leicester, UK). The trial drug manufacturer produced the randomisation sequence using a block size of 10 with equal allocation between active and placebo groups. Drug boxes were supplied to centres in blocks of 10 thus ensuring an equal allocation between active and placebo groups. Treatment packs were held by the pharmacy at each centre and patients randomised sequentially by allocating them to the next numbered treatment pack held at the centre. Patients, clinical and research staff were unaware of which arm of the study a patient was allocated. Active and placebo treatment packs and their contents were identical in appearance. The protocol allowed for emergency un-blinding in the event of significant concerns about patient safety.

Subjects could withdraw from the trial or the trial treatment at any time without prejudice. If a subject withdrew from the trial treatment, then they were followed-up wherever possible and data collected as per protocol until the end of the trial. The only exception to this was where the subject also explicitly withdrew consent for follow-up.

5.2.4 Drug administration

Subjects received either 300,000 IU [7.5mg] of oral cholecalciferol (vitamin D₃) or placebo 3-18 days prior to planned oesophagectomy. The trial drug was administered and observed being taken by subjects by a qualified member of the trial team.

5.2.5 Primary Outcome

The primary outcome was change in extravascular lung water index (EVLWI) measured by PiCCO[®] thermodilution catheter at the end of surgery (measured within one hour post-operatively). Full details are given in sections 3.2 and 3.3. The changes in lung compliance that are a cardinal feature of ARDS occur as a result of accumulation of EVLW. The PiCCO[®] EVLWI is an indirect measure of fluid in the interstitium and alveolar space⁸⁷ and has been shown to be an independent risk factor for mortality in ARDS,^{210,256} and used as an outcome measure in other clinical trials in ALI^{51,61,114} as well as post-thoracotomy.²⁵⁷

5.2.6 Secondary outcomes

The trial secondary outcomes were clinical markers indicative of lung injury: PaO₂:FiO₂ ratio, development of ARDS during the first 28 days, ventilator and organ failure free days, survival (28 and 90 day), safety and tolerability of vitamin D supplementation and plasma vitamin D status (25(OH)D₃, 1,25(OH)₂D₃, vitamin D binding protein (DBP) levels). Further PiCCO® parameters of pulmonary vascular permeability index (PVPI) post-operatively as well as EVLWI and PVPI at day 1 post-operatively (measured at 9am on Day 1) were collected. PVPI was a measurement not stated *a priori* in the published trial protocol prior to commencing the study. However PiCCO® readings determine a value for PVPI at the same time as EVLWI and it was felt that this would be an important outcome to capture relevant to lung injury that can differentiate non-hydrostatic pulmonary oedema.^{209,211}

Since commencing recruitment the new Berlin criteria for the diagnosis of ARDS have been defined¹¹ and this was used to identify post-operative ARDS as 1) presence of bilateral opacities—not fully explained by effusions, lobar/lung collapse, or nodules, 2) respiratory failure not fully explained by cardiac failure or fluid overload 3) PaO₂/FiO₂ ratio < 300mmHg (40kPa) Hg and ventilation with positive end-expiratory pressure (PEEP) or CPAP > 5 cm H₂O (may be non-invasive ventilation). Ventilator free days were defined in accordance with the ARDSnet criteria as the number of calendar days after initiating unassisted breathing to day 28 after randomisation.²⁵⁸ Organ

failure free days are defined in a similar manner to ventilator free days with an organ failure free day being a day without evidence of non-respiratory organ failure. Organ failure was defined as a sequential organ failure assessment (SOFA) score of greater than 3.²⁵⁹ Hospital mortality was assessed as the number of patient deaths at 30 and 90 days. Furthermore an exploratory study of plasma indices of endothelial and alveolar epithelial injury and plasma inflammatory response were measured by ELISA.

5.2.7 Perioperative Care and PiCCO® measurements

Patients were managed according to routine anaesthetic and surgical practice of the medical teams involved. A conservative fluid strategy was adopted. A PiCCO® catheter was inserted into the right femoral artery in the anaesthetic room prior to surgery commencing and the first readings (preoperative) taken prior to knife-to skin. Post-operative readings were taken after skin closure within 1 hour post-operatively and postoperative day 1. Full details are provided in section 3.2 and 3.3.

5.2.8 Data collection

Demographic and background data were collected from electronic and paper patient medical records and by direct patient consultation. Operative data was collected by direct observation and from the anaesthetic charts. Post-operative data were collected from critical care charts, electronic and paper medical records. All the data was recorded in each subjects case report form (CRF). Physiological parameters were recorded daily for the first 7 days. All

post-operative physiological parameters were recorded as those taken at 8am on the morning of the day concerned. Chest radiographs were reviewed for radiological evidence of ARDS where the PaO₂/FiO₂ ratio was less than 300mmHg and there was absence of cardiac failure prior to un-blinding.

5.2.9 Sample collection and processing

Blood was drawn pre-trial drug administration, immediately pre- and post-operatively, on the morning of day 1 and day 3 or 4. These were immediately transferred to the laboratory and processed as described in section 3.4.1 and 3.5.1.

5.2.10 Vitamin D status

25(OH)D₃ was measured by tandem mass spectrometry using the appropriate vitamin D External Quality Assessment Scheme (DEQAS) control as described in section 3.8.1. 25(OH)D₃ levels below 50nmol/l (20 ng/ml) were regarded as deficient. 25(OH)D₃ levels between 50 and 75nmol/l (30 ng/ml) were regarded as insufficient, with levels above 75nmol/l (30ng/ml) designated sufficient levels.²⁶⁰ 1,25(OH)₂D₃ levels were measured by enzyme immunoassay (EIA) (immunodiagnostic systems Ltd, UK) as described in section 3.8.1 and DBP was measured by ELISA (immundiagnostik, Germany).

5.2.11 Biological indices of lung injury and systemic inflammation

Plasma inflammatory cytokines (IL-6, IL-8, TNF- α , IL1- β and IL-10) were measured by Magnetic Luminex® Performance Assays (R&D Systems, UK).

Soluble receptors for advanced glycation products (sRAGE), TNF-1 and TNF-2 were measured by MILLIPLEX® MAP assays (Millipore, USA). Detailed methods described in section 3.8.3.

5.2.12 Statistical analysis

There are no direct data to predict the effect size of vitamin D replacement upon EVLWI. In our groups preliminary data our patients undergoing oesophagectomy had a mean post-operative EVLWI of 10.1 ml/kg and standard deviation of 2.9 ml/kg. For the study to be able to detect an increase of 20% EVLWI from pre-post-operative with a power of 80% at a significance level of 0.05 we required 34 patients in each arm to reach the primary endpoint. An additional six patients were recruited to each arm to allow for dropouts such as open/close cases, unexpected deaths, and other difficulties with data collection. Thus we intended to recruit 40 patients to each arm of the study.

Data were analysed using graphpad PRISM 6® software. Normality was tested using the D'Agostino-pearson omnibus test. Differences between continuously distributed data, was tested using unpaired t-tests or Mann-Whitney tests for non-parametric equivalents. Paired samples were tested using paired t-test or the Wilcoxon ranked sign test. Chi-squared tests (or Fisher's Exact tests) were used for categorical variables. Two-tailed p-values of 0.05 were considered as significant. Results from normally distributed data are shown as mean \pm standard deviation and presented as bar charts or

dot-plots. Non-parametric results are presented as median with interquartile range (IQR) and box and whisker plots with Tukey's distribution. Transformation of non-parametric data was not deemed appropriate due to the outliers and clear non-normality of the variables from normality testing.

5.3 RESULTS

5.3.1 Patient recruitment and characteristics

A total of 79 patients were enrolled in the study by January 26, 2015. Follow-up of outcomes, collation of endpoints and chest radiographs continued until June 2015. A total of 39 patients were assigned to the placebo arm and 40 to vitamin D. Participants CONSORT flow diagram is shown in Figure 5.1. All patients received their intended randomised trial treatment. One patient (2.6%) in the placebo arm withdrew prior to surgery and 2 (5%) in the vitamin D arm did not proceed to surgery post-randomisation due to medical reasons (one developed unrelated ischaemic leg and the other was deemed unfit for surgery post adjuvant chemotherapy). Thirty-five patients in the placebo arm and 33 in the vitamin D arm went on to complete oesophagectomy. One patient (2.6%) in the placebo arm and 3 (7.9%) in the vitamin D arm had inoperable malignancy. Two patients (5.3%) in the placebo arm and 1 (2.6%) in the vitamin D arm were converted to gastrectomy intra-operatively with 1 further patient (2.6%) in the vitamin D arm in whom we were unable to place a PiCCO₂[®] catheter due to anatomical reasons. These patients were withdrawn from the study as they did not receive OLV and meet primary endpoint (EVLWI reading at the end of surgery). However since they received treatment they were included in the analysis of efficacy and safety of high-dose vitamin D replacement.

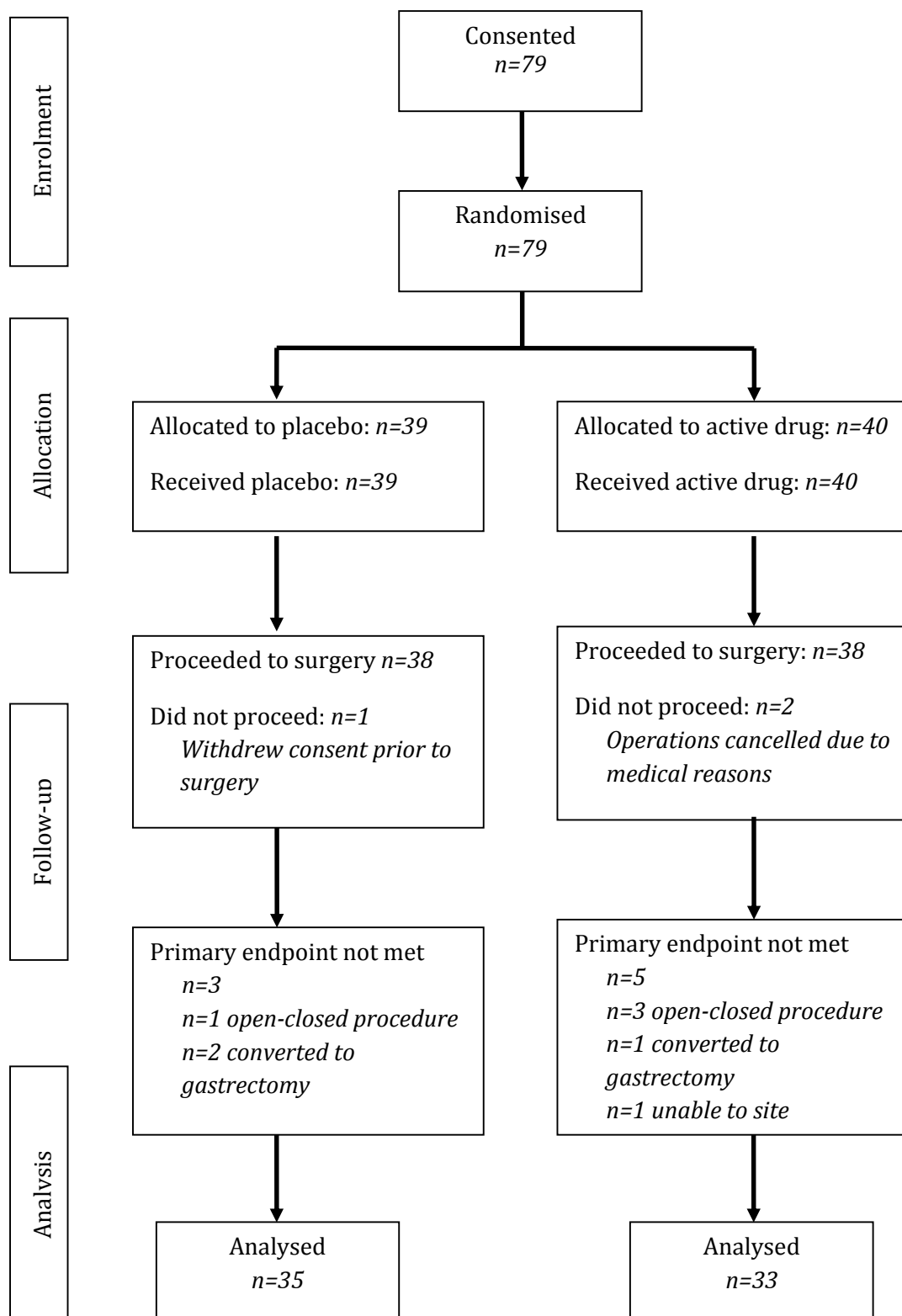


Figure 5.1: Patient CONSORT flow diagram.

Patient baseline characteristics were well matched for age, sex, smoking status and lung function (Table 5.1). All procedures were performed for malignancy and the predominant cell type of adenocarcinoma. There was no significant difference between placebo and vitamin D arms in baseline pre-drug 25(OH)D₃ (mean (SD) 48.8(22.8) vs. 46.8(26.3)nmol/L, p=0.44) and 1,25(OH)₂D₃ (mean (SD) 98.1(37.2) vs 82.8(34.3)pmol/L, p=0.08) concentrations.

In those that completed surgery, groups were also well matched for operative and anaesthetic management as detailed in Table 5.2. Duration of surgery and OLV, maximum tidal volumes, peak airway pressure and cumulative fluid balance were similar between arms.

		Placebo (n=39)	Vitamin D ₃ (n=40)
Age, years	Mean (SD)	65.3 (8.7)	63.1 (10.0)
Sex	Male - n (%)	31 (79.5)	36 (90.0)
Ethnicity	Caucasian - n (%)	36 (92.3)	40 (100.0)
BMI, kg/m²	Mean (SD)	26.6 (4.5)	28.4 (5.6)
Smoker			
Pack years	Median (IQR)	20 (0-40)	15 (0-36.2)
	Never - n (%)	10 (25.6)	11 (27.5)
	Former - n (%)	24 (61.5)	20 (50.0)
	Current - n (%)	4 (10.3)	8 (20.0)
	Unknown - n (%)	1 (2.6)	1 (2.5)
Diagnosis	Adenocarcinoma - n (%)	32 (82.1)	35 (87.5)
	Squamous cell - n (%)	7 (17.9)	5 (12.5)
TMN staging tumour	n - (%)		
	1	2 (5.1)	7 (17.5)
	2	2 (5.1)	4 (10.0)
	3	34 (87.2)	27 (67.5)
	4	1 (2.6)	2 (5.0)
TMN staging nodes	n - (%)		
	0	17 (43.6)	13 (32.5)
	1	19 (48.7)	24 (60.0)
	2	1 (2.6)	3 (7.5)
	3	2 (5.1)	0 (0)
Preoperative chemotherapy	n - (%)	33 (84.6)	32 (80.0)
Lung function			
FEV₁, L/s	Mean (SD)	2.9 (0.6)	3.2 (0.7)
FVC, L	Mean (SD)	4.3 (0.8)	4.5 (0.8)

Table 5.1: Patient baseline characteristics.
SD, standard deviation; IQR, interquartile range

		Placebo (n=35)	Vitamin D ₃ (n=33)
Tumour location	n - (%)		
	Mid oesophagus	3 (8.6)	6 (18.2)
	Oesophageal/gastric junction	32 (91.4)	27 (81.8)
Surgical approach	n - (%)		
	Open	22 (62.9)	22 (66.7)
	MIO	13 (37.1)	11 (33.3)
ASA grade	n - (%)		
	I	1 (2.9)	1 (3.0)
	II	29 (82.8)	22 (66.7)
	III	4 (11.4)	10 (30.3)
	IV	1 (2.9)	0 (0)
Duration of one-lung ventilation, minutes	Median (IQR)	151.0 (124.5 – 196.3)	150.0 (130.0 – 183.8)
Duration of surgery, minutes	Mean (SD)	357.4 (69.7)	386.5 (67.2)
Cumulative fluid balance at end of surgery, Litres	Median (IQR)	2.7 (2 – 3.7)	3.3 (2.5 – 4.3)
Maximum FiO₂	Median (IQR)	0.85 (0.68 – 0.9)	0.80 (0.65 – 0.9)
Maximum tidal volume, ml/kg predicted body weight	Mean (SD)	7.4 (1.5)	7.8 (1.3)
PEEP, cm H₂O	Mean (SD)	5.29 (2.6)	5.17 (2.2)
Peak airway pressure, cm H₂O	Mean (SD)	26.2 (4.3)	26.8 (5.5)

Table 5.2: Anaesthetic and operative characteristics.

SD, standard deviation; IQR, interquartile range; MIO, minimally invasive oesophagectomy; FiO₂, fraction of inspired oxygen; PEEP, positive end-expiratory pressure.

5.3.2 Primary Outcome

There was a significant rise in pre-operative to post-operative absolute EVLWI in the placebo group (from median 6.3 [5.3 - 7.7] to 7.1 [6.0 - 9.5]ml/kg, $p=0.0002$; Wilcoxon signed rank test) compared to the vitamin D treated group (from median 6.2 [5.3 - 7.5] to 6.8 [5.8 - 7.6]ml/kg, $p=0.12$; Wilcoxon signed rank test) in which a significant rise was not seen (Figure 5.2). There was no difference in absolute EVLWI values between placebo and vitamin D arms at preoperative, postoperative or postoperative day 1 time points (Table 5.3).

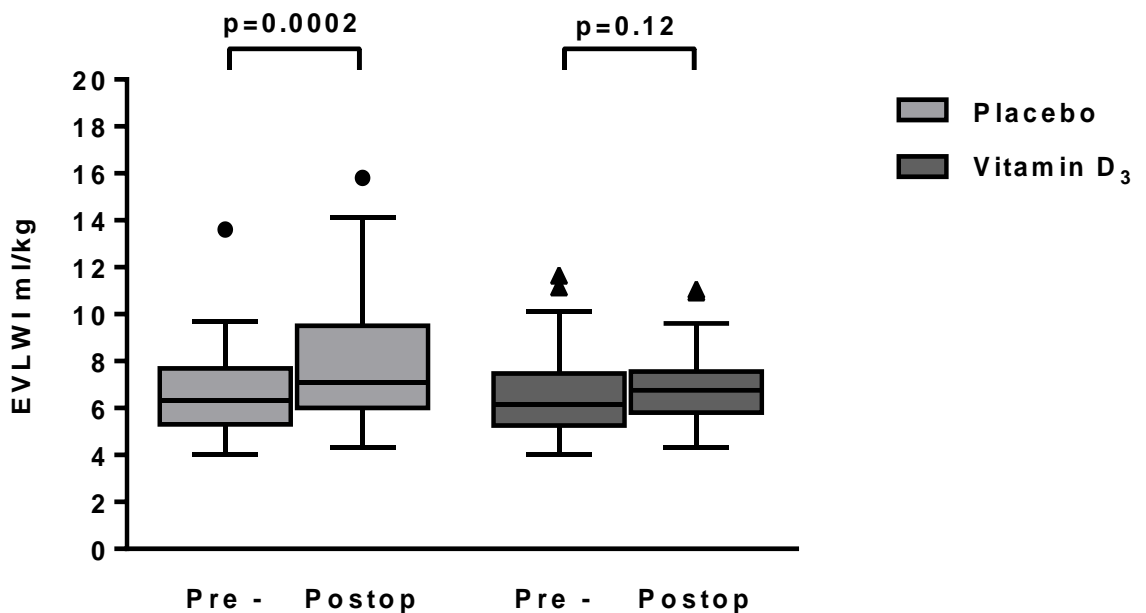


Figure 5.2: Box and whisker plot of absolute extravascular lung water index (EVLWI) values pre to postoperatively.

Placebo $n=35$, vitamin D₃ $n=33$. Data presented as medians with Tukey's distribution. *Circles* and *triangles* represent outlying values. P-values represent Wilcoxon matched-pairs signed rank test.

EVLWI (ml/kg) median (IQR)	Placebo n=35	Vitamin D₃ n=33	p-value
Preoperative	6.3 (5.3- 7.7)	6.2 (5.3 -7.7)	0.819
Postoperative	7.1 (6.0 – 9.5)	6.9 (5.8 – 7.7)	0.269
Day 1	5.9 (5.0 – 7.7)	5.7 (4.9 – 7.6)	0.596

Table 5.3: Absolute extravascular lung water index (EVLWI) values between treatment arms.

Placebo n=35 and vitamin D₃ n=33 at preoperative and postoperative points; placebo n=32 and vitamin D₃ n=31 at day 1 time point due to loss of PiCCO catheter. IQR – interquartile range; p-value represents Man-Whitney test.

Our primary endpoint was EVLWI at the end of the operation. However, to account for the varying baseline preoperative EVLWI values between patients the absolute and fold changes in EVLWI were analysed. . Vitamin D₃ treatment attenuated the increase from baseline in EVLWI seen after surgery, however this effect is not seen at day 1 postoperatively (Figure 5.3); Absolute change in EVLWI median 1.0 [IQR 0.4 -1.8] vs. 0.4 [IQR -0.4 – 1.2] ml/kg, p=0.05 in placebo compared to treatment groups preoperatively to postoperatively and -0.3 [IQR -1.7 – 0.6] vs. -0.4 [IQR -1.8 – 0.7] ml/kg, p=0.78 preoperatively to day 1.

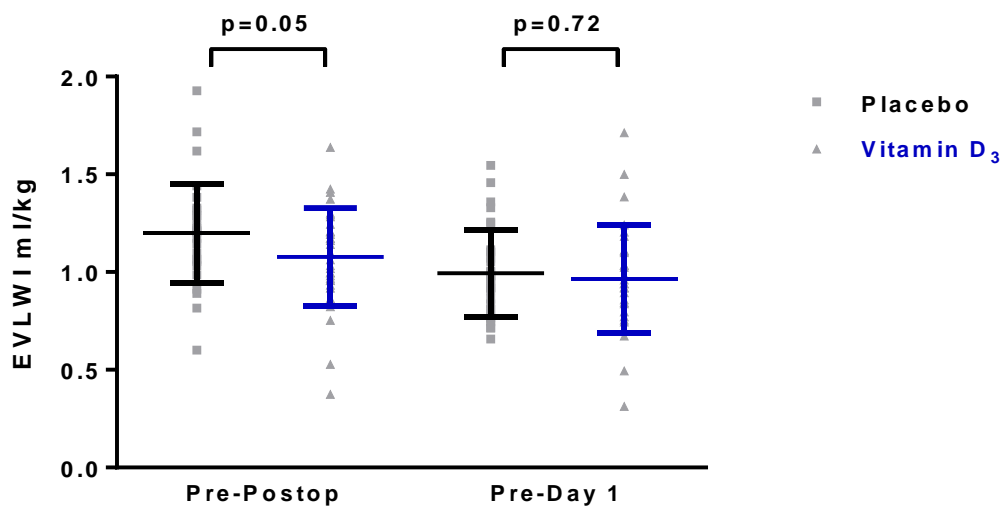


Figure 5.3: Dot-plot showing fold change in extravascular lung water index (EVLWI) between treatment arms.

Fold changes shown from pre to postoperative and preoperative to day 1. Black lines and square dots for placebo (n=35); blue lines and triangle dots for vitamin D₃ treatment (n=33); data presented as medians and interquartile ranges; p-values represent Mann Whitney tests.

5.3.3 Secondary Outcomes

5.3.3.1 Pulmonary vascular permeability index (PVPI)

The rise seen in PVPI was significantly higher pre to postoperative in patients who received placebo (median 1.5 [IQR 1.3 - 1.7] to 2.0 [IQR 1.6 - 2.1], p=0.0002, Wilcoxon ranked sign test) compared to vitamin D₃ (median 1.7 [IQR 1.4 - 2.0] to 1.8 [IQR 1.6 - 2.1], p=0.36, Wilcoxon ranked sign test)(Figure 5.4). There was no difference seen in absolute values of PVPI preoperatively, postoperatively and at day 1 between the two treatment arms (Table 5.4).

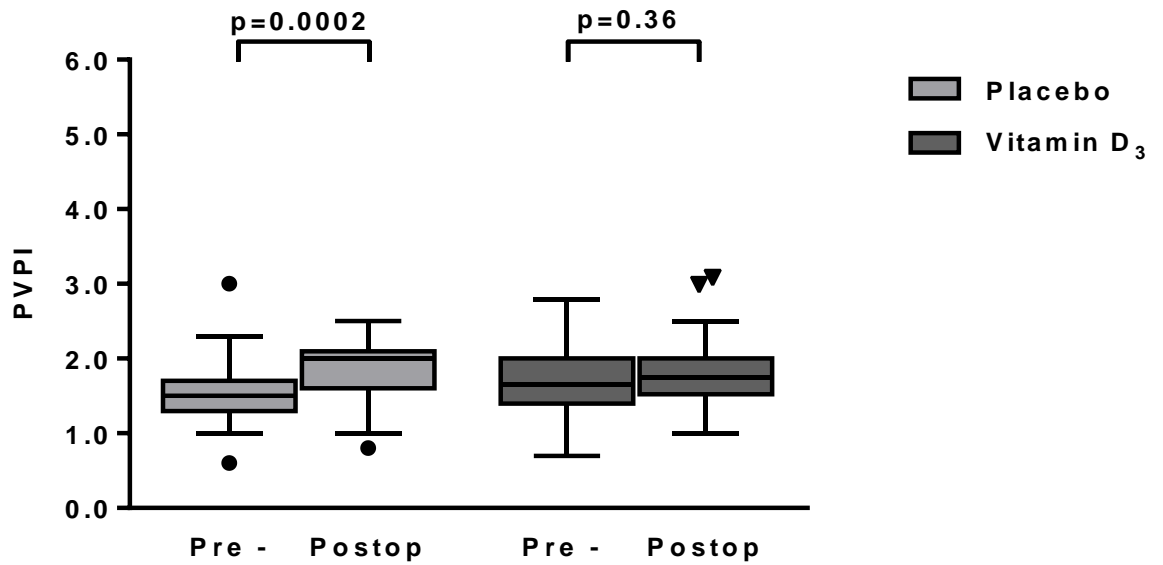


Figure 5.4: Box and whisker plot of absolute values pulmonary vascular permeability index (PVPI) pre to postoperatively.

Placebo n=35, vitamin D₃ n=33. Data presented as medians with Tukey's distribution. *Circles* and *triangles* represent outlying values. P-values represent Wilcoxon matched-pairs signed rank test.

PVPI	Placebo	Vitamin D ₃	p-value
<i>median (IQR)</i>	<i>n=35</i>	<i>n=33</i>	
Preoperative	1.5 (1.3 – 1.7)	1.7 (1.4 – 2.0)	0.06
Postoperative	2.0 (1.6 – 2.1)	1.8 (1.6 – 2.0)	0.31
Day 1	1.4 (1.1 – 1.6)	1.4 (1.1 – 1.9)	0.59

Table 5.4: Absolute pulmonary vascular permeability (PVPI) values between treatment arms.

Placebo n=35 and vitamin D₃ n=33 at preoperative and postoperative points; placebo n=32 and vitamin D₃ n=31 at day 1 time point due to loss of PiCCO₂ catheter. IQR – interquartile range; p-value represents Mann-Whitney test.

In order to account for baseline variability absolute and fold changes from baseline were analysed. Vitamin D₃ treatment significantly reduced the change in PVPI postoperatively compared to placebo, however this effect was not seen at day 1 (Figure 5.5); Absolute change in PVPI in placebo compared to treatment groups median 0.4 [IQR 0 – 0.7] vs. 0.1 [IQR -0.15 – 0.35], p=0.02 preoperatively to postoperatively and -0.1 [IQR -0.3 – 0.2] vs. -0.2 [IQR -0.4 – 0.1], p=0.28 preoperatively to day 1.

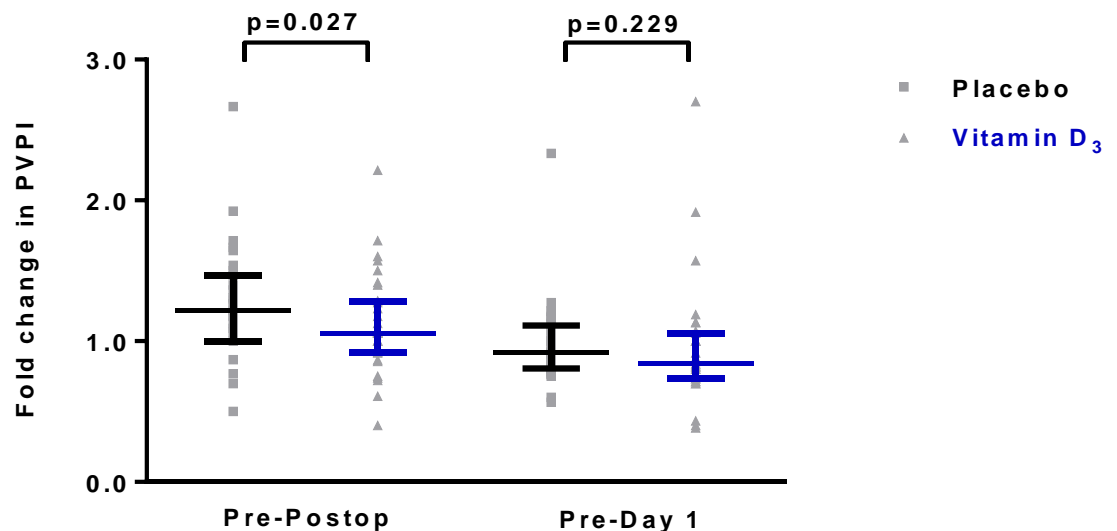


Figure 5.5: Dot-plot showing fold change in pulmonary vascular permeability index (PVPI) between treatment arms.

Fold changes shown from pre to postoperative and preoperative to day 1. Black lines and square dots for placebo (n=35); blue lines and triangle dots for vitamin D₃ treatment (n=33); data presented as medians and interquartile ranges; p-values represent Mann Whitney tests.

5.3.3.2 PaO₂/FiO₂ Ratio

There was no significant difference seen in PaO₂/FiO₂ ratio between the two groups postoperatively and at day 1 (figure 5.6). No significant difference was seen when the results were analysed for those patients that were 25(OH)D₃ deficient post receiving trial drug irrespective of the treatment arm they were allocated.

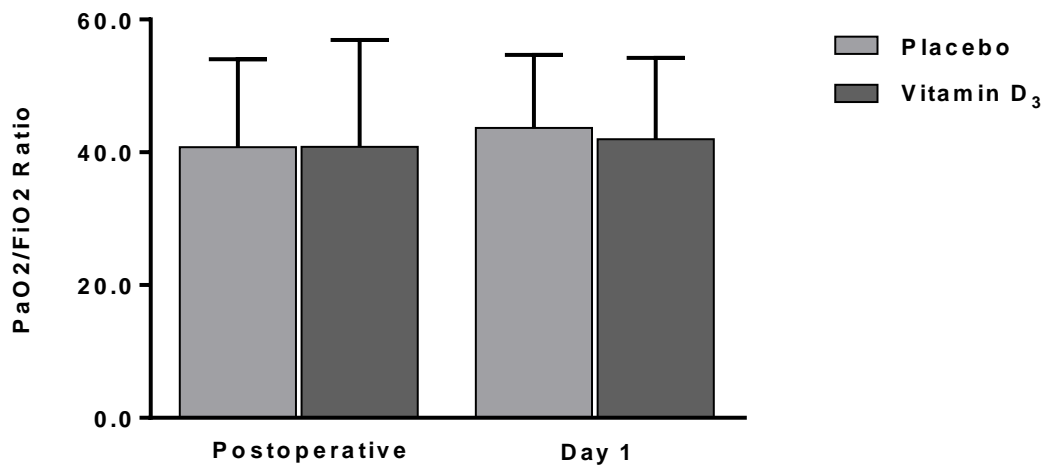


Figure 5.6: Bar chart of PaO₂/FiO₂ ratio by treatment group.

Data presented as mean and standard deviation. Placebo n=35, vitamin D₃ n=33.

5.3.3.3 Development of ARDS

This study was not powered to investigate the development of ARDS but was included as a secondary outcome measure. In total 8 out of 68 (11.8%) patients developed ARDS with all of them occurring within 4 days of surgery with 5/8 (62.5%) within the first 24 hours postoperatively. There was no difference in ARDS rates between placebo and vitamin D₃ treatment arms (placebo 4 [11.4%] of 35 compared with vitamin D₃ 4 [12.1%] of 33; odds ratio 0.94; 95% CI 0.21 – 4.09). There was no significant difference in the post treatment 25(OH)D₃ concentration between the groups (median ARDS 61.5 (IQR 57.7 – 78.5) nmol/L vs. no ARDS median 58.4 (IQR 37.3 – 78.9) nmol/L, p=0.77).

5.3.3.4 Clinical outcomes.

There was an increase in ICU length of stay in the vitamin D₃ treated group compared to placebo group; however hospital length of stay was not significantly different (Table 5.5). Thirty and 90-day survival was similar between the groups with 1 death in the placebo group at 30-days and 2 at 90 days compared with vitamin D₃ arm with no deaths at 30-days and 2 deaths at 90-days (Table 5.5). There was no difference seen in ventilator and organ failure free days (data not shown)

	Placebo <i>n=35</i>	Vitamin D₃ <i>n=33</i>	p-value
ITU LOS, days <i>Median (IQR)</i>	4 (3 – 6)	5 (3 – 10)	0.052
Hospital LOS, days <i>Median (IQR)</i>	13 (10 – 20)	13 (11 – 23)	0.739
30-day mortality, n(%)	1 (2.9)	0 (0)	1.0
90-day mortality, n(%)	2 (5.8)	2 (6.1)	1.0

Table 5.5: Length of stay and survival.

IQR, interquartile range; p-values represent Mann Whitney tests and Fisher’s exact test for categorical data.

5.3.3.5 Safety and tolerability

The trial medication was well tolerated with 4 of 79 (5.1%; 2 placebo and 2 vitamin D₃) patients who received the drug developing self-limiting gastrointestinal upset in the form of episodes of diarrhea and nausea up to 24 hours post drug administration . This was felt in part due to the vehicle Miglyol 812 oil for vitamin D₃ in Vigantol acting as a laxative. There were no episodes of hypercalcaemia post drug administration.

The frequency of serious adverse effects (SAEs) and adverse effects (AEs) was similar between the groups (Table 5.6). There were no reported SAEs related to the trial medication or suspected unexpected serious adverse events (SUSARs).

	Placebo <i>n=35</i>	Vitamin D₃ <i>n=33</i>	p-value
Total SAEs	7 (20.0%)	11 (33.3%)	0.28
Anastamotic leak	8 (22.8%)	6 (18.2%)	0.77
Other surgical complication	2 (5.7%)	5 (15.2%)	0.25
Pneumonia	14 (40%)	14 (42.4)	1.0
Other Respiratory complication	7 (20.0%)	4 (12.1%)	0.51
Sepsis	2 (5.7%)	1 (3.0%)	1.0
Cardiac complication	10 (28.6%)	4 (12.1%)	0.14

Table 5.6: Adverse events summarised by treatment group.

SAEs, serious adverse events; other surgical complications include wound infection and intra-operative surgical complications, para-oesophageal herniation; other respiratory complications include pneumothorax or effusion requiring drainage, undefined respiratory failure, and pulmonary embolus; cardiac complications include arrhythmias requiring chemical or electrical treatment and myocardial infarction.

5.3.3.6 Plasma markers of inflammation and epithelial damage

There were no differences seen in perioperative markers of systemic inflammation (IL-6, IL-8, TNF α or IL-10) between the groups, apart from a greater IL-6 in the cholecalciferol arm which then normalized by day 1. There was no difference in soluble RAGE between the groups, a marker of type 1 epithelial injury. There was also no difference in antimicrobial peptide LL-37 between the two arms at any time point. TNF receptor 1 (p=0.05) and 2 (p=0.02) levels were significantly higher at day 1 in vitamin D₃ treated patients. (Table 5.7)

	Time-point	Placebo	Vitamin D₃	p-value
IL-6 (pg/ml)	Pre-op	9.5 (8.5 – 11.3)	9.8 (8.5 – 11.3)	0.18
	Post-op	296.4 (188.5 – 488.5)	546.3 (202.8 – 920)	0.05
	Day 1	240.5 (133.8 – 444.4)	249.0 (153.4 – 451.4)	0.88
IL-8 (pg/ml)	Pre-op	48.9 (35.6 – 58.9)	44.4 (36.7 – 53.1)	0.22
	Post-op	87.1 (60.9 – 133.1)	102.0 (74.3 – 156.0)	0.17
	Day 1	85.3 (68.0 – 122.0)	85.0 (68.5 – 151.6)	0.97
TNFα (pg/ml)	Pre-op	12.5 (11.0 – 13.5)	12.5 (11.25 – 13.5)	0.96
	Post-op	10.5 (9.5 – 11.5)	11.0 (10.0 – 12.5)	0.14
	Day 1	11.5 (10.4 – 13.6)	12.0 (10.5 – 13.0)	0.54
IL-10 (pg/ml)	Pre-op	15.0 (14.0 – 16.5)	15.0 (14.0 – 17.5)	0.39
	Post-op	66.0 (31.5 – 106.5)	43.0 (31.0 – 76.0)	0.31
	Day 1	27.0 (21.0 – 36.4)	26.5 (23.5 – 37.0)	0.67
TNFR-1 (pg/ml)	Pre-op	392 (254 – 577)	391 (300 – 596)	0.49
	Post-op	806 (520 – 1302)	1181 (683 – 1443)	0.09
	Day 1	724 (468 – 1179)	886 (606 – 1694)	0.05
TNFR-2 (pg/ml)	Pre-op	2365 (2003 – 3677)	2865 (2211 – 4340)	0.26
	Post-op	3112 (2263 – 4879)	4148 (3125 – 5488)	0.12
	Day 1	3807 (2403 – 4980)	5130 (3607 – 6864)	0.02
LL-37 (ng/ml)	Pre-op	3.7 (1.7 – 12.6)	5.6 (2.5 – 11.5)	0.63
	Post-op	5.8 (2.5 – 7.9)	4.6 (2.3 – 11.9)	0.93
	Day 1	14.6 (5.2 – 23.0)	9.4 (3.6 – 19.0)	0.52
sRAGE (pg/ml)	Pre-op	43.0 (36.0 – 58.6)	42.0 (33.9 – 51.3)	0.57
	Post-op	51.0 (37.6 – 67.4)	52.5 (39.0 – 91.0)	0.76
	Day 1	35.3 (28.8 – 44.3)	39.9 (30.6 – 64.1)	0.13

Table 5.7: Comparison of plasma markers of inflammation and epithelial damage.

Data presented are medians (interquartile range): Bold results are significant. P-values represent Mann Whitney tests.

Postoperative sRAGE levels were raised significantly in both groups suggesting Type 1 epithelial damage however there was no difference in sRAGE levels between the arms postoperative, day 1 or at day 3/4.

5.3.4 Efficacy of high dose vitamin D replacement

There was no difference between groups in 25(OH)D₃ levels before trial medication administration (placebo median 43.2 (IQR 35.0 – 69.0) vs. median vitamin D₃ treated 42.7 (IQR 30.2 – 62.1) nmol/L, p=0.65). High dose 300,000IU of cholecalciferol (vitamin D₃) resulted in successful increase in plasma 25(OH)D₃ concentrations to sufficient levels in those that received treatment compared to placebo (median 74.8 [IQR 58.0 – 90.3] compared to 38.6 [IQR 31.3 – 58.6] nmol/L, p<0.0001). By day 3/4 there is a global decrease in 25(OH)D₃ levels however there were persistently higher concentrations present in the vitamin D₃ treated arm (Figure 5.7).

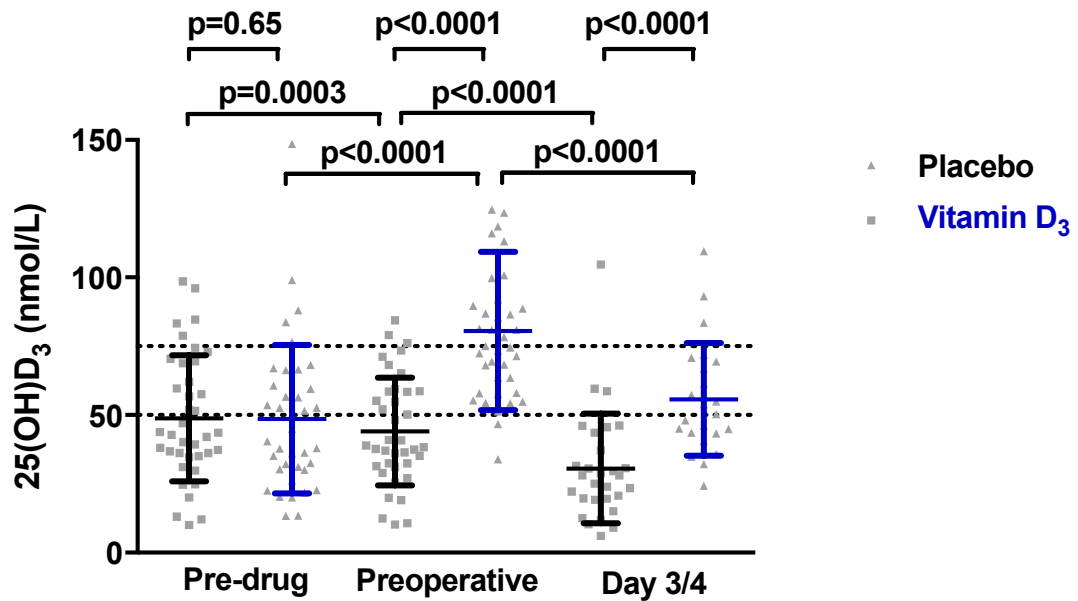


Figure 5.7: Scatter plot of efficacy of high dose vitamin D₃ supplementation on 25(OH)D₃ concentrations.

Black lines and square dots for placebo (n=38 pre-drug, preoperative; n=29 Day 3/4); blue lines and triangle dots for vitamin D₃ treatment (n=38 pre-drug, pre-operative; n=23 day 3/4); Data presented as median and interquartile range, p-values comparing groups represent Mann Whitney tests and within groups Wilcoxon matched pairs rank test. horizontal dotted line at 50nmol/L to show deficiency; 75nmol/L to show sufficiency.

There was no difference between arms in pre-trial medication administration 1,25(OH)₂D₃ concentrations (mean placebo 98.2 (SD 37.2) vs. 84.7 (SD 33.7) pmol/L, p=0.12)(Figure 5.8). Treatment with vitamin D₃ resulted in a significant increase in 1,25(OH)₂D₃ levels but there was no significant difference seen in concentrations between placebo and vitamin D₃ treatment groups (mean 91.9 (SD 32.4) vs. 104.9 (SD 36.9) pmol/L, p=0.12). There was a decrease in 1,25(OH)₂D₃ in the placebo group by day 3/4 however levels were maintained in the vitamin D₃ arm, thus by day 3/4 the levels were significantly lower in the placebo group (mean 66.3 (SD 32.5) vs. 98.03 (SD 51.2) pmol/L, p=0.009)(Figure 5.8)

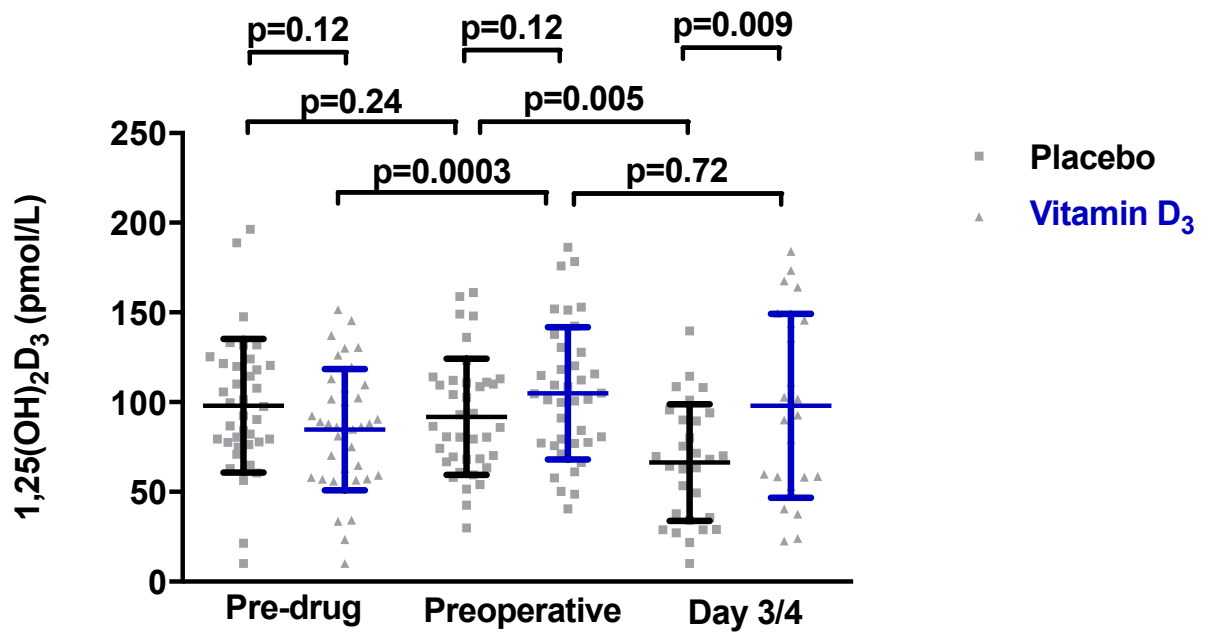


Figure 5.8: Scatter plot of efficacy of high dose vitamin D₃ supplementation on 1,25(OH)₂D₃ concentrations.

Black lines and square dots for placebo (n=38 pre-drug, preoperative; n=29 Day 3/4); blue lines and triangle dots for vitamin D₃ treatment (n=38 pre-drug, pre-operative; n=23 day 3/4); Data presented as mean and standard deviation, p-values between groups represent unpaired t-tests and paired t-tests within groups; horizontal dotted line at 50nmol/L to show deficiency; 75nmol/L to show sufficiency.

There was no difference between arms in DBP levels at any timepoint. There was a significant drop in DBP level at day 3 in both groups. (Figure 5.9)

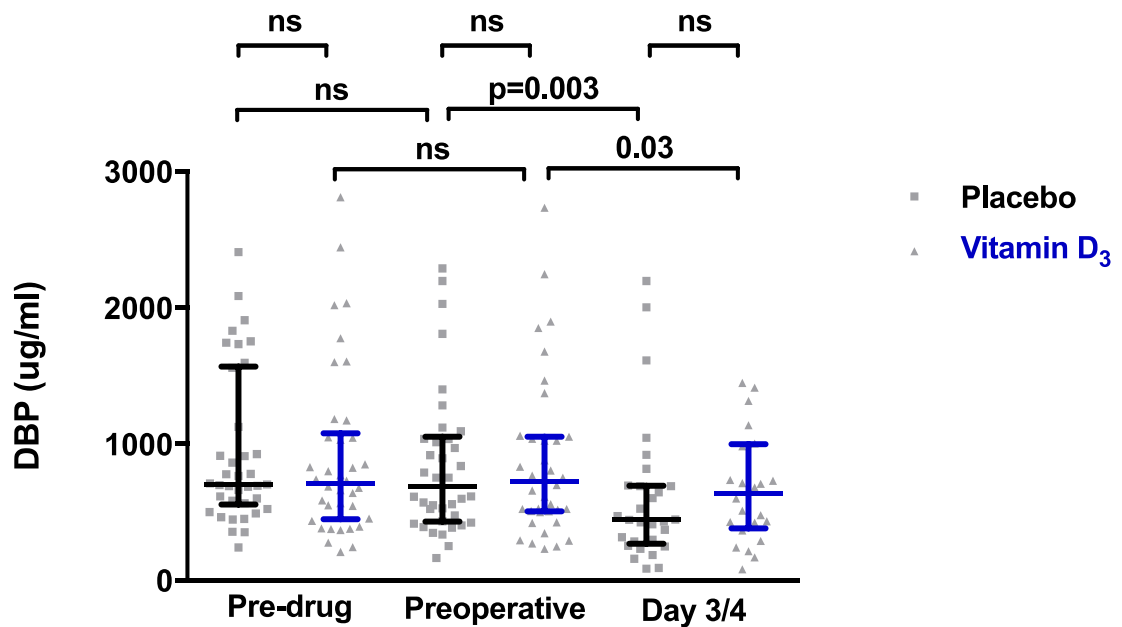


Figure 5.9: Scatter plot of efficacy of high dose vitamin D₃ supplementation plasma vitamin D binding protein (DBP) concentrations.

Black lines and square dots for placebo (n=38 pre-drug, preoperative; n=29 Day 3/4); blue lines and triangle dots for vitamin D₃ treatment (n=38 pre-drug, pre-operative; n=23 day 3/4); Data presented as median and interquartile range, p-values comparing groups represent Mann Whitney tests and within groups Wilcoxon matched pairs rank test.

5.3.5 Post Hoc analysis of EVLWI and PVPI

Only 2 patients who received vitamin D had levels of 25(OH)D₃ <50nmol/L post trial drug administration which is the level below which deficiency is defined. However some patients in the placebo group had 25(OH)D₃ levels above 50nmol/L pre drug administration. Therefore a sub-group analysis of all the patients (placebo and treatment arm) was performed based on 25(OH)D₃ concentration post drug administration to determine if there is a threshold effect above which the beneficial effects of 25(OH)D₃ on EVLWI and PVPI are seen.

Patients who remained deficient in 25(OH)D₃ (<50nmol/L) irrespective of the treatment arm they were allocated to had a significantly higher increase in EVLWI at the end of the operation compared to those that were sufficient (median absolute increase EVLWI 1.2 [IQR 0.9 – 2.0] vs. 0.4 [IQR -0.4 – 1.4] ml/kg, p=0.008)(Figure 5.10).

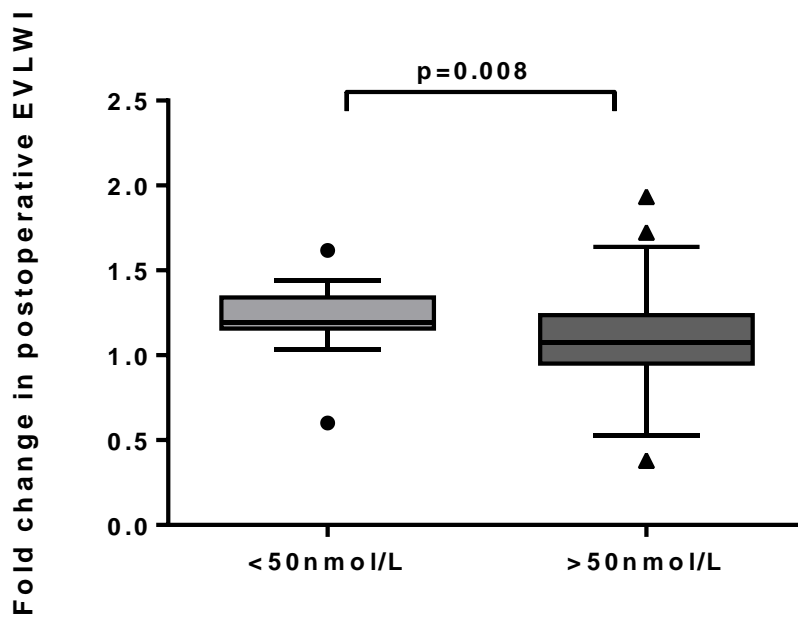


Figure 5.10: Box and whisker plot of fold change in extravascular lung water index in 25(OH)D₃ deficient and sufficient patients.

25(OH)D₃ <50nmol/L n=22; 25(OH)D₃ >50nmol/L n=46. Data presented as medians with Tukey's distribution. *Circles* and *triangles* represent outlying values. P-values represent Mann Whitney test.

Patients who remained deficient in 25(OH)D₃ (<50nmol/L) irrespective of the treatment arm they were allocated to had a significantly higher increase in PVPI at the end of the operation compared to those that were sufficient (median absolute increase PVPI 0.4 [IQR 0.3 – 0.6] vs. 0.1 [IQR -0.1 – 0.4] ml/kg, p=0.014)(Figure 5.11)

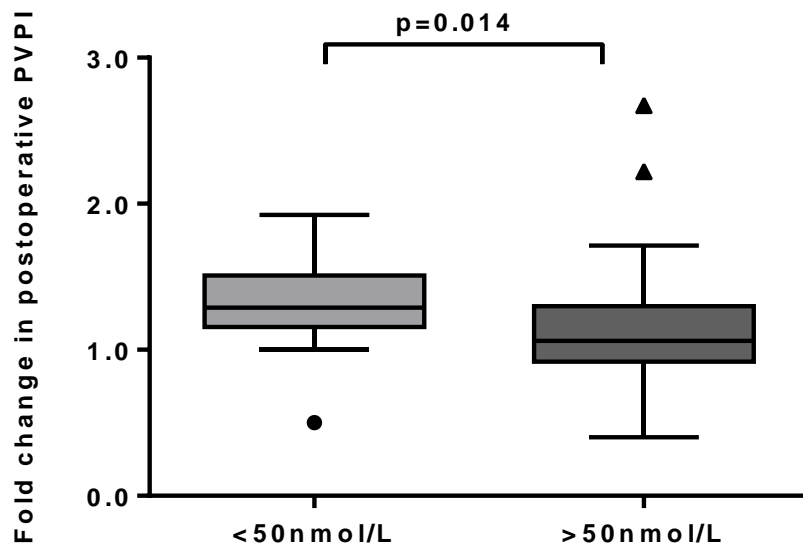


Figure 5.11: Box and whisker plot of fold change in pulmonary vascular permeability index (PVPI) in 25(OH)D₃ deficient and sufficient patients.

25(OH)D₃ <50nmol/L n=22; 25(OH)D₃ >50nmol/L n=46. Data presented as medians with Tukey's distribution. *Circles* and *triangles* represent outlying values. P-values represent Mann Whitney test.

5.4 DISCUSSION

The main finding of this phase II randomised placebo controlled clinical trial was that high dose oral vitamin D₃ (300,000IU cholecalciferol [vigantol®]) treatment pre-oesophagectomy prevented an increase in postoperative EVLWI and PVPI. Although the a priori primary outcome of postoperative EVLWI was not significantly different between placebo and treatment groups, there was a significant rise and fold change in EVLWI and PVPI in the placebo group compared to the treatment group. Furthermore, this effect was more significant in patients with vitamin D₃ deficiency (25(OH)D₃ levels less than 50nmol/L). Thus, suggesting vitamin D₃ may have a protective role on the alveolar epithelial and endothelial membrane and its actions may have a threshold effect.

Vitamin D₃ treatment did not prevent the development of ARDS. The incidence of ARDS in this cohort was 11.7%. This study was not powered to investigate clinical outcomes so it is perhaps not unsurprising that no differences were seen between groups with respect to oxygenation, number of ventilator- or organ failure free days, ICU or hospital length of stay or survival. Importantly treatment was well tolerated with no difference in the rate of serious adverse events.

There are several potential reasons why preoperative treatment with vitamin D₃ and rendering patients non-deficient may attenuate the increase in postoperative EVLWI and PVPI. 1,25(OH)₂D₃ has been shown to upregulate

transcription of proteins required for the formation of claudins and E-cadherin in epithelial cells of the skin¹³⁸ and intestine^{140,141} which are required for normal function of gap, tight and adherens junctions. This may also have a role in the lung protecting the epithelial barrier to prevent alveolar fluid influx and facilitate clearance.⁸⁵ Alveolar epithelial cells possess the ability to convert circulating 25(OH)D₃ to 1,25(OH)₂D₃ and activate VDR responsive genes.¹²³ Recently it has been shown that physiologically relevant doses of 25(OH)D₃ stimulated wound repair, cellular proliferation and reduced sFasL induced cell death of type 2 alveolar epithelial cells *in-vitro*.¹⁹⁸ Both indicating that vitamin D₃ has a direct protective role on the alveolar epithelium. There may also be a protective mechanism on the pulmonary endothelium as 1,25(OH)₂D₃ is able to decrease expression of ICAM-1 and prevent neutrophil adhesion, migration and therefore initiation of lung injury.¹⁴² Once an injury has occurred clearance of neutrophils and alveolar fluid occurs by resident and recruited macrophages. Macrophages constitutively express the VDR and 1 α -hydroxylase (CYP27B1) and vitamin D₃ stimulates the differentiation of mature phagocytic macrophages.^{120,150} Although much of this evidence comes from increased phagocytosis of pathogens vitamin D₃ may increase macrophage resolution of inflammatory cells and debris however more studies are required to elucidate this potential effect.

There was a greater increase in immediate postoperative IL-6 levels in the vitamin D₃ compared to placebo treated arms that had returned to similar

levels by day 1. Other markers of systemic inflammation and epithelial damage were not altered by treatment with vitamin D₃. These results are a surprise as vitamin D₃ has been shown to be anti-inflammatory by reducing NF- κ B signalling and TLR signalling.¹⁵⁴ Overall this study did not show an anti-inflammatory effect of vitamin D₃ treatment on circulating cytokines and confirms the findings of 2 other studies which failed to reveal a relationship between circulating cytokines and 25(OH)D₃ levels²⁶¹ or vitamin D replacement.²⁶² Significantly higher levels of soluble receptors TNFR1 and TNFR2 were found in the vitamin D₃ treated group at day 1 which is slightly more difficult to explain but may explain vitamin D₃ effects on promoting resolution of inflammation.

High dose 300,000IU of vitamin D₃ successfully increased 25(OH)D₃ concentrations to what is classified as sufficient (50nmol/L). This led to a corresponding and sustained rise in 1,25(OH)₂D₃ concentration perioperatively. However plasma levels of 25(OH)D₃ and 1,25(OH)₂D₃ decreased post-operatively by day 3 but continued to remain higher in the vitamin D₃ treated arm. This drop could be explained by increased consumption post-operatively although may also be accounted for by decreasing levels of DBP seen at day 3 or a dilution effect due to perioperative fluid administration. Evidence exists of local synthesis of 1,25(OH)₂D₃ by normal human macrophages on stimulation with IFN-gamma.¹⁵¹ However evidence is lacking of the effects of acute inflammatory

processes on plasma and cellular 25(OH)D₃ levels and its conversion to 1,25(OH)₂D₃.

The major limitation of this study is the change in the 25(OH)D₃ levels of this cohort of patients on which this study design was based. The baseline levels were much higher than those seen in the preliminary observational study on which the trial was powered. Furthermore the magnitudes of changes in EVLWI were also smaller possibly reflecting improved surgical technique and anaesthetic management or may reflect the higher baseline 25(OH)D₃ levels seen in these patients if our hypothesis is correct. Despite this significantly lower change in EVLWI and PVPI were seen in this study. Future vitamin D studies need to carefully consider if vitamin D concentrations and deficiency confirmed prior to enrolment.

In summary this phase II randomised controlled trial demonstrates the beneficial effects of cholecalciferol replacement on biomarkers of lung injury post a high risk insult and provides the first proof of concept that treatment pre-injury may prevent the development of ARDS and large relevant clinical trials are justified.

5.5 ACKNOWLEDGEMENTS

Patients were co-recruited with Dr Rachel Dancer and I am grateful for her assistance with the data collection. Clinical trial cytokine assays were performed by Dr Aaron Scott and Dr Vijay D'Souza.

CHAPTER 6
***IN-VITRO* AND *IN-VIVO* EFFECTS OF VITAMIN D ON**
MACROPHAGE FUNCTION

6.1 INTRODUCTION

Macrophages are important mediators of the acute respiratory distress syndrome (ARDS), forming the first line of response to pulmonary inflammation and infection. The nature of this response is intimately tied to the ongoing inflammatory cascade that ensues in the acute phase of the disease.²⁶³ In recent years macrophage function and phenotype are being increasingly recognised as important in the resolution and repair phase from ARDS.⁹⁰ Human studies of macrophage function in ARDS are limited, however, analysis of serial bronchoalveolar lavage fluid (BALF) from patients with ARDS has shown increased alveolar macrophage number is associated with survival²⁶⁴ and an immature monocyte-like cellular phenotype associated with reduced oxygenation and mortality.²⁶⁵ More recently Brittan *et al.* have confirmed the presence of increased immature monocyte-like cells compared to mature alveolar macrophages post exposure to inhaled lipopolysaccharide (LPS) in healthy volunteers.²⁶⁶ Furthermore patients with neutropenia-related sepsis and ARDS have deactivation of alveolar macrophages which is associated with poor survival²⁶⁷, suggesting macrophages may also play a central role in the development of ARDS.

Ingestion of apoptotic cells by macrophages termed 'efferocytosis' is vital in maintaining lung tissue homeostasis, resolving damaging inflammation and hastening tissue repair.²⁶⁸ Neutrophils have long been considered the key cell type in the pathogenesis and propagation of ARDS.^{1,54} Persistent neutrophilia and dysregulated macrophage clearance of apoptotic neutrophils leads to a

delayed clearance of neutrophilic injury, secondary necrosis and a prolonged inflammatory insult and tissue damage.^{98,269} The release of damage-associated molecular patterns (DAMPs), such as heat shock proteins (HSPs), high mobility group box 1 (HMGB-1) and mitochondrial and DNA peptides by the process of secondary necrosis propagates a second wave of inflammation and tissue damage.²⁶⁸ Inhibition of HMGB-1 in animal models has been shown to attenuate lung injury in pneumonia,^{270,271} ventilator-induced lung injury (VILI),²⁷² and sepsis.²⁷³⁻²⁷⁵ In humans, HMGB-1 concentration at presentation post severe trauma has been shown to predict the risk of developing acute lung injury.²⁷⁶ It is important to note that although neutrophil apoptosis may induce alveolar epithelial barrier dysfunction in early ARDS, apoptosis may also be a pre-requisite in the response to injury by limiting the duration of pulmonary inflammation by sequestering cytokines and endotoxins²⁷⁷ and stimulation of a pro-resolution alternatively activated M2 macrophage phenotype.²⁷⁸ Furthermore, efferocytosis itself induces the production of anti-inflammatory mediators IL-10 and TGF- β that dampen pro-inflammatory responses.²⁷⁹ Therefore, up-regulating efferocytosis and increasing macrophage maturation may be a strategy to reduce inflammation and promote resolution of ARDS.

Tissue macrophages are derived from circulating monocytes and dependent on the microenvironment and stimulus undergo classical M1 activation or alternative M2 activation.²⁸⁰ The M1 phenotype is induced by pro-inflammatory Th1 cytokines such as IFN γ and toll-like receptor (TLR) ligands

such as LPS. M1 macrophages produce high levels of proinflammatory cytokines, reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and have strong bactericidal activity, conversely M2 macrophages are induced by Th2 cytokines, IL-4 and IL-13 and produce anti-inflammatory IL-10 and are characterised by efficient phagocytic activity with a high expression of scavenging molecules.^{278,280,281} Studies to identify subpopulations of human macrophages are limited. However further subsets of M1 and M2 macrophages have been described,⁸⁹ with a discrete subset of alternatively activated (M2c) macrophages induced by macrophage colony stimulating factor (M-CSF) or glucocorticoids that display an increased efferocytosis phenotype and can be identified by reduced CD14 and increased expression of CD163, CD16, CD206, CD200R^{282,283} and the Mer tyrosine kinase (MerTK) efferocytosis receptor.^{284,285} It has also been shown that M1 macrophages can be polarised by granulocyte macrophage colony stimulating factor (GM-CSF) and distinguished by increased CD14 and CD80 expression.^{282,283} Importantly, evidence now suggests that these cells present a huge degree of heterogeneity and plasticity and can be present in continuum and determined by the microenvironment and cytokine and cellular milieu.²⁷⁸

Vitamin D has profound effects on the innate immune system. It stimulates the differentiation of precursor monocytes to mature phagocytic macrophages supported by the differential expression of the vitamin D receptor (VDR) and 1α -hydroxylase during human macrophage

differentiation.^{85,150} Vitamin D is known to rescue and enhance the phagocytic potential of macrophages to bacteria *in-vitro* but its effects upon neutrophil efferocytosis are unclear.^{238,286} Localised synthesis of 1,25(OH)₂D₃ by normal human macrophages on stimulation with IFN γ suggests an intracrine system exists for the action of vitamin D in normal monocytes and macrophages.^{85,143,151} IL-1 and TNF α production induced by TLR-3 agonists from monocyte derived macrophages are inhibited to the same extent by 25(OH)D₃ and 1,25(OH)₂D₃ suggesting that the vitamin D metabolites may have a rapid anti-inflammatory action and that local intracellular activation of 25(OH)D₃ can be anti-inflammatory.^{85,287} The ability of 1,25(OH)₂D₃ to directly inhibit NF- κ B signaling and suppress macrophage TLR expression suggests that vitamin D may also play a key role as a feedback regulator of macrophage responses.^{85,153,154} It has also been shown that 1,25(OH)₂D₃ affects differentiation, maturation and function of monocyte-derived dendritic cells by inhibiting differentiation and maturation into antigen presenting cells.²⁸⁸

Our group has previously reported low 25(OH)D₃ levels in patients undergoing oesophagectomy are associated with elevated postoperative systemic inflammation as measured by levels of IL-6 and HMGB1 and increased alveolar epithelial dysfunction.¹⁹⁸ Unpublished data from the group (PhD thesis, Dr Christopher Bassford, University of Warwick, 2011) has found that *in-vitro* HMGB-1 suppresses human alveolar macrophage efferocytosis in a dose dependent manner suggesting that elevated HMGB-1

may slow the resolution of neutrophilic injury resulting in exaggerated secondary necrosis of neutrophils.

The evidence presented suggests that treatment with vitamin D could have a favorable effect in ARDS by up-regulating macrophage resolution of neutrophilic injury. The primary objective of this study was to investigate if vitamin D₃ can modulate macrophage efferocytosis *in-vitro* and *in-vivo*. The second objective was to investigate the potential role of vitamin D₃ in modulating a monocyte differentiation to a pro-resolving phenotype.

6.2 MATERIALS AND METHODS

6.2.1 *In-vitro* studies

6.2.1.1 Efferocytosis Assays

Lung tissue samples were acquired as part of the Midlands Lung Tissue Collaborative as described in section 3.4.3 and processed to isolate human alveolar macrophages as described in section 3.5.4. Cells were treated for 24 hours and the effects of untreated media control [UTC, RPMI 1640 with 10% foetal bovine serum (FBS, Sigma-Aldrich, Poole, UK)], 25(OH)D₃: 50nmol/L and 100nmol/L (Merck Millipore, Watford, UK), 1,25(OH)₂D₃: 50nmol/L and 100nmol/L (Merck Millipore) and 150ng/mL HMGB-1 (Abcam, Cambridge, UK) on alveolar macrophage capacity to engulf apoptotic neutrophils was assessed by flow cytometry as described in section 3.6. The number of macrophages that had engulfed a neutrophil was calculated as an efferocytosis percentage (E%).

ARDS bronchoalveolar lavage fluid (BALF) from 4 patients (section 3.5.4) was pooled and mixed 1:1 with RPMI 1640 media and 10%FBS (Sigma-Aldrich). Macrophages were incubated with ARDS BALF and 50nmol/L 25(OH)D₃ (Merck Millipore) alone and in combination and efferocytosis assessed after 24 hours. Macrophage apoptosis was assessed to by Annexin V/Sytox staining on the flow cytometry using the same methods as described for murine cell apoptosis in section 3.9.7.

6.2.1.2 Monocyte differentiation and phenotype

Peripheral blood monocytes were extracted from healthy human volunteers and cultured as described in section 3.4 and 3.5. The effect of 50nmol/L 25(OH)D₃ (Merck Millipore), 50ng/mL GM-CSF (PeproTech, Rocky Hill, USA, 50ng/mL) and 50ng/mL M-CSF (PeproTech) with 10ng/mL IL-10 (PeproTech) on monocyte differentiation and phenotype was assessed by cell surface marker expression by flow cytometry as described in section 3.7.

6.2.2 *In-vivo* studies

Patients enrolled in a randomised placebo controlled trial investigating the effects of pre-operative high dose vitamin D₃ (cholecalciferol, 300,000IU) on postoperative extravascular lung water post oesophagectomy¹¹⁶ (described in previous chapter) were recruited to a bronchoscopic sub-study. A further 9 patients that were enrolled in a preliminary dosing study were also included in the correlation analysis (Open label dosing study to optimise vitamin d levels prior to oesophagectomy, REC 11/WM/0330, EudracT 2011-004199-12). Bronchoscopy and bronchoalveolar lavage were performed at the end of the operation as described in section 3.4.2 and alveolar macrophages isolated as described in section 3.5.5.

Blood was taken pre-drug dosing, pre-operative, post-operative, day 1 and day 3/4. Peripheral monocytes were isolated as described in section 3.5.2 Efferocytosis was assessed on alveolar macrophages and monocytes as described in section 3.7.

6.2.3 Statistical analysis

Data were analysed using GraphPad Prism 6.0[©] software (GraphPad Software, La Jolla, California, USA). Data were initially tested for normality using the D'Agostino-Pearson test. Normally distributed data were analysed using t-tests and presented as mean (standard deviation) and bar-charts. Non-parametric data were analysed using Wilcoxon signed-rank test, Mann-Whitney test or Friedman's ANOVA and Dunn's multiple comparison tests when comparing multiple groups. Non-parametric data are presented as median (interquartile range) and box and whisker plot with Tukey's distribution. Linear associations were tested using Spearman's correlation coefficient. A two-tailed p-value of <0.05 was considered statistically significant.

6.3 RESULTS

6.3.1 In-vitro studies

6.3.1.1 Vitamin D and efferocytosis

Alveolar macrophage (obtained from lung resection samples) efferocytosis was increased in response to 25(OH)D₃ (50nmol/L) compared to untreated control, UTC [mean E% 37.1 (23.9) vs. 27.4 (18.3), p=0.014, paired t tests]. The same effect was seen for 1,25(OH)₂D₃ (50nmol/L) [mean E% 33.7 (18.5) vs 27.4% (18.3), p=0.036, paired t-test] after 24 hours incubation. The data is presented in Figure 6.1 as non-parametric as fold change was not normally distributed. The *in-vitro* macrophage studies were not blinded to stimulation and performed on alveolar macrophages isolated from lung resection samples.

Dosing experiments were performed which showed no effect on increasing the concentration of both 25(OH)D₃ and 1,25(OH)₂D₃ to 100nmol/L (Figure 6.2). Therefore a dose of 50nmol/L was used for all the experiments. Ethanol was used as the vehicle carrier for the 25(OH)D₃ and 1,25(OH)₂D₃ *in-vitro* and this did not have an effect on efferocytosis. Due to baseline variability in efferocytosis in untreated control samples between each donor subject cells (Figure 6.3), differences in fold change from control were assessed and paired statistical tests performed.

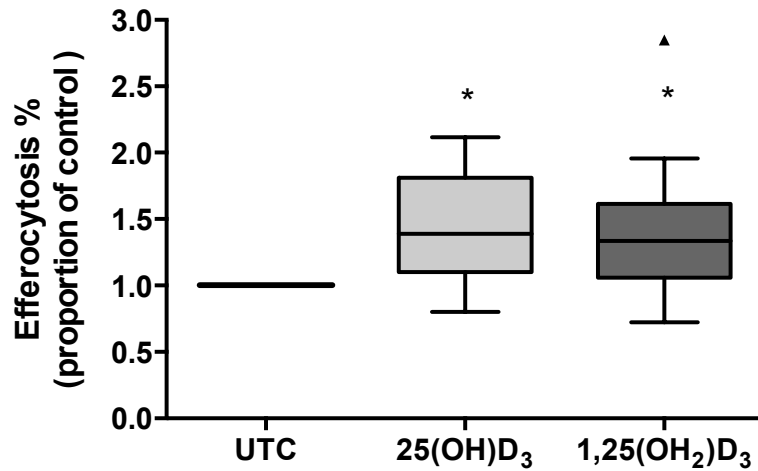


Figure 6.1: In-vitro effect of vitamin D on alveolar macrophage efferocytosis.

Alveolar macrophages obtained from lung resection samples. Results expressed as a proportion of control. Box and whisker plot with Tukeys distribution. Friedman's ANOVA ($p=0.006$, $n=10$) $*p<0.05$ compared to UTC (Dunn's multiple comparisons). 25(OH)D₃ (50nmol/L) and 1,25(OH)₂D₃ (50nmol/L). UTC=untreated media control; Black triangle: outlier

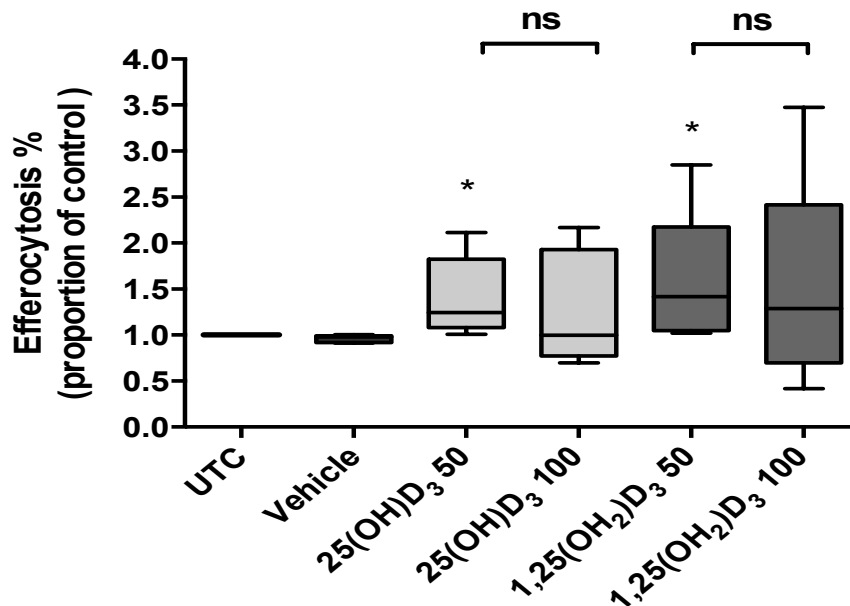


Figure 6.2: Dosing effects of vehicle, 50nmol/L and 100nmol/L of vitamin D.

Alveolar macrophages obtained from lung resection samples. Results expressed as a proportion of control. Box and whisker plots with Tukey's distribution. Friedman's ANOVA ($p=0.07$, $n=5$) $*p<0.05$ compared to UTC

(Dunn's multiple comparisons). Vehicle=Ethanol: UTC; untreated media control.

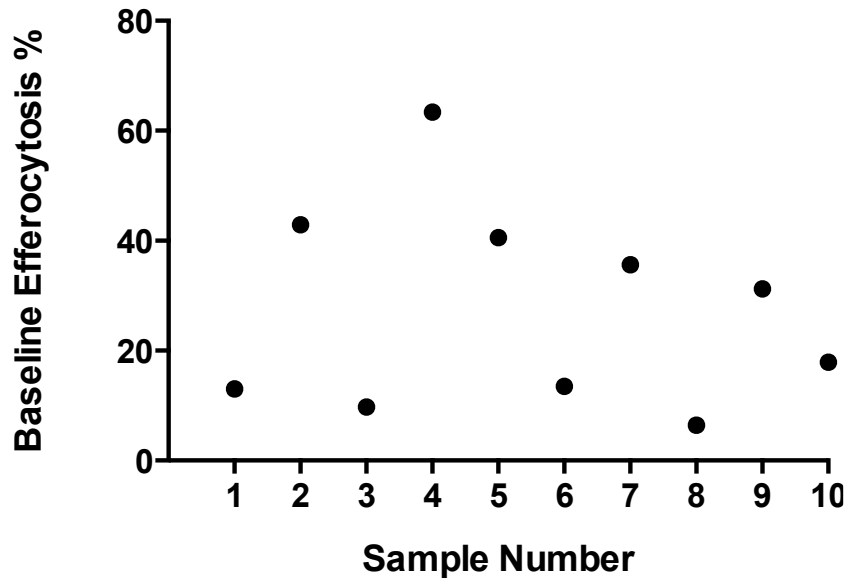
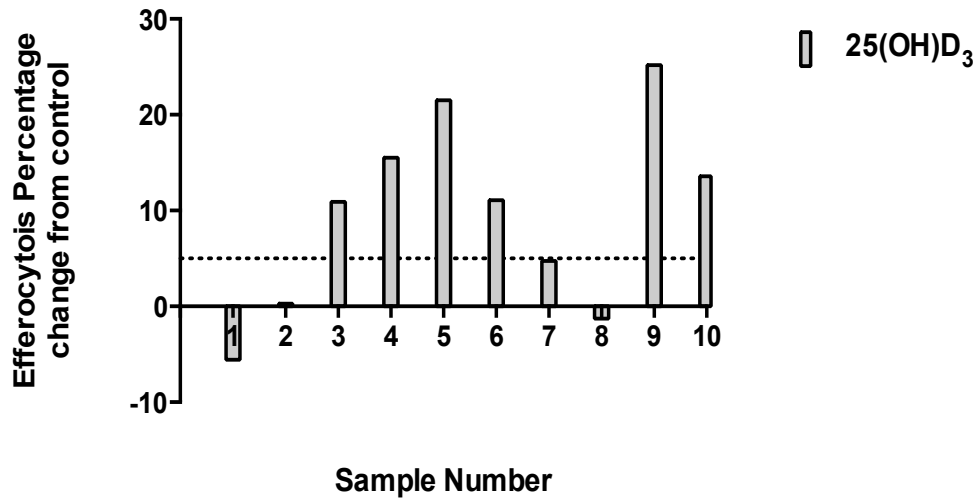


Figure 6.3: Baseline untreated alveolar macrophage efferocytosis variation.

Dot plot shows the huge variation in efferocytosis seen in untreated alveolar macrophages where each number on the x-axis represents an individual patient's sample.

Alveolar macrophages from lung resection specimen also demonstrated heterogeneous responses to exogenous vitamin D at basal state as shown in Figure 6.4. Cellular viability was unchanged between the samples. Unfortunately the vitamin D levels of the donors of lung tissue were not measured. Donor baseline demographics: age, sex, smoking status and lung function did not correlate with efferocytosis in this cohort, data not shown.

A



B

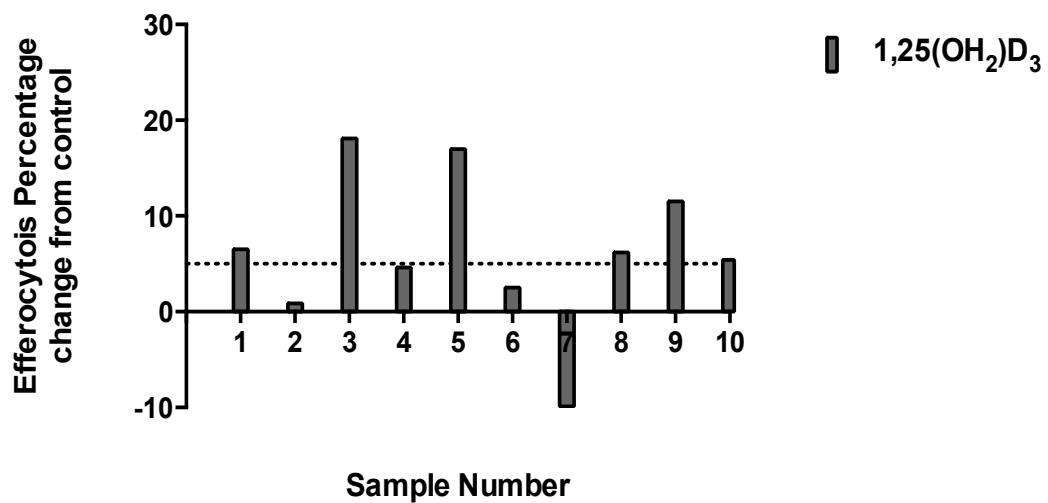


Figure 6.4: Graphs demonstrating the heterogeneous response of alveolar macrophage to exogenous vitamin D₃

A] 50nmol/L 25(OH)D₃; **B]** 50nmol/L 1,25(OH)₂D₃. Dotted line delineates 5% efferocytosis change from control to try and identify responder vs. non-responders to vitamin D₃ treatment.

6.3.1.2 ARDS lavage fluid and vitamin D

ARDS BALF suppressed alveolar macrophage efferocytosis compared to media control (median [IQR] E% 8.6 [7.74 - 13.9] vs 27.3% [18.2 - 32.2], $p=0.031$; Wilcoxon rank signed test). Addition of 50nmol/L of 25(OH)D₃ with ARDS BALF for 24 hours rescued efferocytosis to levels similar to that of 25(OH)D₃ treatment alone (median [IQR] E% 18.4 [14.7 - 36.1] vs 25.1 [18.2 - 38.9], $p=0.218$; Wilcoxon rank signed test) and significantly higher than that of BALF suppression (Figure 6.4). Macrophage apoptosis was assessed after incubation with 50% pooled ARDS BALF to ensure viability and this was confirmed to be maintained at >85%. Although 100% concentration of pooled ARDS dropped cell viability to <50%.

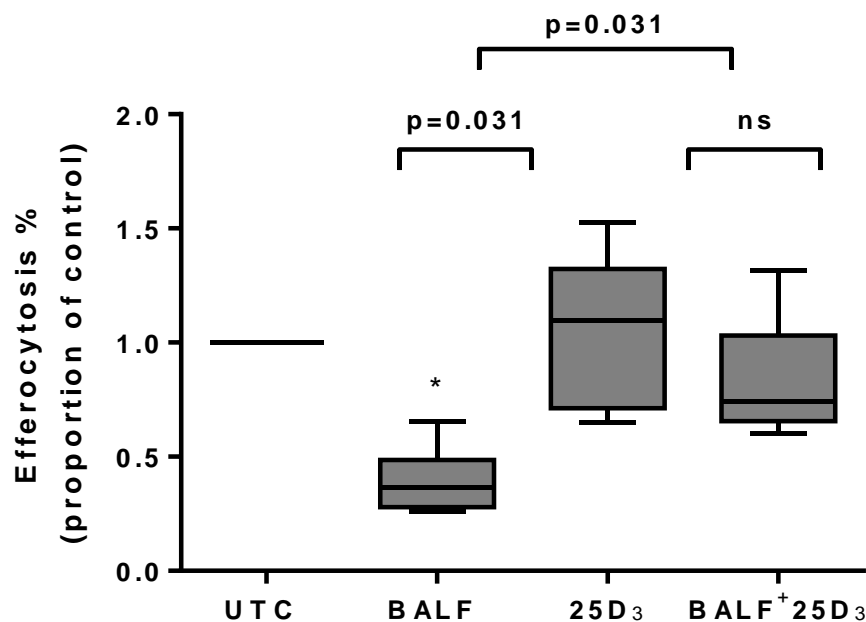


Figure 6.5: The effect of 25(OH)D₃ on ARDS BALF induced suppression of efferocytosis.

Results presented as a proportion of control. Box and whisker plot with Tukey's distribution. Friedman's ANOVA ($p<0.002$, $n=6$); * $p<0.05$ compared to UTC (Dunn's multiple comparisons). Within group analysis by Wilcoxon rank sign paired test. UTC=untreated media control.

6.3.1.3 HMGB-1 and vitamin D

A dosing study confirmed that HMGB-1 suppression of efferocytosis is dose dependent (Figure 6.6). Subsequently, HMGB-1 150ng/ml significantly suppressed efferocytosis compared to cells incubated with media control (median [IQR] E% 8.2 [7.2 – 17.1] vs 22.3 [20.1 – 28.9], $p=0.031$; Wilcoxon rank signed test)(Figure 6.7) but treatment with 50nmol/L of 25(OH)D₃ did not attenuate the HMGB-1 effects, suggesting that vitamin D effects on improving alveolar macrophage efferocytosis are HMGB-1 independent and other mechanisms are in play.

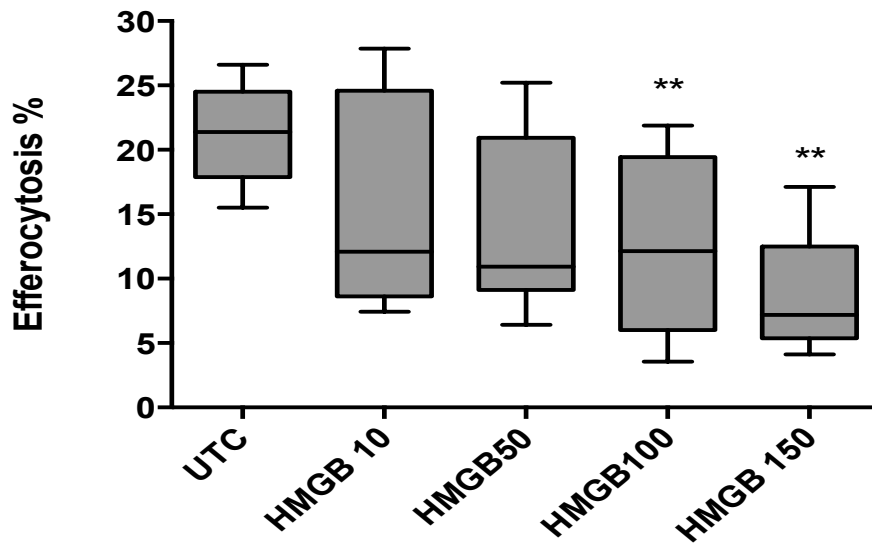


Figure 6.6: Dose response of HMGB-1 on macrophage efferocytosis.

Dose response of 10, 50, 100 and 150ng/mL

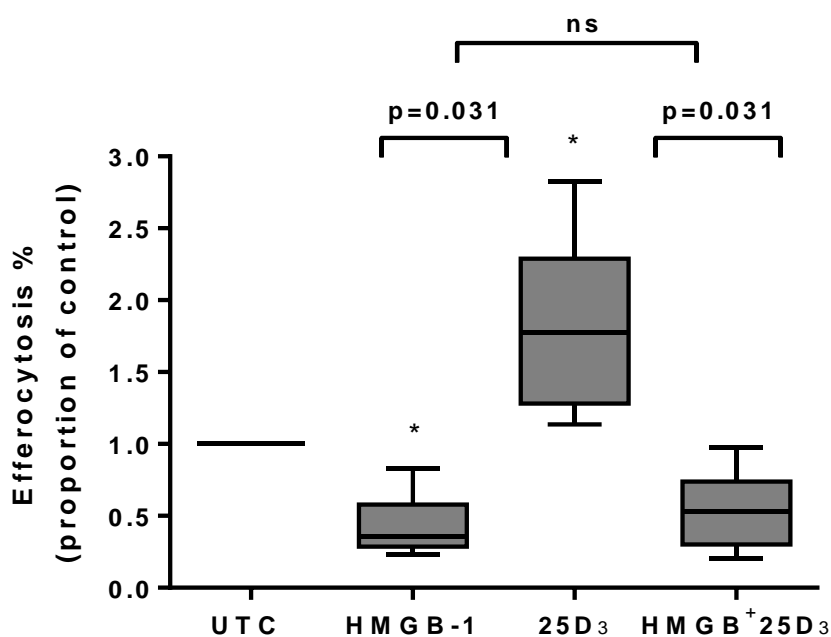


Figure 6.7: Effects of ARDS BALF and 25(OH)D₃ on efferocytosis.

Results presented as proportion of control. Box and whisker plot with Tukey's distribution. Friedman's test ANOVA ($p < 0.001$, $n = 6$); * $p < 0.05$ compared to UTC (Dunn's multiple comparison). Within group analysis by Wilcoxon rank sign paired tests. UTC=untreated media control.

6.3.1.4 Monocyte derived macrophages

To determine if 25(OH)D₃ can differentiate macrophages to an M2 phenotype, normal human monocytes were isolated and incubated with 50nmol/L 25(OH)D₃ for 4 days. 25(OH)D₃ promoted monocyte maturation into a cellular phenotype that was high in the expression of CD16, CD163, CD200R but low in the expression of CD80 (Figure 6.8). These cells had surface marker expression profiles similar to cells stimulated with M-CSF and

Il-10 and not GM-CSF, confirming a typical alternatively activated macrophage. There was no difference in the expression of CD14 and CD206 between the cells and no significant up regulation of the efferocytosis receptor MerTK (Figure 6.9), suggesting that other receptors or pathways are likely to be responsible for the increase in efferocytosis seen in response to vitamin D in the previous experiments.

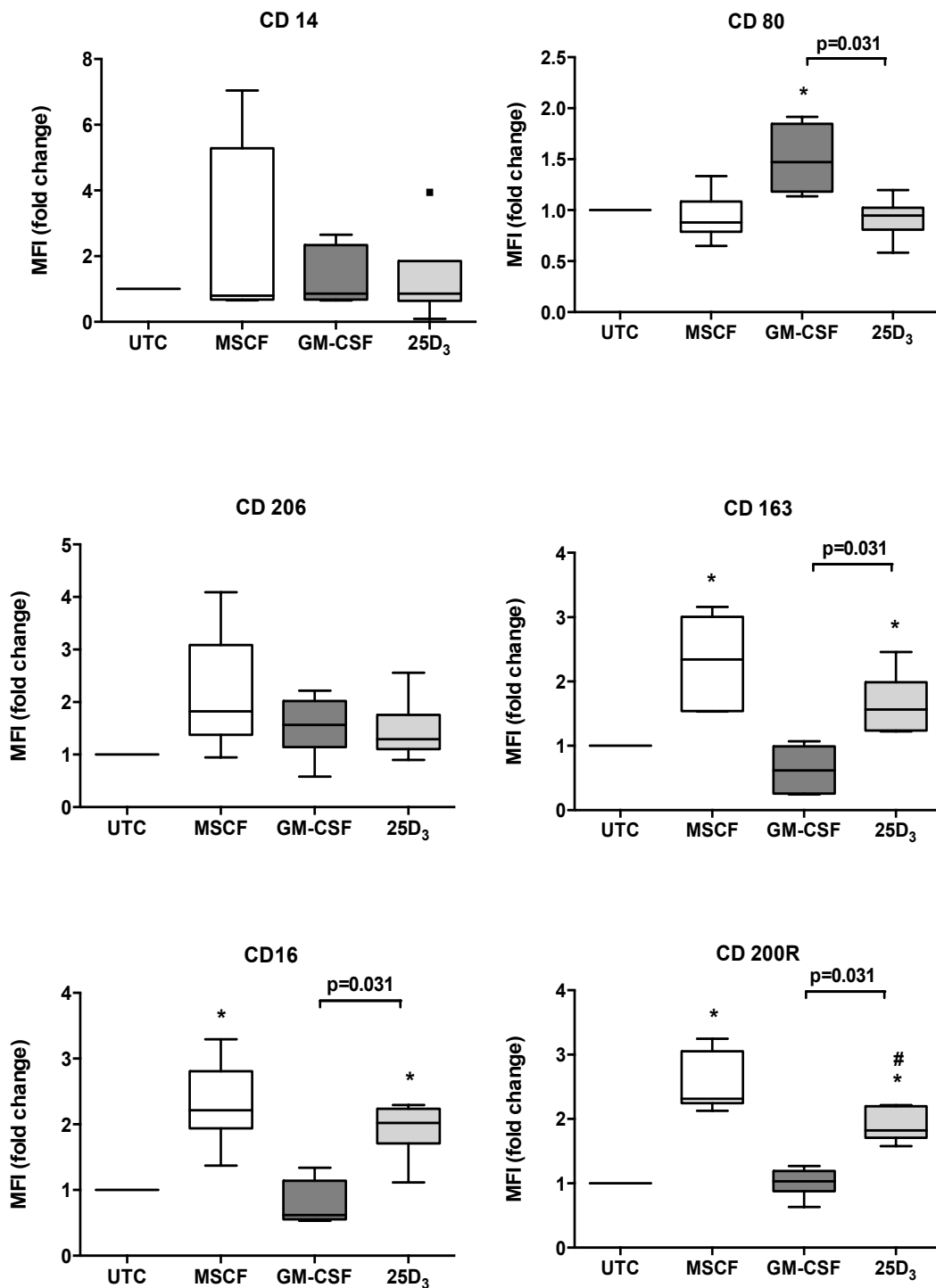


Figure 6.8: Cell surface expression profile of monocyte derived macrophages stimulated with 25(OH)D₃.

Data presented as fold change from UTC. Box and whisker plots with median and Tukey's distribution. Friedman ANOVA's with Dunn's multiple comparisons. n=6, *p<0.05 compared to UTC; #p<0.05 compared to M-CSF. UTC: untreated media control; M-CSF: macrophage colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor.

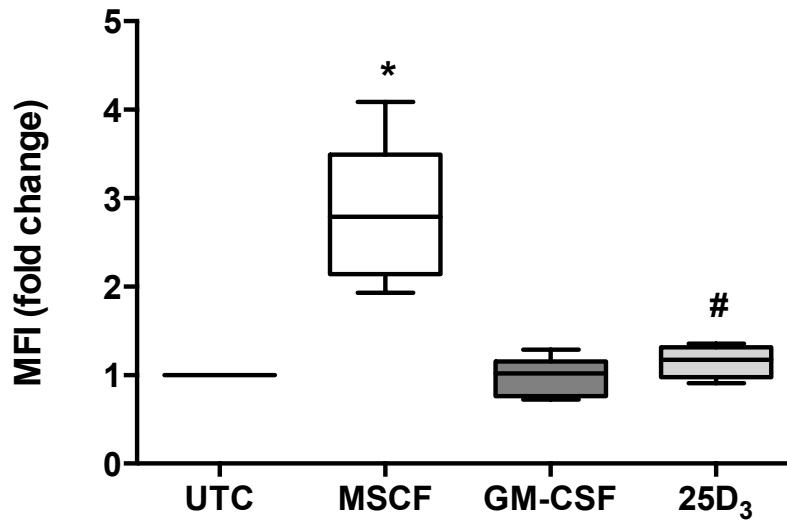


Figure 6.9: MerTK expression is not increased by 25(OH)D₃.

Data presented as fold change from control. Box and whisker plot with median and Tukey's distribution; Friedman's ANOVA ($p=0.007$, $n=6$); $*p<0.05$ compared to UTC; $p<0.05$ compared to M-CSF (Dunn's multiple comparison). UTC: untreated media control; M-CSF: macrophage colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor.

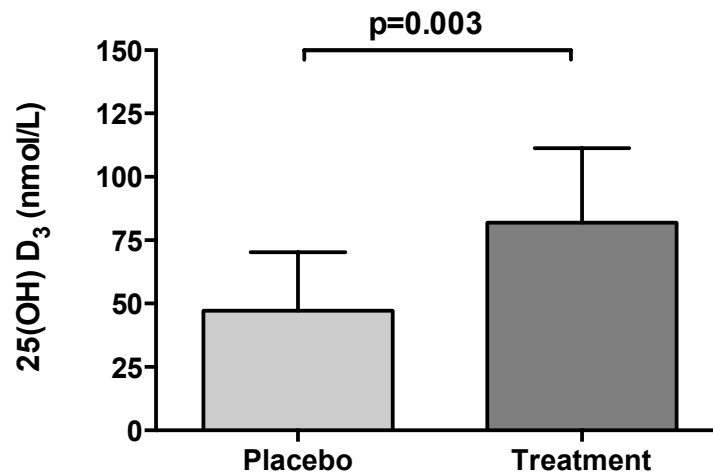
6.3.2 In-vivo studies

Twenty-five patients were recruited to a bronchoscopic sub-study of the vitamin D to prevent acute lung injury following oesophagectomy (VINDALOO) trial. After un-blinding the trial 15 subjects had received placebo and 10 high dose vitamin D treatment (oral liquid cholecalciferol 300,000IU) as per trial protocol. Groups were equally matched in baseline characteristics similar to chapter 5 and therefore data not represented.

Cholecalciferol treatment successfully increased plasma 25(OH)D₃ concentration to sufficient levels compared to patients that received placebo

[mean 81.9 (5.7) vs 47.2 (9.3) nmol/L, $p=0.003$, unpaired t-test)] (Figure 6.10). This was represented in a corresponding rise in $1,25(\text{OH})_2\text{D}_3$ levels compared to placebo [mean 124.5 (11.3) vs 91.0 (7.4) pmol/L, $p=0.016$, unpaired t-test]. Thus, confirming both successful replenishment and physiological effect.

A



B

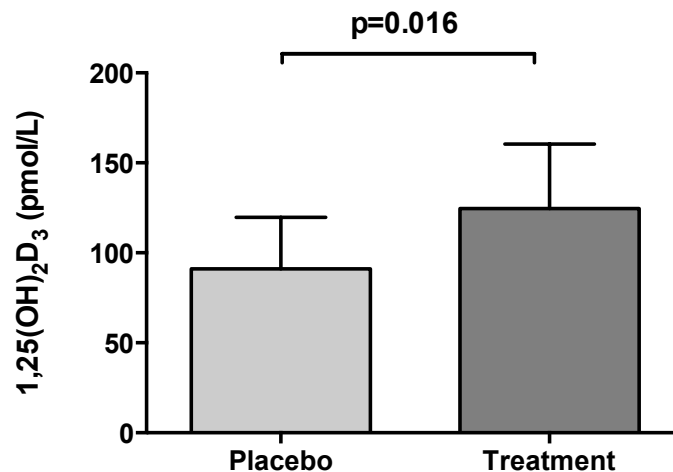


Figure 6.10: Plasma concentration of vitamin D levels post treatment. Bar charts with mean and standard deviations. **A]** $25(\text{OH})\text{D}_3$ levels; **B]** $1,25(\text{OH})_2\text{D}_3$ levels. p-values represent unpaired t-tests.

6.3.2.1 Alveolar macrophage efferocytosis

Alveolar macrophage efferocytosis was not significantly different when comparing placebo to vitamin D treated groups [mean E% 31.3 (3.98) vs 39.6 (8.15), $p=0.32$; unpaired t-test] (Figure 6.11). However the 25(OH)D₃ levels at enrolment in this patient cohort were higher than previously reported¹⁹⁸ with a mean 25(OH)D₃ level of 51.2 (4.86) nmol/L which is classified as insufficient rather than deficient (<50nmol/L).

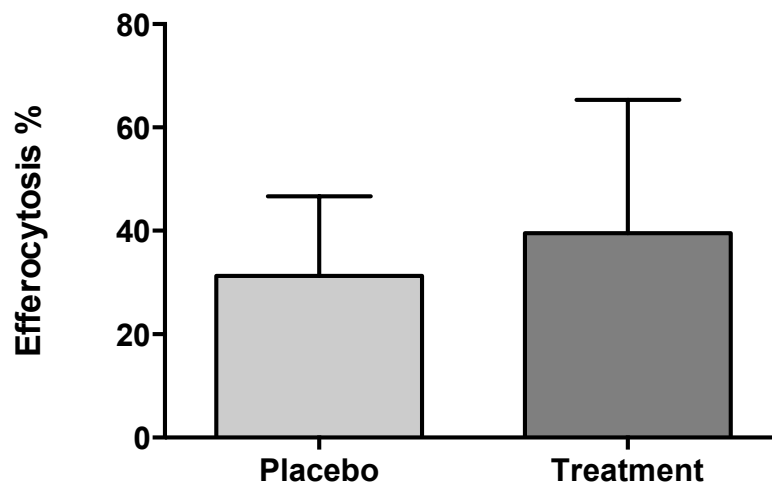


Figure 6.11: Effects of placebo vs treatment on alveolar macrophage efferocytosis.

Bar chart with mean and standard deviation. Placebo n=15, Treatment n=10.

We therefore analysed the results in 2 groups based on 25(OH)D₃ concentration post treatment (deficient <50nmol/L and sufficient >50nmol/L). This demonstrates that if the 25(OH)D₃ concentration was lower than 50nmol/L post treatment then ex-vivo alveolar macrophage efferocytosis was significantly lower than those with levels >50nmol/L [mean E% 18.5 (2.9) vs. 38.9 (4.8), p=0.01; unpaired t-test](Figure 6.12).

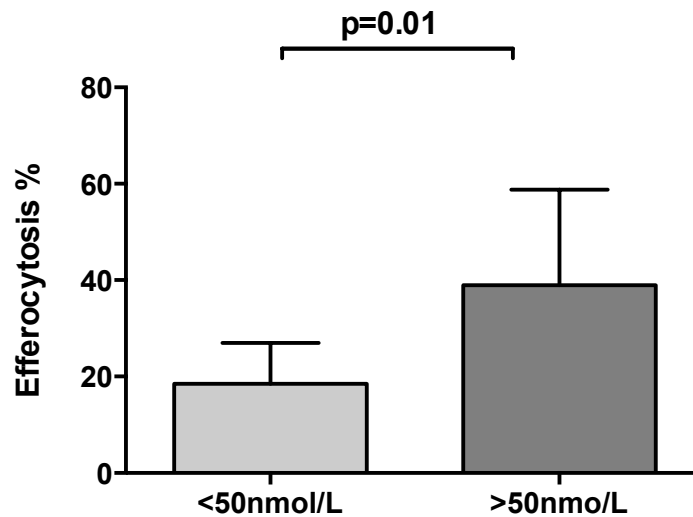


Figure 6.12: Efferocytosis is lower in patients with 25(OH)D₃ deficiency compared to sufficient patients.

Box and whisker plots with median and Tukey's distribution. Deficient <50nmol/L n=8, sufficient >50nmol/L n=17; p=0.01 unpaired t-test.

Due to the imbalance in the proportion of placebo and active treatment in this group, further subdivision of the placebo arm into placebo deficient and placebo sufficient demonstrated a significantly higher efferocytosis percentage in the vitamin D sufficient group (median [IQR] E% 37.6 [34.7-45.5] vs. 15.5 [11.0 - 27.1], p=0.0006, Mann-Whitney test)(Figure 6.13) with

the placebo sufficient group having a similar E% compared to the treated group (median [IQR] E% 35.2 [13.3 – 64.8], $p=0.69$). Furthermore, plasma 25(OH)D₃ levels post treatment and pre-operatively showed a weak positive correlation to efferocytosis ($n=34$, Spearman $r=0.469$ $p=0.005$) (Figure 6.14). This correlation included 9 further experiments from a preceding open label dosing study¹⁹⁸ that weren't included in the analysis of the samples obtained from the randomised control trial as this was a dose escalation study. Samples and experiments were processed in the same standardised methodology described.

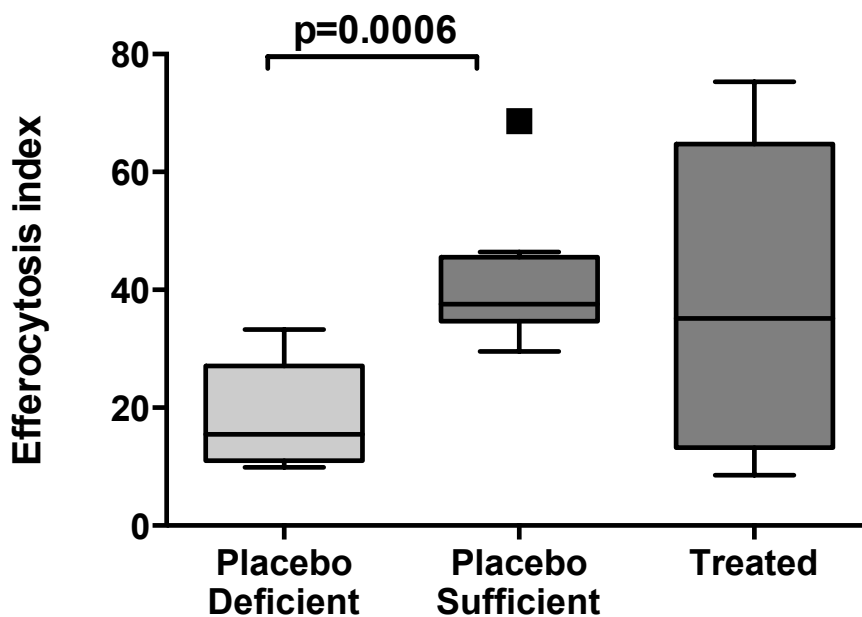


Figure 6.13: Placebo treated patients with sufficient 25(OH)D₃ levels have higher efferocytosis compared to deficient.

Box and whisker plot with median and Tukey's distribution. Placebo deficient <50nmol/L $n=7$, placebo sufficient >50nmol/L $n=8$ and Treated $n=10$. $P=0.0006$, Mann-Whitney test.

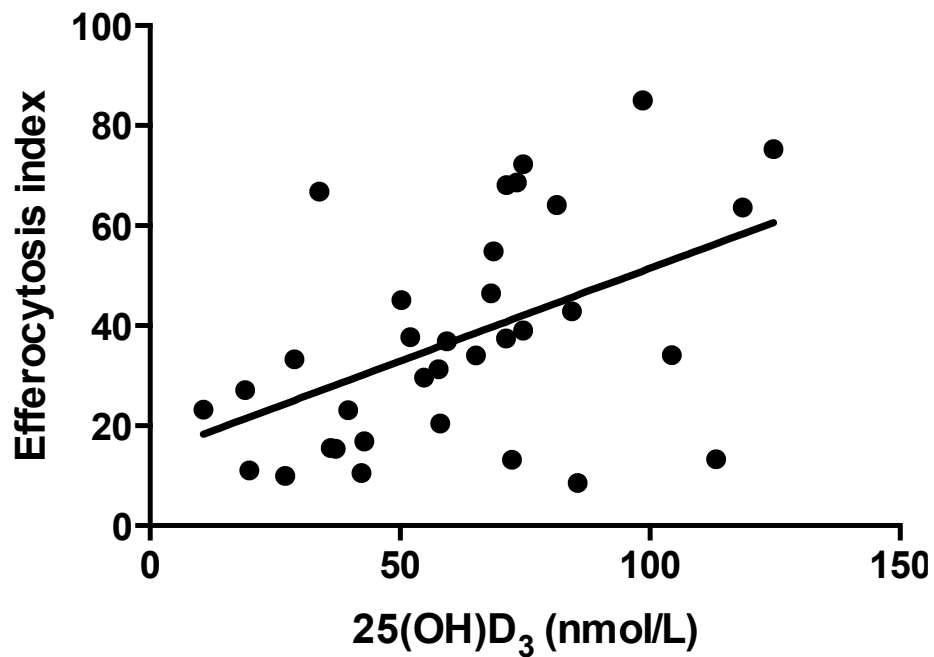


Figure 6.14: Correlation of post dose 25(OH)D₃ concentration and efferocytosis. n=34, Spearman r=0.469, p=0.005.

6.3.2.2 Monocyte efferocytosis

Pre-drug administration, pre-operative and day 3/4 monocytes were isolated and efferocytosis was assessed after 24 hours of culture for 23 patients (11 placebo; 12 treatment arm) There was no difference seen between placebo and cholecalciferol treated groups in *ex-vivo* cultured monocyte efferocytosis at 24 hours at any time-point. Post-treatment 25(OH)D₃ concentration irrespective of allocated treatment arm and level of deficiency did not relate to monocyte efferocytosis as seen with alveolar macrophages.

6.4 DISCUSSION

There are no studies reported investigating the effects of vitamin D₃ (25(OH)D₃) on human alveolar macrophage function and specifically efferocytosis in the context of acute inflammation. In this study we have shown that 25(OH)D₃ and 1,25(OH)₂D₃ at a dose of 50nmol/L can increase the efferocytosis potential of macrophages *in-vitro* and 50nmol/L of 25(OH)D₃ promotes pro-resolving M2 differentiation of peripheral monocyte derived-macrophages. At basal un-stimulated state macrophage response is heterogeneous. Unfortunately in this study we were unable to account for the vitamin D levels of the donors of lung tissue that may account for the variable macrophage responses seen to vitamin D treatment. Smoking and chronic obstructive airways disease (COPD) are known to reduce efferocytosis.^{289,290} However, this small study was not powered to detect a difference between smoking status, lung function and efferocytosis.

Most of the *in-vitro* studies to date that have investigated the effects of vitamin D₃ on the modulation of the innate immune system have concentrated on treatments using 1,25(OH)₂D₃ or synthetic analogs²⁹¹. However, it is evident from macrophage/monocyte responses to infection that actions of vitamin D₃ are likely to be due to the local activation of 25(OH)D₃, the major circulating form and accepted determinant of vitamin D status.¹⁹⁶ Local cellular 25(OH)D₃ activation may be influenced by genetic polymorphisms of both the 1- α hydroxylase enzyme (CYP27B1) and the vitamin D receptor (VDR) in individuals. This is evident in patients with

tuberculosis where improved sputum conversion time was seen in a specific subset of patients with a single nucleotide polymorphism (SNP) within the VDR gene and not in the overall cohort that were supplemented with vitamin D₃.²⁹² Therefore the varying efferocytosis responses seen in this study may be accounted for in part by individual genetic polymorphisms of elements of the vitamin D metabolic pathway.

We have demonstrated for the first time that alveolar macrophage efferocytosis is suppressed by ARDS BALF and HMGB-1. Treatment with 25(OH)D₃ attenuated the effect of ARDS BALF but not HMGB-1, suggesting that the actions of vitamin D on rescuing efferocytosis are independent of HMGB-1. Whilst this is a surprising result as 1,25(OH)₂D₃ has been shown to dampen NFκβ induced Type-1 pro-inflammatory cytokine release,^{154,156} there are many other determinants of efferocytosis that may be implicated including induction of other efferocytosis receptors (e.g. Lipoxin A₄, ALX, Stabilins), bridge molecules (Gas6, protein S and collectins), enhancing Rac 1 GTPase signalling, activation of the peroxisome proliferator-activated receptor (PPARγ) and retinoid X receptor (RXR).²⁷⁹ The effects of 25(OH)D₃ are mediated by activation of the VDR. VDR and its ligand 1,25(OH)₂D₃, dimerise with the retinoid X receptor (RXR) and attach to specific genomic sequences termed vitamin D response elements (VDRE).⁸⁵ The transcription of VDR target genes results in cell growth inhibition, induces apoptosis, controls proliferation and differentiation.^{118,120,121,293} The mechanism by

which efferocytosis is attenuated with 25(OH)D₃ treatment *in-vitro* warrants further investigations.

This study shows that 25(OH)D₃ induced monocyte maturation towards an M2 alternatively activated phenotype as defined by cell surface markers. This is in keeping with previous studies that have shown that vitamin D can modulate monocyte maturation to mature phagocytic macrophages.¹⁵⁰ This effect would be in keeping with our hypothesis that vitamin D₃ acts to promote an anti-inflammatory and resolution phenotype and also supports the increased efferocytosis seen in the alveolar macrophages in this study. However the expression of the efferocytosis receptor MerTK was not increased in this study and therefore other mechanisms and pathways as discussed above need to be further investigated.

The *in-vivo* study of high dose cholecalciferol replacement did not show a difference in alveolar macrophage efferocytosis between placebo and cholecalciferol treatment arm. However, subjects who had vitamin D deficiency (<50nmol/L) irrespective of treatment arm had lower macrophage efferocytosis. Furthermore, subjects in the placebo group that had 25(OH)D₃ concentrations of >50nmol/L had efferocytosis levels similar to that of the treatment arm. Suggesting that vitamin D actions on alveolar macrophage efferocytosis are dependent on being deficient and sufficient circulating concentrations may act as a negative feedback loop on the cellular actions of

vitamin D. This finding would support the idea that vitamin D metabolism in the macrophage has an intracrine control mechanism.¹²⁷

There was no effect of *in-vivo* cholecalciferol on monocyte efferocytosis. This may be due to the fact that these cells were only incubated for 24 hours and may have represented immature poorly differentiated cells or the actions of vitamin D₃ on promoting monocyte efferocytosis require further adequate inflammatory stimulation. In hindsight these experiments should have been done after 3/4 days of incubation similar to the phenotype experiments. To elucidate and investigate this further efferocytosis could have been determined *in-vitro* on monocyte-derived macrophages with different inflammatory stimuli and potential attenuating effects of vitamin D₃ tested.

In conclusion, this study has demonstrated for the first time that vitamin D₃ enhances macrophage efferocytosis *in-vitro* and *in-vivo* and is dependent on baseline vitamin D status. Vitamin D₃ also increased the expression of markers of a pro-resolving phenotype on monocyte-derived macrophages. Further studies are required to confirm this and investigate the potential mechanistic pathways and whether vitamin D₃ can modulate already differentiated alveolar macrophages to a pro-resolving phenotype. These results support and may go some way in explaining the reduced change in extravascular lung water seen post-oesophagectomy in the cholecalciferol arm of the VINDALOO Trial (chapter 5).

CHAPTER 7

THESIS SUMMARY

7.1 OVERVIEW

ARDS is a severe inflammatory condition that remains an important cause of respiratory failure and morbidity and mortality in the critically ill. The paucity of effective pharmacological therapies for ARDS combined with the public health impact make the development of effective therapies a major unmet health need. Identifying and treating modifiable pre-determinants of ARDS is a potential important strategy for the management of this condition.

Vitamin D deficiency is common, is associated with sepsis and ARDS and replacement therapy cheap and easy. However, whether this association is causal is yet to be discerned. This thesis investigated three important questions related to the mechanistic relevance of vitamin D deficiency and its treatment on ARDS: 1] Does vitamin D deficiency exaggerate the inflammatory response and lung injury in a murine model of sepsis? 2] Can vitamin D replacement improve biomarkers and therefore prevent ARDS in a relevant human model? 3] Does vitamin D influence the innate immune response and resolution of neutrophilic injury?

7.2 MURINE MODEL OF SEPSIS

In this study of CLP induced polymicrobial sepsis, mice that were vitamin D₃ deficient pre-injury demonstrated an increase in bacterial load in the peritoneum with associated increased bacteraemia and bacterial translocation to the lung. We observed reduced levels of the murine cathelicidin antimicrobial peptide, CRAMP (in blood and alveolar

compartments) in vitamin D deficient mice that might explain the reduced bacterial load seen. This confirms findings of studies of other cell types and models of the role of vitamin D₃ in modulating antimicrobial peptide release. However this is not reflected in our human study.

Although the organisms grown in the bacterial cultures were not qualified, we have shown defective *ex-vivo* macrophage phagocytosis of *E.Coli* bacteria from vitamin D₃ deficient mice. The effect of vitamin D₃ on macrophage phagocytosis of *mycobacteria* has been established however; this is the first study to our knowledge to demonstrate this effect on *E.Coli*. This may be due to reduced levels of cathelicidin, which has been shown to increase macrophage phagocytosis of *pseudomonas*²⁹⁴ and *mycobacterium*²³⁸ or secondary to suppressed macrophage TLR and PRR responses to bacterial challenge.¹⁵⁴

We have also demonstrated an increased number of apoptotic neutrophils in vitamin D deficient peritoneal lining fluid, suggesting defective clearance by macrophages, termed efferocytosis or increased neutrophil apoptosis. Prolonged presence of apoptotic neutrophils can perpetuate inflammation due to secondary necrosis and further release of pro-inflammatory cytokines that then cause further damage of the surrounding epithelial-endothelial barrier and organ dysfunction. Furthermore vitamin D₃ deficient mice had increased protein permeability in bronchoalveolar and peritoneal lavage fluid, suggesting defective barrier integrity of the epithelial-endothelial

membrane, which may also account for the increased bacterial translocation seen in the vascular and alveolar compartments. Surprisingly we did not observe an exaggerated pro-inflammatory cytokine response in vitamin D₃ deficient mice suggesting that vitamin D₃ effects are primarily driven by its action on cellular and barrier function.

Although there was evidence of early alveolar capillary leak, this was small and we can confirm that this model of murine sepsis at this time point does not induce the pathophysiological changes of ARDS and cannot be recommended for future studies of the condition. It is however a good model of early sepsis and maybe useful in ARDS studies if used in conjunction with secondary injurious models such as an intra-tracheal acid/LPS challenge/pneumonia.

Since the mice were deficient in vitamin D₃ prior to the initiation of sepsis our findings add strength to the argument that vitamin D₃ is causal rather than an effect of sepsis or critical illness that has been suggested by observational studies to date. These experiments also support the hypothesis that vitamin D deficiency is a mechanistic driver of sepsis and inflammation by decreasing cellular macrophage phagocytosis and barrier integrity and the need for more translational studies and a clinical trial of vitamin D treatment in sepsis.

7.3 VITAMIN D TO PREVENT ACUTE LUNG INJURY TRIAL

This study investigated the hypothesis that a single high-dose of oral cholecalciferol (vitamin D₃) pre-operatively would prevent the increase seen in extravascular lung water post-oesophagectomy. The secondary aim was to evaluate the effect of cholecalciferol on other biomarkers of lung injury, clinical outcomes and vitamin D status (circulating concentrations of 25(OH)D₃, and 1,25(OH)₂D).

The randomised placebo controlled trial demonstrated that patients treated with cholecalciferol had significantly lower increases in EVLWI post-oesophagectomy. This occurred in parallel with a reduced increase in PVPI, a marker of alveolar epithelial capillary leak. Increases in EVLWI and PVPI were more pronounced if post-treatment 25(OH)D₃ concentration, irrelevant of study arm was <50nmol/L. This is a level that is widely regarded as being the cut-off for deficiency and may represent a threshold level of benefit. Treatment with cholecalciferol conferred no benefit on clinical outcomes and incidence of ARDS, however this trial was not powered to investigate these outcomes. This study did not show a convincing effect on pro-inflammatory cytokine response which supports our finding of no difference in pro-inflammatory cytokines between vitamin D₃ deficiency and sufficiency in the murine sepsis model.

This trial provides the first proof of concept that in humans at risk of ARDS secondary to oesophagectomy preoperative treatment with cholecalciferol

successfully replenishes 25(OH)D₃ to sufficient concentrations and reduces biomarkers of alveolar epithelial injury. This adds support for the need for a large multi-centre trial powered to investigate patient clinical outcomes.

The level of vitamin D₃ deficiency, incidence of ARDS and magnitude of increases in EVLWI in the current study were much lower than our previous cohorts. This may in part reflect improved nutrition pre-operatively and better perioperative surgical and anaesthetic management. This makes the feasibility of further studies in this model more complex and has already informed the design of follow-on studies of statin therapy in this patient group (Dr Murali Shyamsundar, Queen's University Belfast). Despite this it does prove the mechanistic importance of vitamin D sufficiency in protecting the alveolar epithelial barrier from injury and could be translated to a treatment trial in ARDS or prevention trial in groups at risk of ARDS in whom vitamin D₃ status could be measured rapidly to identify deficiency and hence most likely to benefit from treatment.

Finally we can confirm that a high dose of 300,000IU cholecalciferol can successfully replenish 25(OH)D₃ concentration to levels deemed sufficient and that this correlates with an increase in the active 1,25(OH₂)D₃ metabolite. However there is a drop in these levels post-operatively by day 3 and suggests that future replacement trials should investigate whether a regular interval-dosing regime has greater clinical efficacy.

7.4 IN-VITRO AND IN-VIVO MACROPHAGE STUDIES

Clearance of apoptotic neutrophils is an important macrophage function in the resolution of inflammation. We have for the first time demonstrated *in-vitro* increased efferocytosis of primary human alveolar macrophages in response to incubation with 25(OH)D₃ and 1,25(OH₂)D₃. Interestingly basal state efferocytosis and response to vitamin D₃ stimulation is heterogeneous and may reflect the vitamin D status of the donors of the cells or genetic polymorphisms in the VDR and regulating enzymes of the vitamin D pathway. We have confirmed that BALF from patients with ARDS suppresses efferocytosis and shown that treatment with 25(OH)D₃ can attenuate this effect suggesting a possible mechanistic role for vitamin D₃ in the resolution of inflammation in ARDS. HMGB-1 has been found to be present in ARDS BALF and is released from activated macrophages and necrotic cells. This study confirms HMGB-1 dose-dependent decreased efferocytosis *in-vitro* that is not rescued by 25(OH)D₃. Therefore it is likely that 25(OH)D₃ increases efferocytosis by means of other pathways that need further elucidating. In support of the findings of increased efferocytosis we have shown for the first time that 25(OH)D₃ increases the expression of M2 pro-resolution phenotype surface markers on peripheral blood monocyte differentiated macrophages *in-vitro*.

In parallel to the randomised control trial, the *in-vivo* effect of cholecalciferol (vitamin D₃) on alveolar macrophage efferocytosis was assessed. No difference was seen in efferocytosis between placebo and cholecalciferol

treated groups, however patients who were deficient (<50nmol/L) irrespective of treatment arm had lower efferocytosis. This is supported by the finding of a positive correlation between 25(OH)D₃ plasma concentration and alveolar macrophage efferocytosis. This also complements the finding of increased EVLWI and PVPI in patients who remained deficient post treatment and suggests that pre-insult vitamin D₃ deficiency confers an increased risk of exaggerated injury and dampened macrophage resolution of neutrophilic injury. Future studies therefore need to be able to identify deficiency prior to treatment, as these patients are most likely to benefit. Finally a similar effect was not seen on monocyte efferocytosis suggesting that the effects of vitamin D₃ may require cellular differentiation in the presence of sufficient levels of vitamin D₃

7.5 THESIS LIMITATIONS

The work presented in this thesis had a number of limitations that are summarised below.

Due to the severity of the model and local and UK regulations, a major limitation of the murine study was that we were unable to extend the time-point post CLP to induce sufficient lung injury. At 24 hours mice were too unwell and therefore we had to reduce this to 16 hours and therefore we did not observe significant cell recruitment and neutrophilic alveolitis. Lung histology and markers of endothelial damage may have provided evidence of early lung injury. Furthermore although we have shown the effects of vitamin

D₃ deficiency in a murine model of sepsis and potential mechanisms this needs to be confirmed in other sepsis and murine models. Finally pre-injury replacement of vitamin D₃ would have provided much more strength to the evidence and argument of its potential functional importance in preventing sepsis.

The clinical trial was limited by the fact that the baseline vitamin D₃ deficiency of the patient cohort, magnitude of changes in EVLWI and incidence of ARDS were much lower than the cohort that this study was powered on and designed. A major weakness of the study is that lung water measurements were only carried out until post-operative day 1 and may have missed patients developing ARDS beyond this time-point. This study was a phase II biomarker study and therefore although provides proof of concept and mechanistic insights needs validation in other models and a large clinical trial of prevention and treatment of ARDS.

The macrophage cells studies are limited by the fact that donor vitamin D levels were unknown and may have influenced the efferocytosis responses seen. This should be tested in further studies. Furthermore despite the *in-vivo* assays being blinded the *in-vitro* studies were not blinded.

7.6 FUTURE RESEARCH

The work presented in this thesis provides evidence and a compelling argument for the role of vitamin D deficiency in promoting exaggerated responses to sepsis and acute inflammation and the potential beneficial effects of replacing vitamin D₃ but requires more work to determine mechanistic pathways, cellular responses to vitamin D₃ and determine clinical efficacy in disease:

1] Murine treatment studies are required in valid models of sepsis. Combining a 2 hit model of CLP and intra-tracheal pneumonia may better serve to model all the elements of ARDS and sepsis. Assessing physiological measures of injury (e.g. oxygen saturations, lactate) may be beneficial and more translatable.

2] A dosing study in sepsis and ARDS is required to assess the optimal dosing and maintenance strategy in this critically ill population as vitamin D₃ concentrations have been shown to drop on admission to critical care and confirmed in our oesophagectomy population.

3] Genetic studies of the variable responses to treatment and replacement are required as there may be some patients who are more likely to benefit over others.

4] Measuring vitamin D₃ status takes at least a few days and developing a bedside test may be useful to determine at the point of admission to hospital patients that may benefit from treatment as this may only be beneficial in patients who are deficient below a threshold.

5] More studies are required to ascertain the 'sufficient' level that confers a cellular benefit as this may reflect why some vitamin D₃ replacement trials have proven negative.

6] A large clinical trial with relevant clinical outcome measures is required in both ARDS and sepsis.

7] More mechanistic studies are required to determine the and pathways of increased efferocytosis and to determine if the pro-resolution phenotype seen in monocyte derived macrophages confers a functional benefit.

7.7 CONCLUSIONS

In conclusion, the studies presented in this thesis aimed to elucidate if vitamin D deficiency is a mechanistic driver of ARDS and if treatment with vitamin D₃ may protect against exaggerated inflammation and promote its resolution. Using murine and human models complemented with *in-vitro* cellular studies it has been demonstrated that vitamin D deficiency promotes defective resolution of infection and inflammation and treatment protects lung barrier integrity and improves cellular resolution of neutrophilic injury. It is hoped that the results from these studies will add credence to the argument that vitamin D deficiency is not simply a consequence of critical illness but a mechanistic driver of inflammation, and lead to large clinical trials in ARDS and sepsis that may lead to a simple, cheap and safe treatment.

CHAPTER 8

ABBREVIATIONS

AECC	American European consensus conference (on ARDS)
ALI	Acute lung injury
AM	Alveolar macrophage
ANOVA	One-way analysis of variance
APACHE	Acute Physiology and Chronic Health Evaluation
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
BALF	Broncho-alveolar lavage fluid
BALTI	Beta-Agonist Lung Injury Trial
BMI	Body mass index
BSA	Bovine serum albumin
CI	Cardiac index
CFU	Colony forming unit
CLP	Caecal ligation and puncture
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
CO ₂	Carbon dioxide
CPAP	Continuous positive airway pressure
CRAMP	Cathelicidin related antimicrobial peptide
CRP	C-reactive protein
CV	Coefficient of variation

CXR	Chest radiograph
Cy7	Cyanine
DAMPs	Danger-associated molecular patterns
DBP	Vitamin D binding protein
DNA	Deoxyribose nucleic acid
E%	Efferocytosis percentage
ECMO	Extracorporeal membrane oxygenation
E.Coli	Escherichia Coliform
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EVLW	Extra vascular lung water
EVLWI	Extra vascular lung water index
FBS	Foetal bovine serum
FEV1	Forced expiratory volume in one second
FiO ₂	Fraction of inspired oxygen
FiTC	Fluorescein Isothiocyanate
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony- stimulating factor
GEDI	Global end diastolic index
GEDV	Global end diastolic volume
HFOV	High Frequency Oscillation Ventilation
HMGB-1	High mobility group box protein-1
HRP	Horse radish peroxidase

HRQOL	Health related quality of life
ICAM	Intercellular adhesion molecule
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
ITBV	Intra thoracic blood volume
ITTV	Intra thoracic thermal volume
LIPS	Lung injury prevention score
LOS	Length of stay
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
M-CSF	Macrophage colony-stimulating factor
MDM	Monocyte derived macrophage
MFI	Mean fluorescent index
MIP	Macrophage inflammatory protein
MLTC	Midlands lung tissue collaborative
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MPO	Myeloperoxidase
n	Number
NF- κ β	Nuclear factor-kappa beta
NLR	NOD-like receptor

OD	Optical density
OLV	One lung ventilation
OR	Odds ratio
PAF	Platelet activating factor
PAMPs	Pathogen-associated molecular patterns
PAOP	Pulmonary artery occlusion pressure
PaO ₂	Arterial oxygen pressure
PBS	Phosphate buffered saline
PBV	Pulmonary blood volume
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEEP	Positive end-expiratory pressure
P:F ratio	Ratio of plasma oxygen pressure to inspired oxygen pressure
PiCCO ₂	Pulse contour continuous cardiac output 2
PLF	Peritoneal lavage fluid
PMN	Polymorphic neutrophil
PRR	Pattern recognition receptor
PTV	Pulmonary thermal volume
PBV	Pulmonary blood volume
PVPI	Pulmonary vascular permeability index
ROS	Reactive oxygen species
RAGE	Receptor for advanced glycation end products
RPMI	Roswell Park Memorial Institute (cell culture media)
rpm	Rotations per minute

RR	Relative risk
RXR	Retinoid X receptor
SD	Standard deviation
SOFA	Sequential organ system failure assessment
TNB	Tetramethylbenzidine
TLR	Toll like receptor
TNFR	Tumour necrosis factor receptor
TNF- α	Tumor necrosis factor alpha
UK	United Kingdom
USA	United States of America
UTC	Untreated control
VDD	Vitamin D deficient
VDS	Vitamin D sufficient
VDR	Vitamin D receptor
VDRE	Vitamin D response elements
VEGF	Vascular endothelial growth factor
VFD	Ventilator free day
VILI	Ventilator induced lung injury
VINDALOO	Vitamin D to prevent acute lung injury post oesophagectomy
V _T	Tidal volume
vWF	von Willebrand factor
WT	Wild-type

CHAPTER 9

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CHAPTER 10

PUBLICATIONS

10.1 PAPERS

Parekh D*, Patel JM*, Scott A, Lax S, Dancer RC, Perkins GD, Thickett DR.

Vitamin D deficiency in human and murine sepsis.

Accepted and in press *Critical Care Medicine*

Dancer RC*, Parekh D*, Lax S, D'Souza V, Zheng S, Bassford CR, Park D, Bartis DG, Mahida R, Turner AM, Sapey E, Wei W, Naidu B, Stewart PM, Fraser WD, Christopher KB, Cooper MS, Gao F, Sansom DM, Martineau AR, Perkins GD, Thickett DR. **Vitamin D deficiency contributes directly to the acute respiratory distress syndrome (ARDS).** *Thorax* 2015 Jul;70(7):617-24.

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BMJ 2014 Jul 10;349:g4171

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Vitamin D deficiency and acute lung injury

Inflamm Allergy Drug Targets 2013;12:253-61

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Vitamin D to prevent acute lung injury following oesophagectomy (VINDALOO): study protocol for a randomised placebo controlled trial

Trials 2013;14:100.

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Acute lung injury

Clinical medicine 2011;11:615-8.

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10.2 ABSTRACTS

10.2.1 Oral presentations

Parekh D, Perkins GD, Thickett DR

Vitamin D stimulates macrophage efferocytosis and encourages a pro-resolution phenotype. ERS Lung Science Meeting 2016

Dancer RC*, Parekh D*, Thickett DR

Vitamin D supplementation reduces perioperative systemic and alveolar inflammation in patients undergoing oesophagectomy: Results of the VINDALOO Trial. BTS Winter Meeting 2015

Parekh D, Lax S, Dancer RCA, Perkins GD, Thickett DR. **Vitamin D deficiency and bacterial load in a murine model of sepsis-induced lung injury.** *The Lancet*. 2014 Feb. Vol 383: Page S15

Parekh D, Wang Q, D'Souza VK, Dancer R, Patel JM, Bartis D, Gao F, Lian Q, Jin S, Thickett DR. **Lipoxin A4 improves efferocytosis via inhibition of the HMGB1 in human alveolar macrophages.** British Thoracic Society Winter Meeting. *Thorax* 2014: 69, A54-A55

Dancer RC, Parekh D, Perkins GD, Thickett DR
Long Term Survival In Patients Who Undergo Oesophagectomy Is Lower In Patients Who Develop Post-operative Acute Respiratory Distress Syndrome. British Thoracic Society Winter Meeting. *Thorax* 69 (Suppl 2), A52-A53

Parekh D, Dancer R, Park D, Perkins GD, Thickett DR. **Salmeterol prevents pneumonia and reduces biomarkers of inflammation/epithelial damage but not acute lung injury following oesophagectomy- the results of BALTI-prevention trial.** European Respiratory Society Annual Congress September 2013. *European Respiratory Journal* 42 (Suppl 57), 1818

*Denotes Joint First Author

10.2.2 Poster presentations

Parekh D, Dancer RC, Perkins GD, Thickett DR, Lax S. **Therapeutic Vitamin D Reduces the Severity Of Lipopolysaccharide-Induced Murine Lung Injury.** American Thoracic Society Congress 2014. *Am J Respir Crit Care Med*. 189, A5736

Parekh D, Dancer RC, Perkins GD, Lax S, Thickett DR. **Effects Of Vitamin D Deficiency In A Murine Model Of Sepsis-Induced Lung Injury.** American Thoracic Society Congress 2014. *Am J Respir Crit Care Med*. 189, A1646

Dancer RC, Parekh D, Calfee CS, McAuley DF, Perkins GD, Thickett DR.
Current smokers face increased risk of Acute Lung Injury post oesophagectomy compared to former smokers-implications for therapy and trial design? BTS Winter Meeting. *Thorax* 2013;68, A142-A142

Dancer RC, Parekh D, Marntineau A, Perkins GD, Thickett DR
High dose vitamin D supplementation improves extravascular lung water index and in-vivo treg and LL37 responses post-oesophagectomy. BTS Winter Meeting. *Thorax* 2012; 67 (Suppl 2), A38-A38

10.3 PRIZES/FELLOWSHIPS

European Respiratory Society Lung Science Bursary	2015
American Thoracic Society International Trainee Scholarship	2014
British Lung foundation/NAPP ERS Travel Fellowship	2013
Medical Research Council Research Training Fellowship	2012

APPENDIX

Acute lung injury

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Acute lung injury (ALI) and the more severe acute respiratory distress syndrome (ARDS) are the pulmonary manifestations of an acute systemic inflammatory process characterised clinically by pulmonary infiltrates, hypoxaemia and oedema. It occurs predominantly in young, previously healthy people, and is responsible for thousands of adult and paediatric deaths annually worldwide. Both ALI and ARDS confer a considerable long-term illness and disability burden on the individual sufferer and on society.

Historical background

In 1967 Ashbaugh *et al* published the first description of 12 patients with similar clinical, physiological, radiographic and pathology findings, later described as ARDS.¹ These patients had acute respiratory distress, cyanosis refractory to oxygen therapy, decreased lung compliance and diffuse pulmonary infiltrates on chest x-ray. It is, however, clear that patients with ARDS had been described before, particularly in the context of battlefield trauma. Thus, post-traumatic lung injury has been described as 'wet lung' in World War II, 'shock lung' or 'Da-Nang lung' after a bloody battle during the Vietnam War.

Definitions

ALI and ARDS are clinical syndromes characterised by the acute onset (<7 days) of severe hypoxaemia and bilateral pulmonary infiltrates in the absence of clinical evidence of left atrial hypertension. The severity of the hypoxaemia differentiates ALI from ARDS. The American/European Consensus Conference defined patients as having ALI or ARDS according to the ratio

of partial pressure of oxygen in arterial blood (PaO_2) to the inspired fraction of oxygen (FiO_2) being less than 300 (ALI) or less than 200 (ARDS) (Table 1).²

Incidence

The overall incidence of ARDS remains unclear, but most studies suggest approximately 2–8 cases per 100,000 population per year. ALI is more common, with rates up to 25 per 100,000 per year reported. Epidemiologic investigations of both have predominantly focused on mechanically ventilated patients in intensive care units (ICU), but recent data suggest about 9% of patients in respiratory isolation wards meet the criteria for ALI at some point during their admission. Patients managed on the wards only had a much better prognosis than those who have to be managed in ICU.³

Clinical risk factors for acute lung injury

Certain known risk factors such as sepsis, trauma or multiple traumatic injuries may lead to the development of ALI and ARDS. The mode of lung injury can be either direct or remote to the lung. A list of common causes is shown in Table 2. However, only a relatively small proportion of patients with risk factors actually develop ALI, research suggesting that genetic, demographic (age), social (smoking, alcohol abuse) and other factors play a role in determining who develops ALI.^{4,5}

Table 1. The diagnostic criteria for acute lung injury (ALI).

ALI and ARDS are defined as:

- 1 Acute onset
- 2 Bilateral pulmonary infiltrates on chest x-ray
- 3 Pulmonary capillary wedge pressure <18 mmHg (2.4 kPa) or absence of left atrial hypertension clinically
- 4 $\text{PaO}_2/\text{FiO}_2^* <300$ mmHg (40 kPa) = ALI
- 5 $\text{PaO}_2/\text{FiO}_2 <200$ mmHg (26.7 kPa) = ARDS

ARDS = acute respiratory distress syndrome.

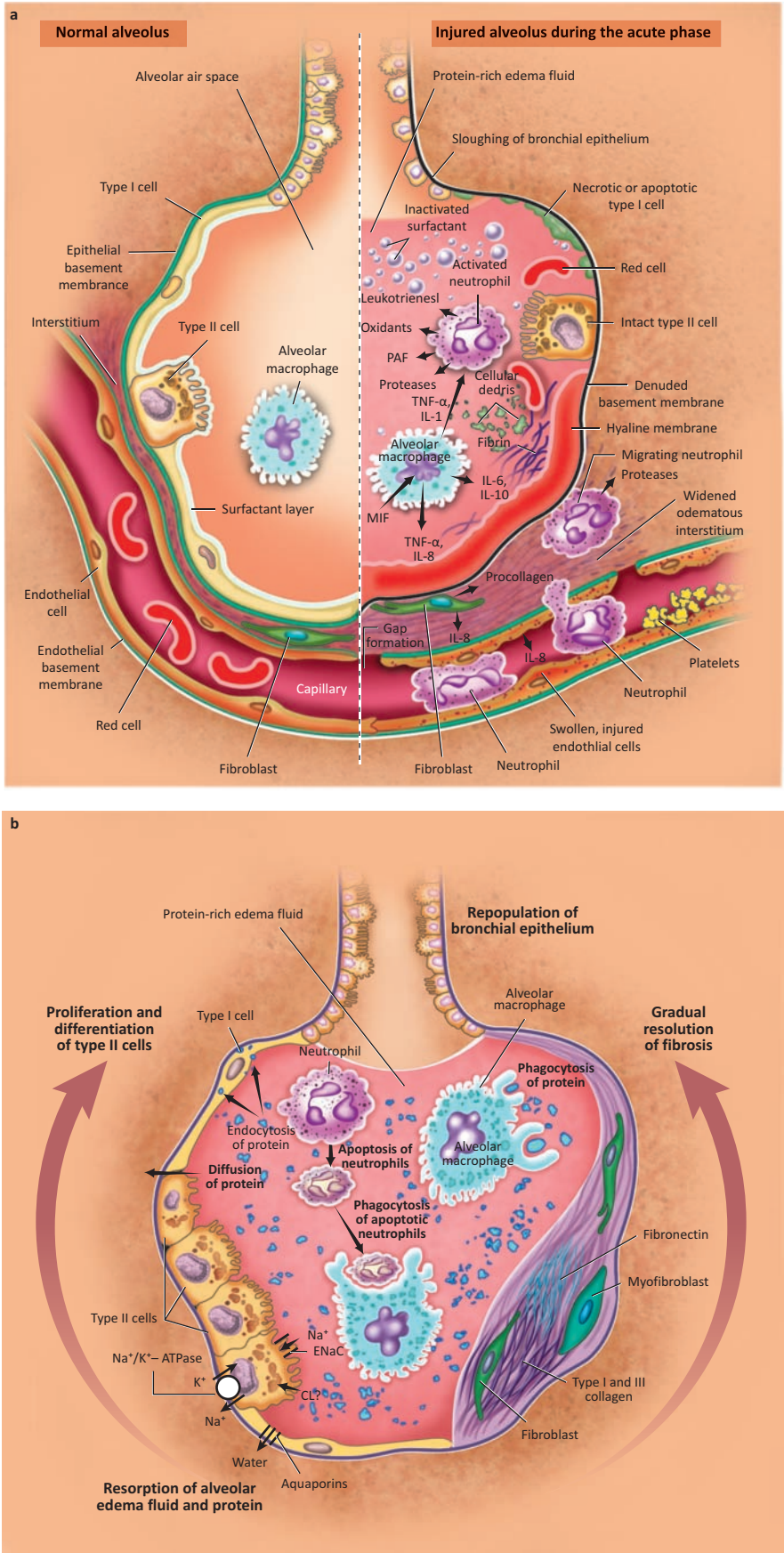
In patients in hospital with septic shock, ALI is associated with delayed goal-directed resuscitation, delayed antibiotics, transfusion, alcohol abuse, recent chemotherapy, diabetes mellitus and baseline respiratory rate.⁶ As discussed earlier, onset of ALI/ARDS is acute, with a diagnosis being made after a median of one day after hospital admission. Patients who develop ALI/ARDS with pulmonary conditions generally do so more quickly than extrapulmonary patients.

Pathophysiology

ALI is characterised by neutrophil recruitment to the lung, with both alveolar and systemic release of chemokines (eg CXCL-8, ENA-78), pro-inflammatory cytokines (eg interleukin (IL)-1, IL-6, tumour necrosis factor), acute-phase reactants (eg C-reactive protein, lipocalin), and matrix remodelling enzymes (eg MMP-9). Exaggerated neutrophilic inflammation is believed to damage the alveolar-capillary barrier,^{7–9} leading to the development of non-cardiogenic pulmonary oedema which impairs gas exchange, causing the need for mechanical ventilation (Fig 1a). The subsequent course of ARDS is variable. In some patients there is reabsorption of alveolar oedema fluid and repair of the injured region of the alveolar epithelium, followed by clinical recovery from respiratory failure (Fig 1b). In other patients alveolar oedema persists, followed

Table 2. Conditions associated with the development of acute lung injury.

Lung injury	
Remote	Direct
Sepsis	Pneumonia
Multiple trauma	Aspiration of gastric contents
Cardiopulmonary bypass	Swine flu
Post-oesophagectomy	Near drowning
Acute pancreatitis	Smoke
Inhalation	
Massive blood transfusion	Pulmonary contusion
	Post lung transplantation



by organisation of hyaline membranes and gradual appearance of intra-alveolar fibrosis and scarring.⁵

Clinical evaluation

Clinical features suggestive of ALI are severe dyspnoea, tachypnoea, and resistant hypoxaemia plus the clinical features of the initiating injury (eg bowel perforation and sepsis syndrome). All patients should be assessed for an underlying cause (particularly pneumonia, sepsis, pancreatitis or transfusion related lung injury) and treated promptly. Rigorous examination is required to look for occult sources of sepsis such as deep soft tissue infections, and appropriate diagnostic tests (eg blood/urine cultures, bronchoalveolar lavage) performed. If no underlying diagnosis is found, invasive lung biopsy is appropriate to rule out conditions such as diffuse alveolar haemorrhage that may respond to immunosuppression.¹⁰

Initial management of cases

There is no current licensed therapeutic treatment for ALI per se. Critical care support is usually required and treatment of the underlying condition to remove the underlying initiating stimulus for the injury.

Protective ventilation

The ARDSnet trial of lung protective ventilation strategy compared a control ventilatory strategy with a tidal volume (V_t) of 12 ml/kg (based on predicted body weight (PBW)) to a lung protective strategy using a V_t of 6 ml/kg PBW.¹¹ The study was stopped early when an interim analysis revealed that the mortality rate in the lung protective group was 22% lower than in the

Fig 1. (a) The normal alveolus (left) and the injured alveolus (right) in the acute phase of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS); (b) mechanisms important in the resolution of ALI and ARDS. ATPase = adenosine triphosphatase; ENaC = epithelial sodium channel; IL = interleukin; MIF = macrophage inhibitory factor; PAF = platelet activating factor; TNF = tumour necrosis factor. Reproduced with permission from the New England Journal of Medicine.⁹

Table 3. Failed therapies for acute lung injury/acute respiratory distress syndrome (ARDS).

Trial	Therapy	Outcome
KARMA ¹⁵ (n=234)	Ketoconazole	Stopped early due to lack of efficacy
LaSRS ¹⁶ (n=180)	Methylprednisolone for late-phase ARDS	No mortality benefit
LARMA ¹⁷ (n=235)	Lisofylline	Stopped early due to lack of efficacy
ALTA (n=282)	Nebulised salbutamol	Stopped early due to lack of efficacy
BALTI-2 (n=250)	iv salbutamol	Stopped early due to lack of efficacy

iv = intravenous.

control group. These beneficial results seemed to hold across a wide spectrum of patients, including septic and non-septic patients, and also those with different degrees of lung dysfunction as assessed by respiratory system compliances. A lung protective ventilation strategy should therefore be initiated for all cases of ALI.

Fluid management

Another advance in supportive therapy was recently reported by the National Heart, Lung and Blood Institute ARDS Network with the results of a prospective, randomised clinical trial evaluating the use of a liberal versus conservative fluid strategy in patients with ALI.¹² The latter resulted in a significant increase in ventilator-free days and a non-significant decrease in mortality (3%). The conservative fluid management strategy used diuretics to target a central venous pressure less than 4 mmHg or a pulmonary artery occlusion pressure below 8 mmHg.

Non-conventional ventilation

Non-conventional methods of ventilation, including high frequency ventilation and liquid ventilation, have not so far proven effective. An alternative modality, high frequency oscillation ventilation (HFOV), is increasingly used in some centres. Although its use other than as a salvage treatment remains debatable, a recent updated meta-analysis suggests that it improves oxygenation, risk of treatment failure and 30-day mortality compared with conventional ventilation.¹³ Ongoing phase 3 studies of HFOV versus a conven-

tional lung protective ventilation strategy are due to report next year and should provide definitive answers about its efficacy. In contrast, efficacy studies of liquid ventilation have been disappointing and its optimal clinical use has yet to be defined.

Extracorporeal membrane oxygenation (ECMO) has been studied in a UK trial (CESAR) of 180 patients with refractory hypoxaemia. Patients were randomised to transfer to a tertiary care centre where 77% received ECMO or to remain at the referring centre and be treated with non-protocolised ventilator strategies. Compared with the control group, transferring adult patients with severe but potentially reversible respiratory failure to a single centre specialising in the treatment of severe respiratory failure for consideration of ECMO significantly increased survival without severe disability.¹⁴ Considerable debate is ongoing as to whether this trial proves the benefit of ECMO per se or the benefits of managing severe respiratory

failure in specialist centres of excellence.

Failed pharmacological therapies for acute lung injury

Numerous pharmacological therapies for established ALI have failed to show benefit in multicentre clinical trials (examples given in Table 3). Despite corticosteroids being the most studied drugs for ALI, overall current evidence to support their use for treating early or late ALI is limited because of concern about increased neuromyopathic events with no mortality benefit.¹⁶ Nevertheless, a subsequent trial with low-dose corticosteroids in early ARDS reported significant improvement in hypoxaemia and lung injury scores as early as days 1 and 2.¹⁸

There remain uncertainties and conflicting evidence that still need answers from an adequately powered randomised trial. Therefore, at present steroids should be considered only in severe life-threatening refractory hypoxaemia and not after 14 days of ventilation.

Key points

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain major causes of morbidity, mortality and healthcare burden in the critically ill patient

The mode of lung injury can be either direct or remote to the lung and is defined as acute onset of severe hypoxaemia and bilateral infiltrates in the absence of left atrial hypertension

A lung protective ventilation and conservative fluid management strategy should be adopted in the management of ALI

High frequency oscillation ventilation and referral for extracorporeal membrane oxygenation (ECMO) should be considered in severe ARDS

To date no pharmacological therapies have shown benefit in large clinical trials

KEY WORDS: acute lung injury, acute respiratory distress syndrome

The future

Since delayed goal-directed therapy of sepsis is a risk factor for lung injury, greater clinical benefit may derive from initiating therapy prior to the onset of severe respiratory failure. Thus there is renewed interest in accurate characterisation of high-risk patient populations and identification of patients in the early stage of ALI prior to the need for mechanical ventilation. Clinical trials are underway such as the Beta Agonist Lung Injury Trial Prevention (BALTI-Prevention) which is examining the efficacy of the long-acting beta-agonist salmeterol for preventing lung injury in patients at high risk of ALI (eg those undergoing oesophagectomy).¹⁹

Ongoing research is evaluating the value of many new therapies, including simvastatin, keratinocyte growth factor and mesenchymal/amniotic fluid stem cells. Neuromuscular blocking agents have recently been re-evaluated with favourable results. A recent trial has shown that cisatracurium given for 48 hours early in the course of ARDS with low V_t ventilation may improve outcomes without significantly increasing the incidence of muscle weakness.²⁰ Further studies are required before these approaches can be adopted in routine practice.

Outcomes

The advent of lung protective ventilation has resulted in reduced mortality in patients with ALI. Nevertheless, ALI carries an enormous burden in both social and fiscal cost,^{21,22} with mortality rates still around 40% and significant pulmonary impairment in up to 50% of survivors.⁹ Debilitating long-term sequelae in survivors include pulmonary, psychological and neurologic impairment²³ (discussed in more detail in the accompanying article *Sequelae and rehabilitation after critical illness* (pp xxx–xxx)).

Conclusions

Our understanding of the pathophysiology of ALI has improved dramatically since the first defining reports. Large-scale clinical trials have generated ventilatory strategies which have resulted in reduced mortality. Effective specific drug therapy for ALI has

remained elusive despite the establishment of large clinical trials networks. Hopefully emerging therapy to prevent or treat ALI currently under investigation will provide additional strategies to reduce the burden of this devastating disease.

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Vitamin D Deficiency and Acute Lung Injury

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Abstract: Acute Lung Injury (ALI) and the more severe form Acute Respiratory Distress Syndrome (ARDS) remain a significant cause of morbidity and mortality in the critically ill patient. It is characterised by a severe inflammatory process resulting in diffuse alveolar damage, influx of neutrophils, macrophages and a protein rich exudate in the alveolar spaces caused by endothelial and epithelial injury. Improvements in outcomes are in part due to restrictive fluid management and protective lung ventilation however successful therapeutic strategies remain elusive with promising therapies failing to translate positively in human studies.

The evidence for the role of vitamin D in lung disease is growing - deficiency has been associated with impaired pulmonary function, increased incidence of viral and bacterial infections and inflammatory disease including asthma and COPD. Studies have also reported a high prevalence of vitamin D deficiency in the critically ill and an association with adverse outcomes. Although exact mechanisms are yet to be discerned, vitamin D appears to impact on a variety of inflammatory and structural cells within the lung including macrophages, lymphocytes and epithelial cells. To date there are few directly supportive clinical studies in ALI; this review explores the compelling evidence suggesting a role for vitamin D in ALI and the mechanisms by which it could contribute to pathogenesis.

Keywords: Acute lung injury, adult respiratory distress syndrome, cholecalciferol, etiology, pharmacology.

INTRODUCTION

Acute Lung Injury

Acute Lung Injury (ALI) and the more severe Acute Respiratory Distress Syndrome (ARDS) are life threatening clinical syndromes of acute respiratory failure characterised by a severe inflammatory process resulting in diffuse alveolar damage, ventilation perfusion mismatch, hypoxaemia and poor lung compliance [1]. Aetiology varies and the mode of injury can be direct or remote to the lung with sepsis, pneumonia, surgery, and trauma with multiple transfusions accounting for the majority of cases [2]. ARDS was first described in 1967 by Ashbaugh *et al.* as a rapid onset of respiratory distress, cyanosis refractory to oxygen therapy, loss of lung compliance and diffuse pulmonary infiltrates on chest radiograph (CXR) [3]. However, specific criteria to identify patients with ARDS were only established in 1994 by the American European Consensus Conference Committee (AECC) [4]. Despite remaining a crude screening tool with recognised limitations relating to specificity and reproducibility [5], the AECC criteria has been widely accepted as a simple diagnostic tool for identifying patients. They include the presence of acute severe hypoxaemia (defined as a ratio of arterial oxygen tension over fractional inspired oxygen, $\text{PaO}_2/\text{FiO}_2 < 200\text{mm Hg}$ (26.7kPa) for ARDS, $\text{PaO}_2/\text{FiO}_2 < 300\text{mm Hg}$ (40kPa) for ALI) and

bilateral infiltrates on CXR in the absence hydrostatic pulmonary oedema (pulmonary artery wedge pressure of $< 18\text{mmHg}$ (2.7kPa) or no clinical evidence of left atrial hypertension).

The overall incidence of ARDS remains unclear due to the limitations in diagnostic criteria and heterogeneity of the populations and underlying causes, but studies suggest an ARDS rate of 58 per 100,000 person years [6] with a higher ALI rate of 79 per 100,000 person years [7]. Reported mortality rates vary between 36-44% [8]. However the use of lung protective ventilation, conservative fluid strategies and improved advanced support of multi-organ failure has resulted in improved survival and more recent clinical trials suggest that mortality may be lower at 19-23% [9]. Despite this, morbidity remains high and although lung function parameters may recover well in ARDS patients who survive, there is significant residual physical limitations and poorer quality of life [10]. Depression, post-traumatic stress and neuro-cognitive impairment are not uncommon leading to a significant impact on both the individual and society as a whole [10]. Despite over 30 years of research therapeutic strategies remain elusive with many anticipated therapies failing to translate from animal to human studies.

Vitamin D Biology

Vitamin D is a secosteroid hormone produced predominantly from precursors within the skin through the action of ultraviolet B (UVB) radiation on 7-dehydrocholesterol. This process and classical vitamin D biology are illustrated in Fig. (1). In addition, an increasing number of tissues have been found to express 1α -

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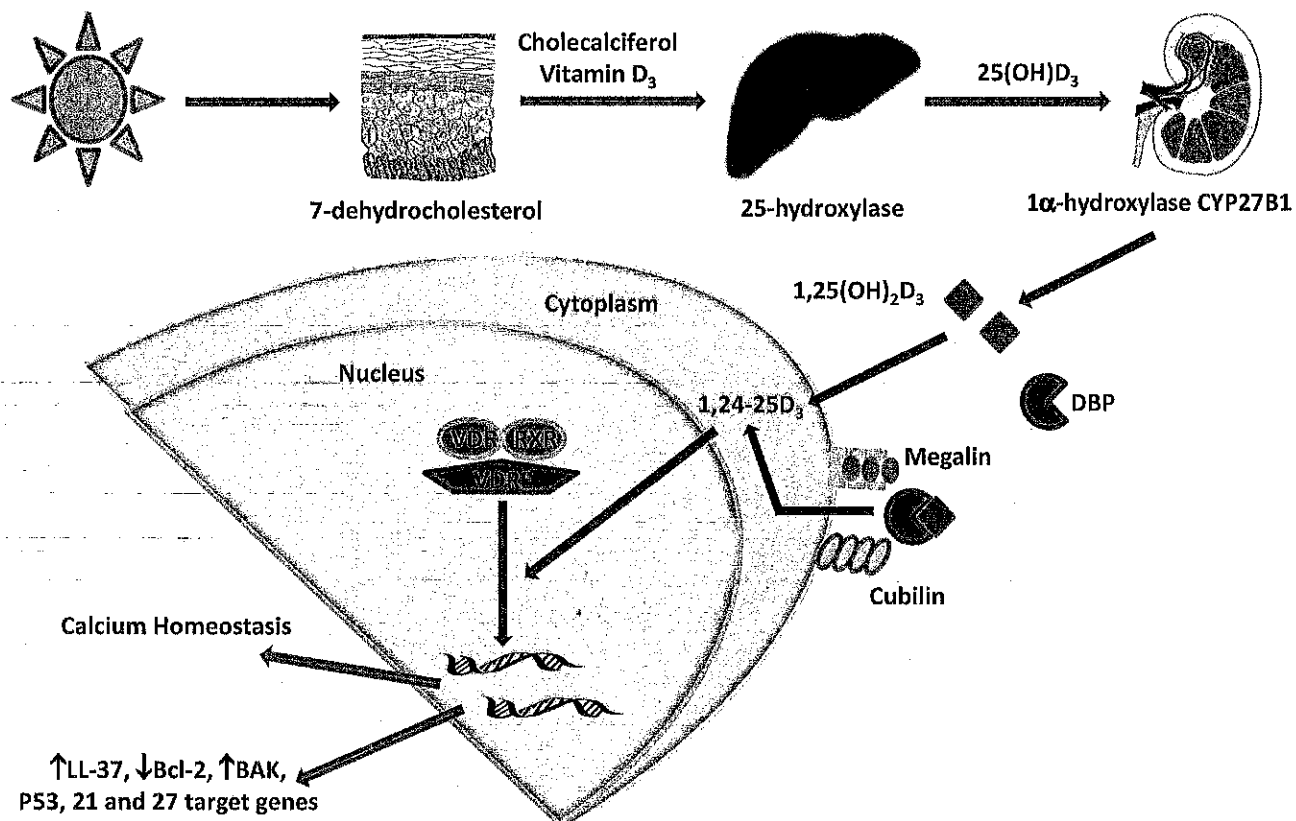


Fig. (1). Vitamin D biology. Synthesised or dietary vitamin D is hydroxylated in the liver to produce 25-hydroxyvitamin D [$25(\text{OH})\text{D}_3$], the major circulating form of vitamin D and widely accepted measure of vitamin D status. In the classical model of vitamin D metabolism $25(\text{OH})\text{D}$ undergoes further hydroxylation in the kidney to the biologically active $1,25$ -dihydroxyvitamin D₃ [$1,25(\text{OH})_2\text{D}_3$] by the mitochondrial enzyme 1α -hydroxylase (CYP27B1). This active metabolite acts on the kidney, gut and bone to maintain calcium homeostasis. The effects of vitamin D are mediated through the vitamin D receptor (VDR) which is a ligand dependant transcription factor. VDR binds to its ligand $1,25(\text{OH})_2\text{D}_3$, dimerises with the retinoid X receptor (RXR) and attaches to specific genomic sequences termed vitamin D response elements (VDRE). The transcription of VDR target genes results in cell growth inhibition, induces apoptosis, controls proliferation and synthesis of antimicrobial peptides, such as LL-37 (adapted from Chisimba *et al.* [16]).

hydroxylase (CYP27B1), including lung epithelial cells [11] and cells of the immune system including macrophages [12], lymphocytes [13] and dendritic cells [14]. Vitamin D metabolites are bound in the circulation to vitamin D binding protein (DBP) which has a high affinity for $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ and therefore regulates free circulating concentrations of vitamin D metabolites [15]. DBP is a serum protein which has immunomodulatory functions relevant in the lung, predominantly relating to macrophage activation and neutrophil chemotaxis [16]. Vitamin D is inactivated by a ubiquitous enzyme, 24 -hydroxylase (CYP24A). The effects of vitamin D are mediated through the vitamin D receptor (VDR). It has therefore been suggested that local production of $1,25(\text{OH})_2\text{D}_3$ along with the presence of the VDR is evidence of a local paracrine/autocrine action in various tissues. Vitamin D regulates more than 900 genes including genes for cellular proliferation, differentiation and apoptosis [17].

Worldwide prevalence of vitamin D deficiency is on the increase [17a, 18]. In a study of middle-aged adults in the United Kingdom, 40% had serum $25(\text{OH})\text{D}$ concentrations

above 30ng/l in the summer months but this fell to less than 17% in the winter [19]. According to several other studies, 40-100% of U.S and European elderly adults are deficient in vitamin D [17a]. Epidemiological studies have suggested a role for low vitamin D status in the risk of developing both viral and bacterial infection [20]. A recent study has further demonstrated the anti-inflammatory effects of vitamin D in patients with pulmonary tuberculosis [21]. In addition, asthma, chronic obstructive airways disease (COPD), interstitial lung disease and cystic fibrosis show correlations between severity and level of vitamin D deficiency [22]. Further details on the role of vitamin D in these diseases can be found in other manuscripts within this theme issue [23].

Published data suggest that vitamin D deficiency is also common in critically ill patients [24] and recent prospective studies associated it with increased morbidity and mortality [25]. As there are currently no human studies of vitamin D supplementation in ALI to understand the potential role of vitamin D deficiency this review will focus on the pathological processes involved and relate these to actions of vitamin D both on inflammation and other diseases of clinical relevance.

ALI PATHOPHYSIOLOGY

Despite the varied precipitating events for ALI the histological changes are identical and represent a final common pathway of response. ALI is characterised in the acute phase by an overwhelming inflammatory process leading to diffuse alveolar epithelial and endothelial injury with alveolar flooding with protein rich exudates due to increased vascular permeability. It is this breakdown in the alveolar epithelial and endothelial barrier, inappropriate influx of inflammatory cells and platelets, uncontrolled inflammation and activation of the coagulation pathways that are the key pathophysiological elements of ARDS and ALI [2], illustrated in Fig. (2).

Neutrophils are thought to play a key role in the progression of ALI. Endothelial and epithelial injury is accompanied by an influx of neutrophils into the interstitium and alveolar space causing an alveolar and systemic release of inflammatory mediators [26]. Alveolar macrophages are

the first line in detection of injurious pulmonary stimuli and initiation of the inflammatory cascade in ALI. Pathogen-associated molecular patterns or PAMPs (e.g. lipopolysaccharide) or danger-associated molecular patterns or DAMPs (e.g. heat shock proteins or high mobility box group protein 1, HMGB1) activate macrophages via toll-like receptors (TLRs), NOD-like receptors (NLRs) and other pattern recognition receptors (PRRs) leading to release of ROS, antimicrobial peptides (cathelicidin or LL37) and early response cytokines (TNF α , IL-6, IL-1) in an NF- κ B dependent way [27]. This pro-inflammatory activity is counterbalanced by the alternatively activated phenotype which is involved in down regulating inflammation and promoting wound repair by the release of anti-inflammatory cytokines (e.g. IL-10, IL-4) and phagocytosis of apoptotic neutrophils, supporting an anti-inflammatory, resolving and tissue remodelling phenotype by the release of TGF β , IL-10 and vascular endothelial growth factor (VEGF) [28]. Furthermore the mediators released by macrophages have

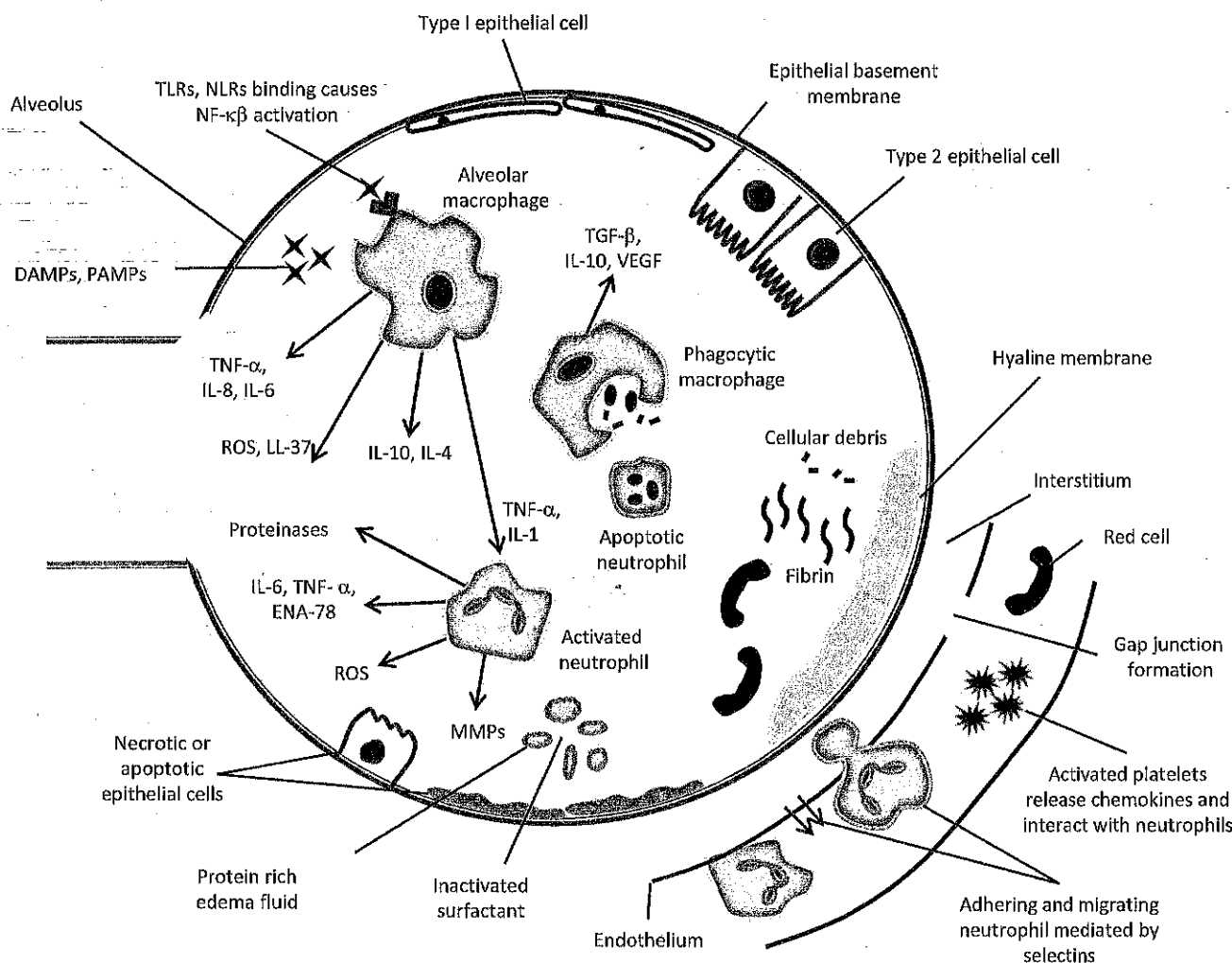


Fig. (2). ALI pathophysiology. Neutrophil recruitment causes both alveolar and systemic release of chemokines (e.g. CXCL-8, ENA-78), pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF α), acute phase reactants (e.g. CRP, lipocalin), proteases, reactive oxygen species (ROS) and matrix remodelling enzymes (e.g. MMP-9) leading to further tissue damage. Apoptosis and necrosis of Type I alveolar epithelial cells contribute to the pulmonary oedema and expose the basement membrane to further insult. Impaired surfactant synthesis due to injury to Type II epithelial cells causes alveolar collapse and decreased lung compliance. An increase in extra-vascular lung water is also seen during this phase, as pulmonary capillary permeability increases.

been shown to abrogate alveolar neutrophil recruitment and mediate the effects of regulatory T-cells (T_{reg}) [29].

The subsequent course of ARDS is variable. In some patients there is reabsorption of alveolar edema fluid and repair of the injured region of the alveolar epithelium, followed by clinical recovery from respiratory failure. However, in other patients alveolar oedema persists followed by organisation of hyaline membranes and gradual appearance of intra-alveolar fibrosis and scarring [30].

ROLE OF VITAMIN D IN ALI?

Barrier integrity of the alveolar epithelium is essential not only to prevent pulmonary oedema but also to facilitate removal of fluid from the air space. The epithelial cell-cell functional complex is formed by gap, tight and adherens junctions. $1,25(OH)_2D_3$ has been shown to upregulate transcription of proteins required for the formation of connexin 43, claudin-1, -2 and E-cadherin in epithelial cells of the skin and intestine [31]. Although this has yet to be confirmed in the lung it suggests that vitamin D mediated action may play a role in stabilising the epithelial junction complex.

Vitamin D is a potent stimulator of antimicrobial peptides in innate immunity [32] and the production of LL-37 (cathelicidin) and some defensins (human β -defensin-2) is dependent on sufficient circulating $25(OH)D_3$ [33]. $1,25(OH)_2D_3$ has the ability to induce the release of LL-37 within the lung and LL-37 is stored at high concentrations in specific granules of neutrophils, and can also be produced by macrophages and epithelial cells. It can be detected in airway secretions and is upregulated in response to infection and inflammation [34]. In addition to direct antimicrobial capability, *in vitro* and *in vivo* studies suggest a broad range of activities that could modify innate inflammatory processes and adaptive immune responses. LL-37 can bind to and neutralize lipopolysaccharide (LPS), and functions as a chemoattractant for neutrophils, monocytes and T cells through a formyl peptide receptor [34]. Furthermore, respiratory epithelial cells can convert $25(OH)D_3$ to $1,25(OH)_2D_3$ and activate VDR responsive genes increasing the production of TLR co-receptor CD14 and *hCAP18* from which LL-37 is cleaved within 24 hours [35]. In the context of ARDS, LL-37 is elevated significantly in the BAL fluid of these patients in comparison with normal controls [36]. In terms of the pathophysiology of ALI this could be important as LL-37 may drive epithelial repair responses as well as being an anti-microbial peptide [37]. Elevating local LL-37 may also be important as a downstream immunomodulator of vitamin D since it has recently been shown to reduce TLR agonist-mediated neutrophil-derived increases in IL-1 β , IL-6, IL-8 and TNF- α in addition to stimulating bacterial phagocytosis [38]. Further studies are needed to determine if LL-37 is causative of inflammation or a response to injury in ALI.

The concept of the ability of vitamin D to stimulate the differentiation of precursor monocytes to mature phagocytic macrophages was supported by observations showing differential expression of the VDR and 1α -hydroxylase during human macrophage differentiation [39]. This together with the evidence of localised synthesis of $1,25(OH)_2D_3$ by

normal human macrophages on stimulation with interferon gamma (IFN γ) is suggestive of an intracrine system for the action of vitamin D in normal monocytes and macrophages [32, 40]. IL-1 and TNF α production induced by TLR3 agonists from monocyte derived macrophages are inhibited to the same extent by $25(OH)D_3$ and $1,25(OH)_2D_3$ after 24 hours suggesting that the vitamin D metabolites may have a rapid anti-inflammatory action and that local intracrine activation of $25(OH)D_3$ can be anti-inflammatory [41]. Hydrogen peroxide secretion in human monocytes is also activated by $1,25(OH)_2D_3$ resulting in increased oxidative burst potential [42]. The ability of $1,25(OH)_2D_3$ to directly inhibit NF- κ b signalling and suppress macrophage TLR expression suggests that Vitamin D may also play a key role as a feedback regulator of macrophage responses [43]. Furthermore, $1,25(OH)_2D_3$ has also been shown to reduce the production of inflammatory cytokines and chemokines (IL-8 and CXCL-10) from stimulated epithelial cells by modulating the NF- κ b signalling [44]. Any or all of these actions are likely to be relevant in the lung and ALI. Neutrophils also express the VDR [45] however, little is known about vitamin D regulation of neutrophil function.

Recent data have further implicated vitamin D in adaptive immunity because of its influence upon the differentiation of T cells between the regulatory T cell (T_{reg}) and the pro-inflammatory T helper 17 (T_H17) subsets [46]. T_H17 cells are known to stimulate tissue inflammation and neutrophil chemotaxis, both of which are seen in ALI, predominantly by IL-17 production. It also appears that expression of markers of T_{reg} cells (Foxp3) or T_H17 cells (IL-17) by T cells may not be stable and that there is a greater degree of plasticity in their differentiation than previously appreciated [46b]. Recent evidence has further suggested T_{reg} cells are important in resolution of experimental ALI [29]. This suggests that local lung regulation of the balance between T_{reg} and T_H17 cells may be a determinant of resolution/persistence of neutrophilic inflammation which is known to be associated with a poor prognosis in human ALI.

Vitamin D also promotes autophagy, a cellular process that ensures the synthesis, degradation and recycling of intracellular macromolecules and inclusions in mononuclear cells [47]. It represents an inducible response to stress in lung cells. Agents that trigger autophagy that are particularly relevant to lung cell biology include hypoxia, particle and cigarette smoke exposure, proinflammatory states, and conditions that promote ER stress or oxidative stress [48]. Autophagy has been shown to be both protective and injurious in a variety of different models, suggesting that its role in human diseases is complex. Relatively few studies have been done in the lung and the functional importance of autophagy in ALI is yet to be explored.

MMPs are upregulated in activated cells and can facilitate tissue remodelling and repair [30]. They can also control cytokine and chemokine processing, apoptosis and antimicrobial peptide cleavage and activation [49]. They are however a complex family of enzymes with MMP-2, MMP-7 and MMP-9 shown to be involved in alveolar epithelial repair in experimental models of lung injury [30, 50] and increased levels of lung injury in MMP-9 deficient mice exposed to ventilator induced lung injury [51]. In contrast MMP-3 deficient mice were protected against bleomycin

induced lung injury [52] and MMP-8 has shown opposing effects depending on the model of lung injury employed. 1,25-(OH)₂D₃ has been reported to down-regulate the expression of protein for MMP-9 in airway smooth muscle cells from asthma patients [53] and inhibit the expression and activity of a number of MMP during *in vitro* mycobacterium tuberculosis (MTB) infection [54]. This limited data support a role for vitamin D in suppressing MMP. Although blockade of specific MMPs during the early stages of ALI may be beneficial to prevent initial destruction of the basement membrane this may be detrimental to the resolution process [55]. Thus, further investigation of the possible effect and mechanisms of vitamin D on MMP function is needed in appropriate ALI models.

Vitamin 1,25D₃ at physiological sufficient levels can reduce the activation of lung microvascular endothelial cells, reducing adhesion molecule expression (ICAM-1 and ELAM-1), reducing iNOS expression and NO release, and attenuating platelet activating factor (PAF) induced neutrophil adhesion to pulmonary microvascular endothelial cells suggesting that sufficient vitamin D levels may attenuate/prevent an important initiating event of acute lung injury [56].

Apart from its specific role in being the carrier protein for 25(OH)D₃, DBP exerts several other important biological functions, from actin scavenging to fatty acid transport and macrophage activation [57]. Tissue injury and cell death release actin into the circulation. In the extracellular compartment, G-actin polymerizes into F-actin filaments. This may cause vascular obstruction and organ dysfunction. Severe cell or tissue loss lowers the DBP-serum level. The degree of reduction correlates with the development of organ dysfunction, respiratory failure, hematologic failure and sepsis [58] and low concentrations of DBP have been

reported in ARDS [57, 59], further suggesting a role for the vitamin D axis in ALI. These pathological concepts linking vitamin D and ALI are summarised in Table 1.

CLINICAL AND ANIMAL MODEL EVIDENCE IN ALI OR ITS CAUSATIVE DISEASES

There are limited human studies of vitamin D in critically care patients. Cecchi *et al.* reported Vitamin D levels to be lower in sepsis patients than in trauma and age related controls but this was not related to outcome [60]. In contrast in a large cohort of critically ill patients, it has been demonstrated that deficiency of 25-hydroxyvitamin D at the time of critical care initiation is a significant predictor of all-cause patient mortality [25b]. Findings that are also true in surgical patients admitted to critical care [61]. The only study looking at vitamin D levels in patients with severe sepsis suggests that these patients have a lower serum vitamin D level than ITU control patients. This was associated with lower plasma levels of LL-37, suggesting that this deficiency is of functional importance *in vivo* [62]. Sepsis has direct relevance to ALI and the pathophysiological changes leading to multi-organ failure may be similar and therefore highly relevant.

The few animal studies to date investigating the role of vitamin D deficiency and its supplementation in ALI have conflicting results. 1,25(OH)₂D₃ inhibited neutrophil but not monocyte recruitment by 40% in a hamster model of ALI, thought to be in part due to its inhibitory effects on IL-8 [63]. It also ameliorated lung damage secondary to ischaemia reperfusion injury after femoral artery ligation in rats [64]. A further study has shown reduced interstitial inflammation and collagen deposition post radiotherapy in rats administered vitamin D [65]. Conversely, a recent study of

Table 1. Pathological Concepts Linking Vitamin D and ALI

Process/Cell Type	Role of Vitamin D	Potential Effect in ALI	Relevant References
Antimicrobial peptide production	Increased LL-37 release by macrophages and epithelial cells	Promote epithelial repair responses and reduce downstream pro-inflammatory signals	Shaykhiev <i>et al.</i> [37] Alawani <i>et al.</i> [38]
Monocyte differentiation	Stimulates differentiation to mature phagocytic macrophages	Anti-inflammatory actions and prevention of secondary necrosis of apoptotic neutrophils and persistence of inflammation	Kreutz <i>et al.</i> [39]
NF- κ B signalling	Reduces NF- κ B signalling and TLR expression	Dampen inflammatory response	Sadeghi <i>et al.</i> [43b] Hansdottir <i>et al.</i> [35]
Regulatory T-cells	Stimulates differentiation towards regulatory T-cell and away from Th17 cells	Promote resolution of ALI	D'Alessio <i>et al.</i> [29]
MMP driven tissue remodelling	Reduces MMP	Prevent initial destruction of basement membrane but potentially impair resolution	O'Kane <i>et al.</i> [30], Albaiceta <i>et al.</i> [51], Yamashita <i>et al.</i> [52], Song <i>et al.</i> [53]
Neutrophil adhesion to endothelium	Reduces adhesion molecule expression on the endothelium, PAF and NO release	Prevent neutrophil migration and initiation of lung injury	Chen SF [56]
vitamin D Binding Protein (DBP)	Actin scavenging and macrophage activation	Prevent vascular occlusion, tissue damage and organ dysfunction	Dahl <i>et al.</i> [58]
Autophagy	Promotes autophagy	Not known	Wu <i>et al.</i> [47b]
Barrier integrity	Upregulate gap, adherens and tight junctions	Prevent alveolar fluid influx and facilitate removal	Palmer <i>et al.</i> [31c] Kong <i>et al.</i> [31d]

LPS induced lung injury in vitamin D deficient mice found no difference in the degree of lung injury [66]. Issues centred on differing aetiologies of lung injury as well as methodology of when lung injury is measured or defined may go some way to explaining this apparent contradiction.

There are currently no registered clinical trials of vitamin D replacement in patients with established ALI. A phase II randomised double blind placebo controlled trial of Vitamin D to prevent ALI in patients undergoing oesophagectomy (VINDALOO, Current Controlled Trials ISRCTN27673620) [67] is on-going with recruitment due to complete in late 2014. This study is utilising this particular surgery as a model for patients at risk of developing ALI and replacing with high dose vitamin D₃ preoperatively. Results will include exploration of factors influencing response to supplementation, such as genetics, which was found to be key in determining this in TB [68]. Future clinical trials may have to consider specific subgroups of 'responders', or even selectively recruit to optimise power.

CLINICAL EVIDENCE FROM OTHER RELEVANT DISEASES

Primary studies and meta-analyses have identified significant associations between lower serum 25(OH)D₃ levels and impaired lung function [69], decreased survival from lung cancer [70], increased rates of severe asthma exacerbations [71], tuberculosis (TB) [72], acute respiratory tract infections [73] and chronic obstructive airways disease [74]. A recent study has shown that high vitamin D levels are associated with better lung function, less airway hyperresponsiveness and improved glucocorticoid response in asthma [75] and others have shown an interesting potential application of vitamin D to overcome the poor glucocorticoid responsiveness in severe asthmatics by upregulation of IL-10 production from regulatory T-cells [76]. Clinical trials of steroids in ARDS have to date revealed conflicting results despite their potential overwhelming anti-inflammatory effect and could represent an element of glucocorticoid resistance as seen in other inflammatory conditions [77]. Therefore low levels of vitamin D could be a contributory factor in steroid resistance seen in some patients with ARDS.

The mechanisms that link vitamin D with the development of COPD are largely speculative. Its role in exacerbations is likely to be due to its effects on increasing antimicrobial activity however, a recent single centre randomised trial showed no effect of supplementation on exacerbations [78]. Furthermore, MMP-9 has been shown to be increased in the sputum on COPD patients [79] and vitamin D deficiency may lead to increased MMP-9 activity resulting in enhanced degradation of lung parenchyma. However this is contrary to some of the evidence presented earlier of its role in ALI, suggesting a more complex interaction in ARDS that needs further elucidating.

Exposure to sunlight has been known to help with the treatment of TB for more than 100 years, although it is only recently been found that macrophage release of cathelicidin, required for efficient macrophage killing of MTB, requires co-activation of the VDR and TLR [80]. The TLR activation of macrophages and the epithelium by PAMPs to stimulate

local vitamin D expression could theoretically be performed by DAMPs and non-pathogen injurious stimuli in a similar fashion, both having relevance in the multiple aetiologies involved in ALI.

CONCLUSION

The evidence presented supports a profound role of vitamin D in modulating immune responses. Along with evidence from other inflammatory diseases of the lung it is not unreasonable to suggest a potential role for vitamin D in ALI. Mechanistically vitamin D deficiency could lead to an impaired innate immune response to injury to the lung proceeded by an excessive adaptive immune response to the injury with increased production of inflammatory cytokines, exaggerated inflammation as well as impaired resolution and repair of damage. However, many questions remain unanswered and more *in-vitro*, *in-vivo* and human studies are required to ascertain the potential mechanisms of vitamin D deficiency in promoting ALI with a view to larger trials of replacement therapy to treat and prevent it.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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STUDY PROTOCOL

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Vitamin D to prevent acute lung injury following oesophagectomy (VINDALOO): study protocol for a randomised placebo controlled trial

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Abstract

Background: Acute lung injury occurs in approximately 25% to 30% of subjects undergoing oesophagectomy. Experimental studies suggest that treatment with vitamin D may prevent the development of acute lung injury by decreasing inflammatory cytokine release, enhancing lung epithelial repair and protecting alveolar capillary barrier function.

Methods/Design: The 'Vitamin D to prevent lung injury following oesophagectomy trial' is a multi-centre, randomised, double-blind, placebo-controlled trial. The aim of the trial is to determine in patients undergoing elective transthoracic oesophagectomy, if pre-treatment with a single oral dose of vitamin D₃ (300,000 IU (7.5 mg) cholecalciferol in oily solution administered seven days pre-operatively) compared to placebo affects biomarkers of early acute lung injury and other clinical outcomes. The primary outcome will be change in extravascular lung water index measured by PiCCO[®] transpulmonary thermodilution catheter at the end of the oesophagectomy. The trial secondary outcomes are clinical markers indicative of lung injury: PaO₂:FiO₂ ratio, oxygenation index; development of acute lung injury to day 28; duration of ventilation and organ failure; survival; safety and tolerability of vitamin D supplementation; plasma indices of endothelial and alveolar epithelial function/injury, plasma inflammatory response and plasma vitamin D status. The study aims to recruit 80 patients from three UK centres.

Discussion: This study will ascertain whether vitamin D replacement alters biomarkers of lung damage following oesophagectomy.

Trial registration: Current Controlled Trials ISRCTN27673620

Keywords: Acute lung injury, One lung ventilation, Oesophagectomy, Vitamin D

Background

Acute lung injury (ALI) and the more severe acute respiratory distress syndrome (ARDS) are common, devastating clinical syndromes of acute respiratory failure in the critically ill person. The incidence of ALI is 79 per 100,000 person years with a mortality rate of 30% to 65% [1]. Survivors of ARDS experience a significant reduction in health-related quality of life, with 46% reported to be unable to return to work within 12 months.

ALI is the final common pathway of response to a variety of direct pulmonary insults, such as bacterial /viral pneumonia and gastric aspiration, or indirect insults, such as abdominal sepsis or battlefield trauma. Only a relatively small proportion of patients develop ALI, with research suggesting that genetic, demographic (age), social (smoking, alcohol abuse) and other factors play a role in determining who develops ALI [1,2]. There are no current readily available tests that can clearly identify those who are at high risk of ALI and no therapeutic interventions proven to prevent its occurrence.

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One lung ventilation (OLV) as a model for ALI/ARDS

To allow access to the oesophagus during surgery (using the transthoracic technique), one of the lungs is deflated and the subject is ventilated through the other lung. This is known as one-lung ventilation (OLV). There is a high postoperative incidence of ALI/ARDS [3-5] following OLV and unlike most insults leading to lung injury the delivery of OLV is predictably timed, thereby allowing serial studies to be carried out throughout the period of stimulus and development of the condition. Preoperative risk factors including age, respiratory function and cigarette smoking have been found to be related to the incidence of postoperative pulmonary complications [3,6-8]. It is unclear at present why only a percentage of patients undergoing OLV develop lung injury or why the lung injury typically occurs 24 to 48 hours after the cessation of OLV. Our data show that the development of lung injury is, however, associated with a doubling of in-hospital stay and elevated mortality.

We have extensively modeled the local and systemic inflammatory response to transthoracic oesophagectomy in 50 patients undergoing OLV. After OLV, patients have a neutrophilic alveolitis, with a significant alveolar and systemic inflammatory response. This is associated with the release of markers of both endothelial and alveolar epithelial dysfunction and an increase in the permeability of the alveolar barrier. This manifests clinically as increased extravascular lung water and a fall in oxygenation.

Alveolar levels of surfactant protein D and bronchoalveolar lavage fluid (BALF) protein permeability index are highest in those who develop ALI within 72 hours of OLV suggesting that peri-operative alveolar epithelial damage is a risk factor for the subsequent development of ALI. Immediate post-operative plasma markers of neutrophilic activation (myeloperoxidase, and matrix metalloproteinase-9 (MMP-9)) as well as the receptor for advanced glycation end-products (RAGE, a type I epithelial cell marker) are similarly raised in those who develop ALI within 72 hours of OLV. Proposed causative mechanisms for this injury include the ischaemic/reperfusion insult suffered by the collapsed lung, as well as oxidative stress and barotrauma causing epithelial injury to the ventilated lung [9]. These mechanisms are important in the pathogenesis of ALI making OLV a valid model for studying the pathogenesis of ALI in humans and exploring therapeutic strategies for preventing lung injury in a predefined subject population [10,11].

Vitamin D biology

Vitamin D₃, or cholecalciferol, is mainly formed in the skin after exposure to sunlight, then hydroxylated in the liver to 25-hydroxyvitamin D₃ (25(OH)D₃) and subsequently in the kidney to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). When 25(OH)D₃ is sufficiently available, 24,25-dihydroxyvitamin

D₃ (24,25(OH)₂D₃) is formed in the kidney which is further catabolised. Vitamin D metabolites are bound in the circulation to vitamin D binding protein (VDBP) which has a high affinity for 25(OH)D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ and, therefore, regulates free circulating concentrations of vitamin D metabolites. The biologically active metabolite 1,25(OH)₂D₃ can also be generated locally within tissues due to induction of extra-renal cyp27b1 (25(OH)D-1-alpha hydroxylase) and binds to the vitamin D receptor (VDR) resulting in modified gene expression.

Epidemiological studies have suggested a role for low vitamin D status in the risk of developing both viral and bacterial infection [12,13]. A recent study has further demonstrated the pleiotropic anti-inflammatory effects of vitamin D in patients with pulmonary tuberculosis [14]. Published data suggest that vitamin D deficiency is common in critically ill patients [15], and recent prospective studies suggest an association with increased morbidity and mortality [16-18]. Literature on acute vitamin D supplementation in critical illness is lacking but serious adverse events attributable to vitamin D supplementation are rare [18,19].

Is severe vitamin D deficiency a driver of post-OLV ALI?

Vitamin D has profound effects on human immunity acting as an immune system modulator, preventing excessive expression of inflammatory cytokines and increasing the 'oxidative burst' potential of macrophages, thereby enhancing bacterial killing. Vitamin D also stimulates the release of antimicrobial peptides such as LL-37 (cathelicidin) within the lung. LL-37 can also bind to and neutralize lipopolysaccharide (LPS), and functions as a chemoattractant for neutrophils, monocytes and T cells through a formyl peptide receptor-like molecule [20].

Respiratory epithelial cells convert 25(OH)D₃ to 1,25(OH)₂D₃ and activate VDR responsive genes increasing the production of *hCAP18* from which LL-37 is cleaved within 24 hours [21]. In terms of the pathophysiology of ALI this could be important as LL-37 may drive epithelial repair responses as well as being an anti-microbial peptide [22]. Elevating local LL-37 may also be important as a downstream immunomodulator of vitamin D since it has recently been shown to reduce Toll-like receptor (TLR) agonist-mediated neutrophil-derived increases in IL-1 β , IL-6, IL-8 and tumour necrosis factor-alpha (TNF- α) in addition to stimulating bacterial phagocytosis [23]. The ability of 1,25(OH)₂D₃ to directly inhibit nuclear factor-kappaB (NF- κ B) signalling and suppress macrophage TLR expression suggests that vitamin D may also play a key role as a feedback regulator of macrophage responses [24,25].

The only study looking at vitamin D levels in patients with severe sepsis suggests that these patients have a lower serum vitamin D level than healthy control patients. This was associated with lower plasma levels of LL-37,

suggesting that this deficiency is of functional importance *in vivo* [26]. IL-1 and TNF production induced by TLR3 agonists from monocyte derived macrophages are inhibited to the same extent by 25(OH)D₃ and 1,25(OH)₂D₃ after 24 - hours suggesting that the vitamin D metabolites may have a rapid anti-inflammatory action and that local intracrine activation of 25(OH)D₃ can be anti-inflammatory [27].

Recent data have further implicated vitamin D in adaptive immunity because of its influence upon the differentiation of T cells between the regulatory T cell (T_{reg}) and the pro-inflammatory T helper 17 (T_H17) subsets [28-30]. T_H17 cells are known to stimulate tissue inflammation and neutrophil chemotaxis, both of which are seen in ALI, predominantly by IL-17 production. It also appears that expression of markers of T_{reg} cells (Foxp3) or T_H17 cells (IL-17) by T cells may not be stable and that there is a greater degree of plasticity in their differentiation than previously appreciated [29]. Recent evidence has further suggested T_{reg} cells are important in the resolution of experimental ALI. This suggests that local lung regulation of the balance between T_{reg} and T_H17 cells may be a determinant of resolution/persistence of neutrophilic inflammation which is known to be associated with a poor prognosis in human ALI.

Although the above suggest a potentially beneficial effect of vitamin D, we must exercise some caution as some studies have shown potentially adverse effects of vitamin D. In low dose nasal LPS challenge, cellular inflammation is actually lower in vitamin D receptor deficient (VDR KO) mice due to toll-receptor hyporesponsiveness. In addition the chemotactic effects of LL-37 could in theory increase neutrophil recruitment to the lung; albeit in ALI, CXCL-8 and ENA-78 are the main chemokines driving neutrophil recruitment. More recently it has been shown that the plasma LL-37 concentration was decreased in vitamin D supplemented patients with tuberculosis, possibly representing a global suppressive effect of vitamin D supplementation on markers of acute phase response or an indirect response to enhanced microbial killing [14]. Despite these reservations, the predominant biological effects of vitamin D led us to hypothesise that vitamin D deficiency may be a risk factor for ALI, causing elevated inflammation which results in exaggerated epithelial damage in at risk, vitamin D deficient individuals.

The VINDALOO trial is a three centre randomised double blind, placebo-controlled trial aiming to define the safety and effectiveness of a single high dose of vitamin D in preventing ALI in a group of patients at high risk of developing the condition.

Methods/Design

Trial approvals and conduct

The trial is approved by South Birmingham Research Ethics Committee (REC 12/W/M/0092). The trial is

registered on the International Standard Randomised Controlled Trial Registry (ISRCTN27673620). The sponsor organisation for the trial is the University of Birmingham. The trial is funded by the Medical Research Council UK (MRC reference G1100196). The trial will be carried out in accordance with the Medical Research Council (MRC) Good Clinical Practice Guidelines, applicable UK legislation and Standard Operating Procedures of the Perioperative and Critical Care Trials Group at the University of Birmingham. The trial will be reported in line with the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [31].

Outcome measures

Primary outcome

The primary outcome will be the extravascular lung water index (EVLWI) measured by PiCCO thermodilution catheter at the end of the oesophagectomy (measured within one hour post-operatively). In ALI the changes in lung compliance that are a cardinal feature of this disease occur due to the accumulation of extravascular lung water (EVLW). The PiCCO EVLWI has been shown to be an independent risk factor for mortality in ALI and has been used as the primary outcome in several clinical trials in ALI (BALTI-1, HARP) [32,33] as well as post-thoracotomy [34]. In BALTI-1 we demonstrated that the transpulmonary thermodilution technique (PiCCO) is able to detect a significant change in lung water with a treatment that might be expected to achieve this objective. We have tested the CV coefficient of variance of EVLWI measurements previously and found it to be 6.8% over six sequential assessments over two hours. Further, we have studied the perioperative changes in lung water following oesophagectomy and demonstrated that preoperative vitamin D status influences the level of accumulation of EVLW.

Secondary outcomes

The trial secondary outcomes are clinical markers indicative of lung injury: PaO₂:FiO₂ ratio, oxygenation index, development of lung injury/ARDS during the first 28 days, ventilator and organ failure free days, survival (28 and 90 day) and safety and tolerability of vitamin D supplementation.

Lung injury will be defined by the American European Consensus Conference definition [35] as the acute onset of: (1) bilateral infiltrates on the chest x-ray; (2) hypoxaemia with a PaO₂:FiO₂ ratio of <40 kPa; and (3) absence of clinical evidence of left atrial hypertension. Ventilator free days are defined in accordance with the ARDSnet criteria as the number of calendar days after initiating unassisted breathing to day 28 after randomisation, assuming a patient survives for at least 48 consecutive hours after initiating unassisted breathing [36]. Un-assisted breathing is defined as one of at least 48

consecutive hours of: (1) being extubated with face mask, nasal prong oxygen, or room air; (2) T-tube breathing; and (3) tracheostomy mask breathing, CPAP = 5 cm H₂O without pressure support of intermittent mandatory ventilation assistance.

Organ failure free days are defined in a similar manner to ventilator free days with an organ failure free day being a day without evidence of non-respiratory organ failure. Organ failure will be defined as a sequential organ failure assessment (SOFA) score of greater than three [37].

Plasma indices of endothelial and alveolar epithelial function/injury, plasma inflammatory response will be measured by ELISA. Plasma vitamin D status (25(OH)D₃, 1,25(OH)₂D₃, VDBP) and calcium will be assessed peri-operatively (pre-drug dose, pre-operative, post-operative, day 1 and day 3) as well as EVLWI day 1 post-operatively (measured at 9 am on day 1).

Eligibility criteria

Patients will be eligible for the trial if they fulfill the following criteria:

- Planned transthoracic oesophagectomy for oesophageal carcinoma at a participating centre.
- Men over 18 years old on the day of first dose of the investigational medicinal product (IMP).
- Women over the age of 55 or more than 2 years since menopause.
- Women of potential child bearing age (under 55 and less than two years since menopause) may be recruited provided they agree to use contraception during the pre-post-operative period (eight weeks).
- Ability to give written informed consent to participate in the study.

Patients fulfilling any of the criteria below will be excluded:

- Known intolerance of vitamin D.
- Known sarcoidosis, hyperparathyroidism, or nephrolithiasis.
- Known serum corrected calcium >2.65 mmol/L.
- Undergoing haemodialysis.
- Pregnant or breastfeeding.
- Patients with tuberculosis or lymphoma.
- Diagnosis of chronic obstructive pulmonary disease (COPD) with a forced expiratory volume in one second (FEV1) less than 50% predicted or resting oxygen saturations of less 92%.

Power and sample size estimate

There are no direct data to predict the effect size of vitamin D replacement upon EVLWI. In our preliminary work with 50 patients, the patients with 25(OH)D₃

concentrations less than 15 nmol/L had the greatest increases in EVLWI (+3.2 ml/kg, +27%) compared to those patients with less severe deficiency pre-postoperatively (+1.0 ml/kg, +10% $P = 0.013$) suggesting that severe vitamin D deficiency influences EVLWI. As a group, after undergoing oesophagectomy our patients have a mean post-operative EVLWI of 10.1 ml/kg and standard deviation of 2.9 ml/kg. For the study to be able to detect a change of 20% in EVLWI with a power of 80% we will require approximately 34 patients in each arm to reach the primary endpoint ($P = 0.05$). An additional six patients will be needed to allow for dropouts, such as open/close cases, unexpected deaths and other difficulties with data collection. Thus, we intend to recruit 40 patients to each arm of the study.

With 34 patients completing each arm of the study, based upon preliminary unpublished data from 50 oesophagectomy cases, this study would detect a treatment effect of vitamin D upon the PO₂:FiO₂ ratio postoperative (PO) of ± 8.16 ($\pm 20\%$), PO plasma soluble intercellular adhesion molecule-1 (sICAM-1) of ± 16 ng/ml ($\pm 32\%$), plasma C-reactive protein (CRP) ± 53 ng/ml ($\pm 33\%$), PO plasma von Willebrand factor (vWF) $\pm 55\%$ relative to control plasma ($\pm 24.7\%$ change), PO plasma high-mobility group box 1 (HMGB-1) 5.42 ng/ml ($\pm 54\%$), PO plasma myeloperoxidase (MPO) 105 pg/ml ($\pm 57\%$), PO plasma MMP-9 51 ng/ml ($\pm 57\%$), and PO plasma surfactant protein-D (SP-D) 691 ng/ml ($\pm 61\%$). All calculations assume 80% power at a two-tailed significance level of 0.05.

Trial conduct

Approach to patients and obtaining informed consent

Patients will be identified through upper gastrointestinal cancer teams. Eligible patients will be invited to participate by their treating clinician, specialist clinical nurse or research nurse. If agreeable, written informed consent will be obtained, following a face to face discussion about the study.

Randomisation and drug / placebo supply

The trial drug manufacturer will produce the randomisation sequence using a block size of 10 with equal allocation between active and placebo groups. Drug (oral cholecalciferol oily solution Vigantol, 300,000 IU (7.5 mg)) and matching placebo (Miglyol 812 oil, the vehicle for vitamin D₃ in Vigantol) will be supplied and packaged according to the randomisation sequence into numbered treatment boxes by Novalabs (Leicester, UK). Drug boxes will be supplied to centres in blocks of 10 thus ensuring an equal allocation between active and placebo groups to balance any differences in case mix, pre-operative, operative and post-operative care between centres. Patients will be randomised sequentially by allocating them to the next numbered treatment pack held at the centre.

Drug administration

Subjects will receive either 300,000 IU (7.5 mg) of vitamin D or placebo seven days prior to planned oesophagectomy. The drug will be administered by qualified medical or nursing staff.

Concomitant medications

Patients taking the following medications are not eligible for inclusion in the study:

- Taking more than 1,000 IU/day (25 mcg/day) vitamin D supplementation by month preceding enrolment.
- Taking cardiac glycoside, carbamazepine, phenobarbital, phenytoin, primidone or long-term immunosuppressant therapy.
- Patients taking benzothiadiazine derivatives at doses higher than that which is recommended in the British National Formulary (BNF).
- Patients taking a benzothiadiazine derivative in combination with a calcium supplement.

Post randomisation withdrawals and exclusions

Subjects may withdraw from the trial or the trial treatment at any time without prejudice. If a subject withdraws from the trial treatment, then they will be followed-up wherever possible and data collected as per protocol until the end of the trial. The only exception to this is where the subject also explicitly withdraws consent for follow-up.

Blinding/un-blinding

Patients, clinical and research / trial staff will be unaware of the arm of the study to which a patient is allocated. Active and placebo treatment packs and their contents will be identical in appearance. The protocol allows for emergency un-blinding in the event of significant concerns about patient safety. In the unlikely event that un-blinding is required the local investigator will discuss this with the Chief Investigator. All events will be logged.

Monitoring and reporting adverse events

VINDALOO is recruiting a population who are prone to recognized medical and surgical complications. It is expected that many of the patients will experience an event that might be seen as a serious adverse event but is a recognized complication following oesophagectomy. Adverse and serious adverse events which are recognised complications of surgery, for example, medical (pneumonia, sepsis) and surgical (chyle leak, anastomosis leak) will be recorded in the case report form.

Death and other serious adverse events thought to be related to the study drug or serious unexpected serious adverse reactions will be reported to the trial co-

ordinating centre and Chief Investigator within 24 hours of becoming aware of their occurrence. The Chief Investigator will inform the sponsor and regulatory authorities.

Data collection

Data up until hospital discharge will be recorded in each subject's Case Report Form (CRF) by a member of the trials team. Most of the data collected will be obtained from the patient's hospital notes. In the unlikely event that a subject is transferred to another hospital, the study team will ensure that data collection is completed by the receiving hospital.

If the subject remains in hospital at 28 or 90 days, survival at these time points will be recorded by hospital staff. Mortality after hospital discharge will be obtained from the National Health Service (NHS) Statistical Tracking Service (NSTS).

Statistical analysis plan

Data will be analysed with the help of the trial statistician. Data will be analysed using SPSS for Windows 17.0. A detailed analysis plan will be developed during the trial prior to commencement of analysis. In brief, for continuously distributed data, differences between groups will be tested using independent samples t-tests with transformations of variables to Normality if appropriate, or non-parametric equivalents. Chi-squared tests (or Fisher's Exact tests) will be used for categorical variables. A *P* value of 0.05 will be considered as significant. We will test for significant correlations between changes in the biological markers using standard methods. The treatment effect will be analysed on an intention-to-treat basis. For further examinations of relationships between a binary variable and known explanatory variables, the following tests will be applied as appropriate. Logistic regression will be used to provide the estimated risk ratios for the treatment effect with associated 95% confidence interval (CI). Time to event outcomes, such as duration of ventilation or duration of hospital stay, will be analysed by survival methods and reported as hazard ratios and 95% CI. A single final analysis is planned at the end of the trial.

Trial organisation/oversight

Trial oversight will be provided by a Trial Steering Committee (TSC) comprising investigators, clinicians and trialists. An independent data monitoring committee will monitor the safety of participants enrolled in the trial through regular review of adverse event reports. An interim analysis of efficacy is not planned.

Discussion

The preliminary data that this study is based upon suggests a significant role for vitamin D deficiency as a risk factor for the development of acute lung injury. This

trial will look at biomarkers of alveolar epithelial damage and inflammation to determine the proof of concept that vitamin D replacement may have efficacy as a preventative agent for the development of acute lung injury.

Trial status

Patient recruitment commenced in September 2012 and is expected to run for two years (last patient recruited in August 2014).

Abbreviations

ALI: Acute lung injury; ARDS: Acute respiratory distress syndrome; BAL: Bronchoalveolar lavage; CI: Confidence interval; DBP: Vitamin D binding protein; ELISA: Enzyme-linked immunosorbent assay; EVLWI: Extravascular lung water index; IL: Interleukin; IMP: Investigational Medicinal Product; ISRCTN: International Standard Randomised; MMP-9: Matrix metalloproteinase-9; MRC: Medical Research Council; OLV: One lung ventilation; PICCO: Pulse Contour Cardiac Output Monitoring; RAGE: Receptor for advanced glycation endpoints; REC: Research Ethics Committee; TNF- α : Tumour necrosis factor alpha; T_H1: T helper cells; Treg: Regulatory T cells; VDBP: Vitamin D binding protein; VDR: Vitamin D receptor; 25(OH)D: 25-hydroxyvitamin D.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors made a substantial contribution to the protocol development. All authors have read and approved this manuscript.

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