INVESTIGATION INTO A POTENTIAL ROLE FOR VITAMIN D IN THE PATHOGENESIS OF ACUTE RESPIRATORY DISTRESS SYNDROME

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Abstract

Patients undergoing oesophagectomy are at risk of developing Acute Respiratory Distress Syndrome (ARDS), an immune mediated form of severe respiratory failure. The immunomodulatory properties of Vitamin D are increasingly recognised. We hypothesised that preoperative Vitamin D supplementation would reduce levels of perioperative alveolar oedema in patients undergoing oesophagectomy.

Vitamin D deficiency is common in patients with and at risk of ARDS. High dose supplementation with cholecalciferol is a safe and effective method of increasing Vitamin D levels. Supplementation reduces perioperative increases in inflammatory alveolar oedema.

Circulating plasma concentrations of the active form of Vitamin D relate to long term post-operative mortality. Patients who survive at least 2 years post-op have higher preoperative circulating numbers of Natural Killer cells. We did not find any evidence of an effect of Vitamin D on Natural Killer Cells.

In conclusion, preoperative Vitamin D status relates to perioperative changes in inflammatory alveolar oedema and high dose Vitamin D supplementation is a safe and effective method of improving preoperative Vitamin D status. Preoperative cholecalciferol administration should be considered in patients with and at risk of vitamin D deficiency.

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Declaration

No part of this thesis has previously been submitted for the award of any degree at University of Birmingham, or any other institution. Clinical samples used in chapter 4 were archived samples from patients recruited by other investigators within the research group, Dr Gavin Perkins collected samples for the BALTI investigation, Dr Christopher Bassford collected samples for the BALTI-2 investigation and Dr Daniel Park collected samples for the BALTI-prevention study. My work on the open label trial of Vitamin D supplementation and the VINDALOO trial (patient recruitment and data collection) was carried out in conjunction with Dr Dhruv Parekh. For the laboratory work I was assisted by Dr Aaron Scott and Dr Vijay D'Souza. Nevertheless, the work contained in this thesis is the work of Dr Rachel Dancer.

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List of Abbreviations

AAA	Abdominal Aortic Aneurysm
ACE	Angiotensin Converting Enzyme
AECC	American European consensus conference (on ARDS)
ALI	Acute lung injury
APACHE-II	Acute physiology and chronic health evaluation score-version II
APC	Allophycocyanin
ARDS	Acute respiratory distress syndrome
BMI	Body Mass Index
BRDU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
^{o}C	degrees Celcius
CAMP	Cathelicidin Antimicrobial Peptide
CD	Cluster of Differentiation
CI	Cardiac Index
cmH_2O	Centimetres of Water
CO	Cardiac Output
CO_2	Carbon Dioxide
CPAP	Continuous Positive Airways Pressure
CRF	Case Report Form
CRP	C Reactive Protein
CTIR	Centre for Translational Inflammation Research
CV	Coefficient of Variation
CVP	Central Venous Pressure
CXR	Chest Radiograph
CYP	Cytochrome P
DBP	Diastolic Blood Pressure
DEQAS	vitamin D External Quality Assessment Scheme
DNA	Deoxyribose Nucleic Acid
DNAse	Deoxyribonuclease

EAE	Experimental Autoimmune Encephalomyelitis
EVLP	Ex-vivo Lung Perfusion
eGFR	estimated Glomerular Filtration Rate
EIA	Enzyme ImmunoAssay
ELISA	Enzyme Linked ImmunoSorbent Assay
EVLW	Extra Vascular Lung Water
EVLWI	Extra Vascular Lung Water Index
FCS	Foetal Calf Serum
FEV1	Forced expiratory volume in one second
FiO_2	Fraction of inspired oxygen
FITC	Fluorescein
FVC	Forced Vital Capacity
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCS	Glasgow Coma Score
GEDI	Global End Diastolic Index
GEDV	Global End Diastolic Volume
GLP	Good Laboratory Practice
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
HVDRR	Hereditary Vitamin D-Resistant Rickets
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
ITU	Intensive care unit
IQR	Interquartile range
ISRCTN	International Standard Randomised Controlled Trial Number
ITBV	Intra thoracic blood volume
ITTV	Intra thoracic thermal volume
IU	International Units
kPa	kiloPascals

LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LPS	Lipopolysaccharide
M	Molar
MAP	Mean arterial pressure
MHC	Major Histocompatibility Complex
ml	millilitres
mmHg	Millimetres of Mercury
MS	Multiple Sclerosis
µL	microlitres
n	number
ng	nanograms
NHS	National Health Service
NK Cell	Natural Killer Cell
nmol	nanomoles
ns	not significant
OLV	One Lung Ventilation
PACS	Picture Archiving and Communications Systems
PAMP	Pathogen Associated Molecular Pattern
PaO_2	arterial oxygen pressure
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.005% Tween 20
PBV	Pulmonary blood volume
PE	Phycoerythrin
PEEP	Positive end expiratory pressure
P/F ratio	PaO_2/FiO_2 ratio (the ratio of plasma oxygen pressure to inspired oxygen pressure)
$PiCCO_2$	Pulse Contour Continuous Cardiac Output
pmol	picomoles
PTV	Pulmonary Thermal Volume

PVPI	Pulmonary Vascular Permeability Index
qPCR	Real time/quantitative polymerase chain reaction
RAGE	Receptor for Advanced Glycation End products
RNA	Ribonucleic Acid
RNAse	Ribonuclease
ROC	Receiver Operating Characteristic
rpm	Rotations per Minute
RPMI	Roswell Park Memorial Institute (cell culture media)
RVR	Retinoid Receptor
SBP	Systolic Blood Pressure
SD	Standard Deviation
S/F Ratio	the ratio of oxygen saturations to inspired oxygen pressure
SOFA	sequential organ system failure assessment
Strep-PE	Streptavidin-phycoerythin
SVR	Systemic Vascular Resistance
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T Cell
UK	United Kingdom
USA	United States of America
UVB	Shortwave Ultraviolet Radiation
VDBP	Vitamin D Binding Protein
VDR	Vitamin D receptor
VLDL	Very Low Density Lipoprotein
WCC	White Cell Count

CHAPTER 1

INTRODUCTION

1.1 Acute Respiratory Distress Syndrome

1.1.1 Definition

Acute Respiratory Distress Syndrome (ARDS) is a form of rapid onset severe respiratory failure which causes significant mortality and morbidity in critically ill patients. It was first described by Ashburgh and colleagues in 1967 with a case series of 12 previously healthy patients who developed respiratory failure, infiltrates on the chest radiograph and decreased lung compliance in response to a variety of both direct and indirect pulmonary insults (1). Subsequently, ARDS was acknowledged to be an important cause of morbidity and mortality in critically ill patients, but the lack of a precise definition hampered subsequent studies. In 1994 the American European Consensus Committee (AECC) attempted to address this issue by defining ARDS using the following criteria:

1) Acute onset of hypoxemia with PaO_2/FiO_2 (P/F) ratio less than or equal to 300 mm Hg,

2) Bilateral infiltrates on chest radiograph (CXR),

3) No evidence of left atrial hypertension.

In acknowledgment that severity of the syndrome is variable, patients who met all three of the above criteria were diagnosed with Acute Lung Injury (ALI) if the P/F ratio was between 200mmHg and 300mmHg (26.7-40.0kPa) and Acute Respiratory Distress Syndrome (ARDS) if the P/F ratio was less than 200mmHg (26.7kPa) (2).

While the AECC definition of ARDS improved clarity of diagnosis with criteria which are generally easily available at the ITU bedside, its reproducibility is limited due to the highly subjective nature of two elements of the definition (chest radiograph infiltrates and evidence of left atrial hypertension). In addition, ARDS can occur in the presence of left atrial hypertension and these patients were excluded by this definition (3). In 2012 a consensus group suggested the Berlin definition - a new criteria for diagnosis along with a new severity classification. The purpose of this was to simplify the diagnostic process and better prognosticate outcomes (4). It defines three levels of severity (mild, moderate and severe) according to severity of hypoxaemia. The Berlin definition is summarised in table 1.

Table 1.1 Berlin Definition of Acute Respiratory Distress Syndrome. Adapted from (4).

		Acute Respiratory Distress Syndrome
Timing		Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest Imaging (X Ray or CT Scan)		Bilateral opacities not fully explained by effusions, lobar/lung collapse or nodules
Origin of Oedema		Respiratory failure not fully explained by cardiac failure or fluid overload. If no risk factor present, objective assessment such as echocardiography required to exclude hydrostatic oedema
Oxygenation	Mild	200mmHg (26.6kPa) < PaO_2/FiO_2 ≤ 300mmHg (40kPa) with PEEP or CPAP ≥ 5cmH_2O
	Moderate	100mmHg (13.3kPa) < PaO_2/FiO_2 ≤ 200mmHg (26.6kPa) with PEEP≥5cmH_2O
	Severe	PaO_2/FiO_2 ≤ 100mmHg (13.3kPa) with PEEP ≥ 5cmH_2O

While the Berlin definition addresses concerns regarding the didactic nature of the AECC guidelines in relation to radiograph appearances and left atrial hypertension, the requirement for patients to be receiving some form of ventilatory support (PEEP or CPAP ≥ 5cmH_2O) excludes ward based patients including patients with an early form of the disease who can be managed with high concentrations of inspired oxygen alone and patients who are not considered suitable for ventilation. A 2007 study found that only 45% of patients meeting the AECC criteria for ALI were admitted to the intensive care unit, with

no significant difference in mortality between ALI patients treated on the ward or on ICU (5). This has implications for both clinical practice and research as early identification and supportive care of patients with ARDS may impact on clinical outcomes.

1.1.2 Epidemiology

In 2005, the incidence of ARDS in the USA was estimated to be around 190 600 patients annually. The disease was also estimated to result in 3.6 million hospital days in the USA (6). It is likely that this figure underestimates the true incidence as it does not consider incidence of ARDS in children nor allow for increased mortality from ARDS in non-Caucasian populations (7). A 2007 study in three Spanish hospitals found that 5.1% of hospital admissions over a 4 month period had at least one risk factor for the development of ARDS and 6.5% of these patients developed the condition. This is equivalent to an incidence of 27.7 per 100,000 head of population per year (5).

1.1.3 Aetiology

The majority of patients with ARDS develop the disease in the presence of severe sepsis secondary to pneumonia (44%) or a non-pulmonary infection (33%) (6). Other causes include aspiration of gastric contents, haemorrhage, major trauma, pancreatitis and drug reactions. However, not all patients with these diseases develop ARDS, and factors such as smoking and presence of comorbidities may influence the risk of development of ARDS (8, 9).

1.1.4 Pathophysiology

Whether the cause of ARDS is a direct or indirect pulmonary insult, the alveolar inflammation which results represents a final common pathway, mediated by circulating pro-inflammatory chemokines and cytokines and characterised by neutrophil accumulation in the alveoli. Factors generated by micro-organisms, neutrophils and platelet-leukocyte interactions such as lipopolysaccharide (LPS), TNF-α and interleukins (e.g. IL-1β, IL-6, and IL-8) destabilize and disrupt alveolar capillary barrier function (10). Disrupted alveolar capillary barrier function results in increased permeability to water, proteins and immune cells; this is a hallmark of the exudative phase of early ARDS (11). Type 1 alveolar epithelial cells, which make up 90% of the cells in the epithelium, become necrotic resulting in the formation of hyaline membranes on the denuded basement membrane (12). Neutrophils typically dominate the cellular population in the alveoli in the early stages of the disease (13) although ARDS can develop in neutropenic patients suggesting that the presence of neutrophils is not critical for the development of the disorder (12).

As the disease starts to resolve, alveolar oedema is resorbed and repair of the alveolar capillary barrier occurs with clearance of inflammatory cells and exudate from the airspaces predominantly by macrophages (12). Type II alveolar epithelial cells differentiate into type 1 cells as the alveolar epithelium repairs. In some cases ARDS resolves after the initial phase with no radiological evidence of ongoing disease. However, most patients who survive ARDS will have persistently

lower than expected carbon monoxide diffusion capacity (14) and a subset of patients will develop clinically significant fibrotic lung disease post ARDS as a result of proliferation of fibroblasts and myofibroblasts (12).

1.1.5 ARDS Phenotypes

ARDS is a heterogenous disease with patients meeting the criteria for diagnosis having a variety of causes of the disease, timing of onset in relation to that risk factor and severity of illness. Heterogeneity of the illness has been postulated as a reason for the failure of multiple clinical trials into potential treatments of ARDS which showed promise in pre-clinical trials but which did not improve outcomes when applied to a general ARDS population (15).

While the Berlin criteria definition stratifies patients according to severity of hypoxaemia, some researchers have suggested dividing ARDS into phenotypes relating to factors such as direct vs indirect causes of ARDS (16) or timing of development of ARDS in relation to the precipitating illness/event (17). One group have suggested dividing ARDS into two subphenotypes – a hyperinflammatory group and a hypoinflammatory group, based on a combination of biological and clinical markers identified by latent class analysis, and have shown that stratification based on these criteria can identify a group with more severe inflammation and worse clinical outcomes (18). However, currently there is no consensus on any approach to reduce ARDS heterogenicity.

1.1.6 Treatment of ARDS

Supportive care is the mainstay of treatment in ARDS as no pharmacological treatment has been shown to be effective in reducing morbidity or mortality. The only therapy which has been shown to be effective in reducing mortality in a non-stratified ARDS population in low tidal volume ventilation, which aims to reduce barotrauma in the alveoli which can occur if the lungs are hyperventilated (19). Early application of prone positioning has been shown to reduce mortality at 30 and 90 days when used in patients with severe ARDS (20), but other studies which enrolled patients without severe hypoxaemia failed to show any survival benefit (21). Patients with severe ARDS may also benefit from muscle paralysis, with a 48 hour administration of cisatracurium being associated with a decrease in mortality for the most severe hypoxemic patients (22), but other studies have shown that spontaneous modes of ventilation are more advantageous for patients with less severe disease (23).

1.1.7 ARDS prognosis

In 2005 in-hospital mortality from ARDS was estimated at 39%. (6) Advances in ventilation strategies have reduced in-hospital mortality, with a more recent study finding that 24% of patients diagnosed with ARDS died prior to hospital discharge (24). Cause of death in patients who do not survive an episode of ARDS is variable. Estimates of the proportion of patients who die as a result of refractory hypoxaemia vary and are likely to depend on facilities available at the treating institution. In addition, multi-organ failure and refractory shock are

commonly cited causes of death in patients with ARDS and these are likely to be connected to the underlying aetiology (25, 26).

Long term mortality in patients who survive an admission in ARDS is also high, with 22% of patients who survived to hospital discharge having died within 1 year. The most common causes of death in these patients were infection and malignancy with age and presence of comorbidities independent risk factors (24).

In patients who do survive, the majority will require ventilation for 1-2 weeks, although 10% require mechanical ventilation for 30 days or more (27). Survivors can expect a prolonged recovery from the disease, with persistent functional disability common one year after discharge from the intensive care unit and 51% of survivors unable to return to work within 12 months (14).

1.1.8 Models of ARDS

Models of ARDS are useful in order to try to gain further understanding about the pathogenesis of the disease and the effects of potential treatments. However, the heterogeneity seen in ARDS makes modelling the disease difficult and no model replicates the condition perfectly.

1.1.8.1 In-Vitro Models

In-vitro models of lung injury are useful for primary investigations into the effects of substrates of interest on the alveolar epithelium; they use single layers of cultured alveolar epithelial cells to model the epithelial surface of the alveoli.

Models typically focus on alveolar type 2 cells and can use primary type 2 cells isolated from human resected lung specimens or A549 cells, a cultured cell line similar to type 2 cells derived from lung adenocarcinoma. The wound repair model uses a pipette tip to wound the single cell layer, and then looks at the effect of culture with or without the substrate of interest on speed of wound repair. In addition, cell proliferation and viability following culture with or without the substrate can be assessed using Bromodeoxyuridine (BRDU) and Cell titre assays (28).

1.1.8.2 Animal Models

Animal models of ARDS generally attempt to replicate either direct lung injury (e.g using intratracheal administration of LPS or acid to the lungs) or indirect lung injury (e.g. using models of intra-abdominal sepsis such as caecal ligation and puncture or using intravenous administration of LPS to mimic systemic sepsis). The majority of these models involve rodents. While the use of such models has generated useful knowledge with regards to the mechanisms of lung injury, no one model has so far been able to replicate the complex pathophysiology seen in patients with ARDS (29).

1.1.8.3 Human Models

1.1.8.3.1 Ex Vivo Lungs

Ex-vivo human lung models have been used in pre-clinical trial experiments, for example to investigate the effect of mesenchymal stem cells on development of

ARDS (30). The model utilises donor human lungs that are unsuitable for transplantation. Similar models using lungs from small-medium sized animals have been widely used to research respiratory disease in the past (31). They offer a more complex model of lung structure than the single layers of cultured type 2 cells described above, and allow the effects of lung architecture and type 1 cells, which are notoriously difficult to culture, to be studied (27).

The human ex-vivo lung models allow researchers to study the effects of injury to the lung through application of stimuli such as live bacteria or LPS, a component of the outer membrane of gram negative bacteria. Introduction of potential treatments to the system can allow investigation of the possible benefits of the treatment, before proceeding to live human trials. Human lungs have been maintained for up to 12 hours using the EVLP method with no lung injury or loss of function. (27)

1.1.8.3.2 Inhaled LPS

This model uses LPS to induce a local inflammatory response in the lungs of healthy volunteers. 6 hours post inhalation a subclinical inflammatory response can be seen with neutrophil infiltration and local cytokine and chemokine release. In addition, mild systemic inflammatory changes are generated by this model (32, 33). This model has been used to investigate potential treatments for ARDS although, while this model has generated preliminary data for trials of multiple potential therapies including beta-agonists and statins, this has not translated into a positive clinical trial (34, 35).

1.1.8.3.3 Surgical Models

Multiple surgical models have in used to investigate potentially modifiable risk factors for the development of ARDS including cardiac bypass surgery, abdominal aortic aneurysm (AAA) repair and oesophagectomy. Patients undergoing elective surgery are an attractive model as patients can be recruited in advance of a timed uniform insult, thus reducing some of the heterogeneity seen in many ARDS studies. The risk of ARDS is highest in patients undergoing oesophagectomy with one study identifying that 23% of patients develop ARDS post-op (36). Risk of ARDS post cardiac bypass surgery is relatively low (2%) and complicated by the high risk of cardiac dysfunction. Open AAA repair has now been largely superseded by endovascular methods which do not lead to the same levels of inflammation and therefore this model has not been used for clinical trials in recent years.

1.1.8.3.4 Oesophagectomy

Oesophagectomy is a long, complicated procedure with a high risk of post-operative morbidity and mortality. It is typically a two stage operation – first, the abdominal stage to mobilise the stomach, then the thoracic stage to remove a portion of the oesophagus and anastomose the upper part of the oesophagus to a gastric conduit (figure 1.1).

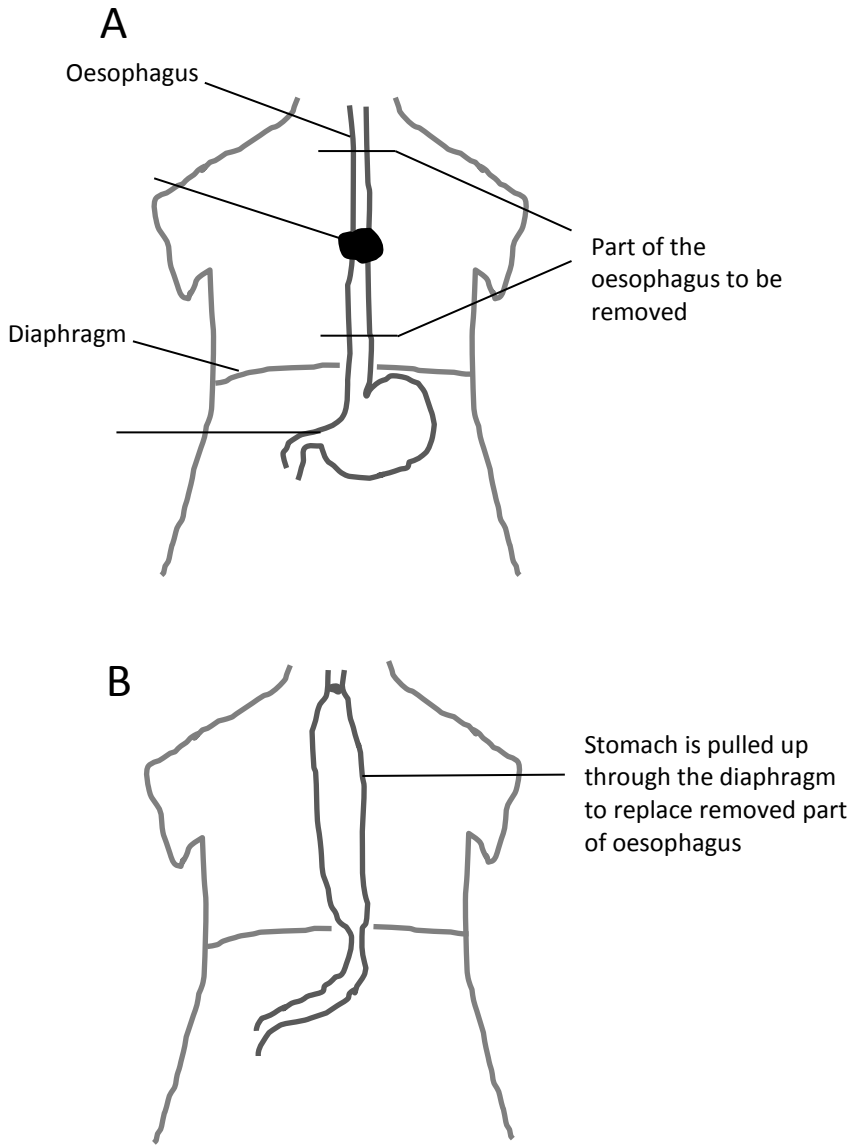


Figure 1.1 Diagram showing before (A) and after (B) a total oesophagectomy

Typically a straightforward operation without complications will take 5-6 hours. The majority of patients undergoing the operation have oesophageal cancer, although sometimes the same operation is carried out for benign causes of oesophageal stricture.

In order to access the oesophagus during the thoracic stage one of the lungs must be collapsed so that all ventilation will occur via one lung (One Lung Ventilation or OLV). OLV causes different stresses to both lungs (see figure 1.2) which combined with a lengthy operation and the inflammatory effects of the abdominal component of the operation and handling of the gut results in high post-operative systemic and local inflammation, resulting in high risk of post-operative ARDS.

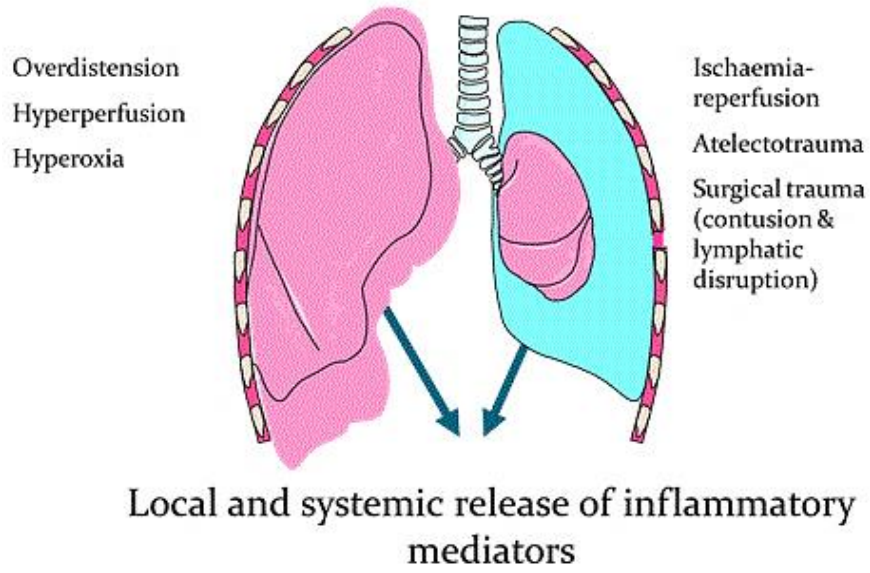


Figure 1.2 Pulmonary insults resulting from one lung ventilation
Unpublished diagram created for the UKALI research group

1.1.9 Quantification of alveolar oedema using PICCO monitoring

Radiological evidence of alveolar oedema is part of the definition of ARDS, but the severity of alveolar oedema and whether the source of the oedema is cardiac or inflammatory can be difficult to determine at the bedside. Estimation of extravascular lung water (EVLW) by transpulmonary thermodilution can assist

with quantification of alveolar oedema (37), while the ratio of EVLW to pulmonary blood volume (Pulmonary Vascular Permeability Index or PVPI) can help differentiate between hydrostatic and inflammatory causes of oedema (38).

The $PICCO_2$ monitor uses transpulmonary thermodilution to calculate EVLW and PVPI. A thermistor is placed in a peripheral artery (femoral, brachial or radial) and a bolus of cold saline injected into the superior vena cava. The cold bolus travels through the heart and lungs into the systemic arteries, where it is detected by the thermistor. The monitor detects the time of transit, changes in temperature of the injected bolus and exponential downslope time of the transpulmonary thermodilution curve (39).

These measurements can be used to measure cardiac output using the Stewart-Hamilton algorithm:

$$CO=[(Tb-Ti) \times Vi \times K]/[\int \Delta Tb \times dt]$$

CO= cardiac output (L/min)

Tb=blood temperature before the injection of cold bolus (^{o}C)

Ti=temperature of the injectate solution (^{o}C)

Vi= injectate volume (mL)

$\int \Delta Tb \times dt$=area under the thermodilution curve

K=correction constants

Intrathoracic thermal volume (total volume of intrathoracic blood and extravascular fluid, ITTV) is the product of cardiac output and mean transit time. Pulmonary thermal volume (total intravascular and extravascular fluid in the

lungs, PTV) is the exponential downslope time of the thermodilution curve multiplied by cardiac output. The Global End-Diastolic Volume (GEDV) is the difference between ITTV and PTV (39).

The total amount of blood in the thorax (ITBV) has been shown to have a linear relationship with GEDV and is calculated by multiplying GEDV by 1.25, and the difference between intrathoracic thermal volume and intrathoracic blood volume is the extravascular lung water (37). PICCO measurement of EVLW has been shown to correlate closely with gravimetric measurement of pulmonary oedema in dogs (40).

The difference between the pulmonary thermal volume and EVLW is the pulmonary blood volume (PBV) and, finally, the ratio of EVLW and PBV is the pulmonary vascular permeability index (PVPI). A high PVPI indicates greater levels of EVLW compared to PBV and is therefore consistent with alveolar oedema due to an active inflammatory process, whereas hydrostatic alveolar oedema is associated with a low PVPI. Use of the PICCO method to calculate PVPI has been shown to be useful in helping differentiate patients with hydrostatic alveolar oedema from patients with ARDS (38).

PICCO measurement of EVLW has been shown to be pharmaceutically manipulatable in the BALTI studies of the effect of beta agonists on prevention and treatment of ARDS (41, 42). Multiple studies have shown that Extra Vascular Lung Water, indexed according to predicted body weight (EVLWI), is an independent predictor of mortality in ARDS (43-45). Early reductions in EVLWI

may be associated with a survival benefit (46). PVPI has also shown to be a predictor of survival in ARDS patients and to be independent of EVLWI (44, 47).

1.2 Vitamin D Biology

The important role of Vitamin D in bone health has long been recognised; Vitamin D is essential for facilitating absorption of calcium from the gut and without it children will develop rickets and adults the painful bone condition osteomalacia. However, many other cells and tissues in the human body express the Vitamin D receptor and the importance of Vitamin D in many other physiological processes is increasingly recognised (48).

1.2.1 Sources of Vitamin D

Humans are able to obtain vitamin D via the skin or the gut. Sunlight is the greatest source of Vitamin D in humans; in the form of UVB radiation (290-315nm) it acts on 7-dehydrocholesterol in the skin to produce Vitamin D_3 (48). Two different isoforms of Vitamin D can be obtained via the gut, Vitamin D_2 is typically obtained from mushrooms (where it is created via exposure to sunlight in a similar manner to photosynthesis of Vitamin D_3 in humans) and Vitamin D_3 is obtained from animal sources such as egg yolk and oily fish (49). Supplements can comprise of either isoform, with Vitamin D_2 being commonly used in the USA and Vitamin D_3 being more available in the UK. Table 1.2 summarises the vitamin D content of different sources of Vitamin D.

Table 1.2. Vitamin D content of different sources of Vitamin D.
Adapted from (49)

Source		**Vitamin D Content**
Salmon	Fresh – Wild (3.5oz) Fresh – Farmed (3.5oz) Canned (3.5oz)	600-1000IU D_3 100-250IU D_3 or D_2 300-600IU D_3
Sardines	Canned (3.5oz)	300IU D_3
Mackerel	Canned (3.5oz)	250IU D_3
Tuna	Canned (3.6oz)	230IU D_3
Shiitake Mushrooms	Fresh (3.5oz) Sun Dried (3.5oz)	100IU D_2 1600IU D_2
Egg Yolk		20IU D_3 or D_2
Cod Liver Oil (1tsp)		400-1000IU D_3
Multivitamin		400IU D_2 or D_3
Sunlight, UVB	0.5 minimal erythemal dose*	3000IU D_3

**About 0.5 minimal erythemal dose of uvb radiation would be absorbed after exposure of the arms and legs to sunlight for 5-10 minutes on average (time of exposure required for a specific dose varies with time of day, season, latitude, skin sensitivity)*

1.2.2 Vitamin D Metabolism

Whether sourced from the skin or gut, Vitamin D enters the circulation and is hydroxylated in the liver to form 25-OH Vitamin D, the main circulating form. 25-OH Vitamin D predominantly circulates bound to Vitamin D Binding Protein (VDBP), although it is also able to circulate bound to albumin or unbound. 1,25-$(OH)_2$ Vitamin D, the physiologically active form, is typically formed in the kidney, although many other cells express 1α-hydroxylase and are capable of converting

25-OH Vitamin D to 1,25-$(OH)_2$ Vitamin D to form higher local concentrations than that seen in the circulation (figure 1.3).

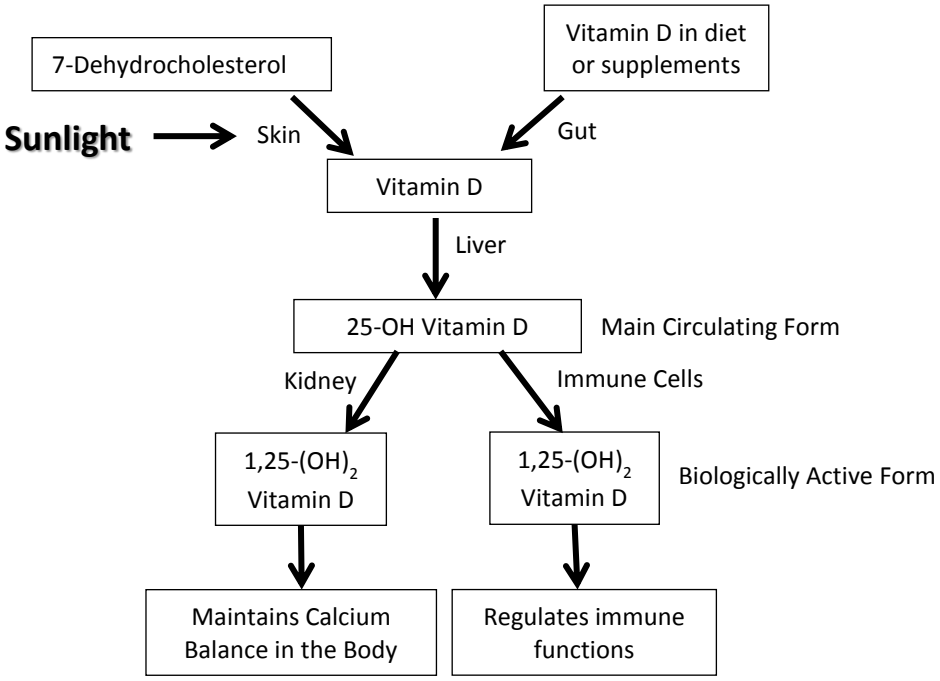


Figure 1.3 Human Vitamin D synthesis and metabolism

While the classical Vitamin D pathway is well described, not all the vitamin D produced by the action of sunlight is converted immediately to the physiologically active form. Studies have shown that excess Vitamin D is stored in fat (ready for use in winter when sunlight levels are lower), but the factors that influence whether Vitamin D is converted to the physiologically active form or not are not well known. One study in post-menopausal women found a correlation between 25-OH and 1,25-$(OH)_2$ Vitamin D plasma concentrations, that was strongest in deficient subjects (i.e. women who had low plasma concentrations of 25-OH vitamin D were also likely to have low plasma concentrations of 1,25-$(OH)_2$ Vitamin D) (50), but this study was cross sectional,

and did not look at the longitudinal effects of changes in vitamin D status. Another study found that 1,25-$(OH)_2$ Vitamin D status is linked to Vitamin D binding protein plasma concentrations and that women with different Vitamin D Binding Protein phenotypes have different relationships between 25-OH and 1,25-$(OH)_2$ vitamin D (51). Other studies have suggested that circulating 1,25-$(OH)_2$ vitamin D plasma concentrations are not solely reliant on 25-OH vitamin D status. For example, a study looking at the effects of sun exposure for 30mins daily over 4 weeks in the spring in elderly residential home residents found that while 25-OH vitamin D plasma concentrations increased significantly, there was no significant change in 1,25-$(OH)_2$ Vitamin D plasma concentrations (52). Another study comparing 12 months treatment with Hormone Replacement Therapy (HRT) with Vitamin D_3 supplementation in post-menopausal women found that despite the groups which received Vitamin D_3 seeing a significant rise in serum 25-OH vitamin D, patients in the group which received HRT only had a greater rise in 1,25-$(OH)_2$ Vitamin D plasma concentrations than patients in the groups that received Vitamin D_3 either on its own on in combination with HRT (53).

1.2.3 Effects of Vitamin D on immune functions

1,25-$(OH)_2$ Vitamin D acts by binding to and activating the Vitamin D receptor (VDR), a nuclear receptor. This complex then forms a heterodimer with the retinoid receptor (RVR) which induces the expression of Vitamin D responsive genes (54). VDR is expressed by numerous cells of the immune system including

neutrophils, macrophages and T and B lymphocytes (55, 56). The effects of vitamin D on human immunity are wide-ranging, acting across various aspects of both the innate and adaptive immune systems in endocrine, paracrine and intracrine manners.

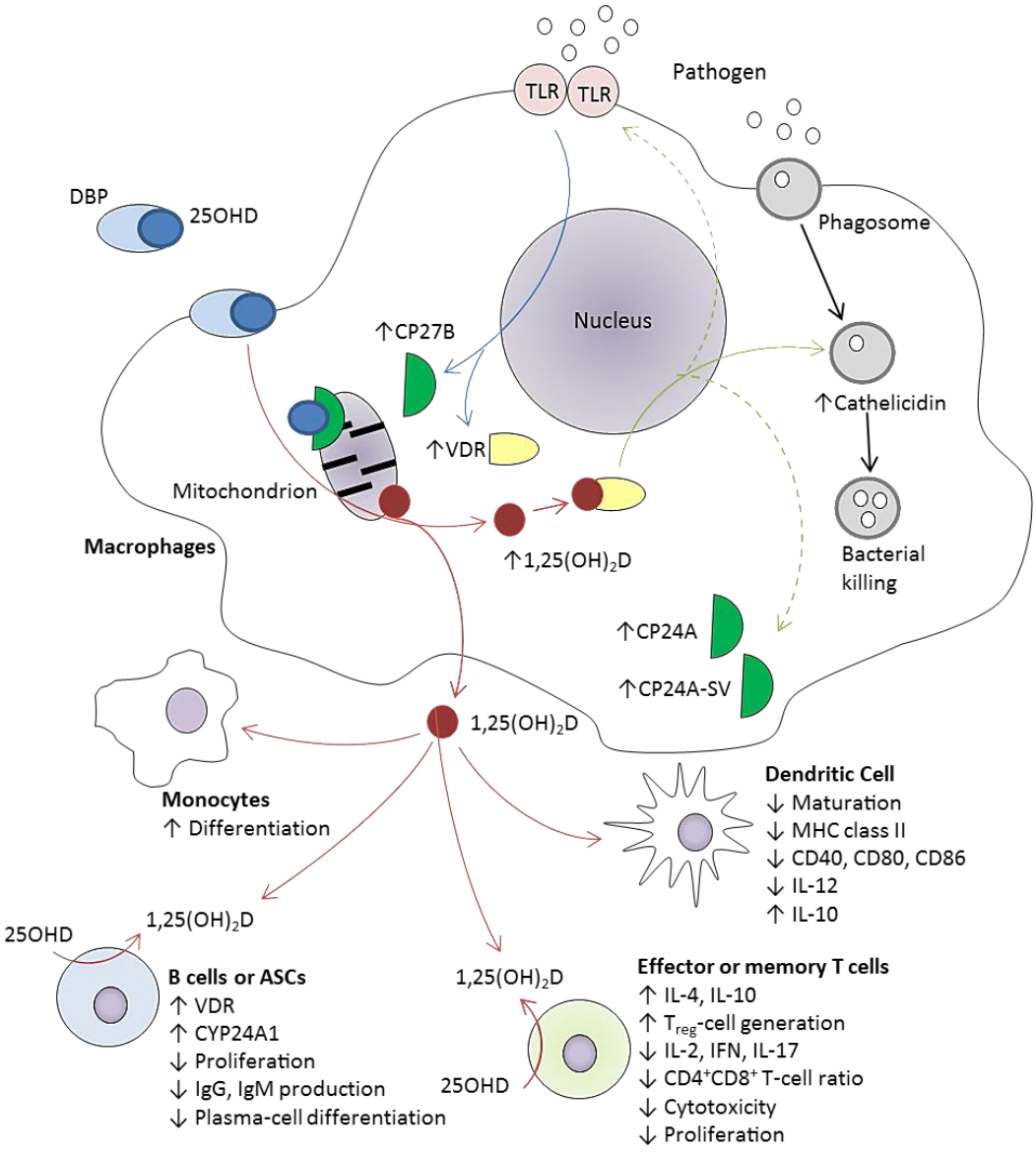


Figure 1.4: Impact of Vitamin D on cells of the immune system

Adapted from figures in (57, 58) Solid lines indicate positive feedback, dashed lines indicate negative feedback.

As illustrated in figure 1.4, monocytes/macrophages and dendritic cells express CYP27B which converts 25-OH Vitamin D to 1,25-$(OH)_2$ Vitamin D, leading to locally increased plasma concentrations of 1,25-$(OH)_2$ Vitamin D compared to circulating plasma concentrations. This process is upregulated when the cell is stimulated with interferon-γ (IFN-γ) (59) or when Toll like receptors (TLRs) sense the pathogen associated molecular pattern (PAMP) (60). This process also downregulates CYP24A which is responsible for inactivating 1,25-dOH Vitamin D by conversion to 24,25-$(OH)_2$ Vitamin D (54).

In monocytes and macrophages, 25-OH vitamin D is converted to 1,25-$(OH)_2$ Vitamin D which interacts with the VDR to induce the intracrine release of antimicrobial peptides such as cathelicidins via binding to Vitamin D responsive elements (VDRE) in the promotor regions of cathelicidin antimicrobial peptide (CAMP) genes. CAMP is expressed by neutrophils, monocytes, dendritic cells, lymphocytes, natural killer (NK) cells and epithelial cells of the skin, respiratory tract and gastrointestinal tract. Cathelicidins not only have broad antimicrobial activity, acting against bacteria, fungi and viruses, they are also capable of inducing wound healing, angiogenesis and modulating apoptosis (61).

As the most numerous circulating immune cell, neutrophils are likely to be the greatest source of cathelicidins. Neutrophils express VDR but not a functional CYP27B enzyme, suggesting that neutrophil release of cathelicidin may be more dependent on the endocrine effect of circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D formed in the kidney. A study in patients with chronic renal

disease found an association between serum plasma concentrations of the cathelicidin LL37 and circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations, but not plasma concentrations of 25-OH Vitamin D (62). However, a study in patients with sepsis (who would be expected to have high neutrophil numbers) found a correlation between circulating LL37 and 25-OH vitamin D (63).

As antigen presenting cells, dendritic cells link the innate and adaptive immune systems. Multiple studies have demonstrated that 1,25-$(OH)_2$ Vitamin D suppresses dendritic cell maturation, leading to reduced secretion of IL-12 and increased secretion of IL10 as a result of decreased expression of costimulatory molecules such as CD-40, CD-80 and CD86 and MHC class II. This results in reduced activation of naïve T cells. As dendritic cells express CYP27B in a similar manner to monocytes and macrophages it is likely that a local high concentration of 1,25-$(OH)_2$ Vitamin D is necessary for these effects (64-66).

Studies investigating the effect of Vitamin D on T cells have established that sunlight exposure or 1,25-$(OH)_2$ Vitamin D supplementation increases circulating CD3+ lymphocytes and CD8+ T cells (67, 68). In vitro, 1,25-$(OH)_2$ vitamin D suppresses the effects of Th1 cells, which secrete cytokines such as IFNγ and Tumour Necrosis Factor-α (TNFα) (69). There is also some evidence that 1,25-$(OH)_2$ Vitamin D increases Th2 mediated secretion of IL4, a cytokine involved in immune regulation (70). In addition, Vitamin D has been shown to increase numbers of Regulatory T cells (Treg) both in vivo (71) and in vitro (72).

The effect of Vitamin D on Natural Killer (NK) cells is less well defined and the few studies available mainly focus on the effects of Vitamin D on NK cell function, not circulating numbers. While some studies have suggested that 1,25-$(OH)_2$ Vitamin D increases NK cell cytotoxicity (73), others have suggested a reduction in cytotoxicity with Vitamin D (74). One study of vitamin D supplementation which found an increase in circulating numbers of Treg found no effect on circulating NK cell numbers (71).

1.2.4 Role of Vitamin D deficiency in autoimmune disease

Vitamin D deficiency has been implicated as a causative factor in multiple autoimmune diseases including multiple sclerosis (MS), inflammatory bowel disease (IBD), type 1 diabetes and rheumatoid arthritis (75). Such diseases are more common in people living in northern latitudes such as Canada and Northern Europe where exposure to sunlight is lower than further south (76). Exacerbations of MS are more likely to occur in the spring, when Vitamin D plasma concentrations are at their lowest, than in other seasons (77, 78). Studies in mice have shown that 1,25-$(OH)_2$ Vitamin D treatment results in reversal of paralysis associated with experimental autoimmune encephalomyelitis (EAE, a mouse model of MS). This was accompanied by a reduction in IL17A secreting CD4+ T cells (79). However, studies have shown that EAE is less severe and onset is delayed in VDR knockout mice, suggesting that VDR plays a role in development of the disease (80). In IBD, VDR knockout mice show more severe symptoms of experimentally induced colitis than wild type mice with high colonic

expression of TNF-α, IL-1α, IL-1β, IL-12, IFN-γ, IL-10, MIP-1α and KC. Treatment with oral or rectal 1,25-$(OH)_2$ Vitamin D reduced inflammation in the wild type mice (81).

Studies in mice have also demonstrated that low plasma concentrations of 1,25-$(OH)_2$ Vitamin D results in hypertension as a result of negative regulation of the renin-angiotensin system by VDR (82). However, in humans, a rare autosomal recessive loss of Vitamin D receptor function results in hereditary vitamin D-resistant rickets (HVDRR). A group of 17 patients with HVDRR aged 6-36 years were studied to investigate the role of VDR in the renin-angiotensin system in humans. The patients were found to have elevated 1,25-$(OH)_2$ Vitamin D plasma concentrations but normal renin and angiotensin converting enzyme (ACE) activity, non-significant mild elevation of angiotensin II, normal aldosterone plasma concentrations and no hypertension (83). No trend towards increases in autoimmune conditions or other extra skeletal effects of Vitamin D deficiency has been detected in patients with HVDRR (84).

A study in middle aged men showed that circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations inversely correlate with hypertension and VLDL triglycerides with 25-OH Vitamin D plasma concentrations correlate with fasting insulin and insulin sensitivity (85). A meta-analysis of studies of Vitamin D in patients with type 2 diabetes concluded that Vitamin D and calcium insufficiency may negatively influence blood glucose plasma concentrations and supplementation with both nutrients may be helpful in optimizing glucose metabolism (86). In MS, lower 25-

OH Vitamin D plasma concentrations are associated with a higher risk of relapse (87, 88) but a systematic review of trials of Vitamin D therapy in patients with MS was inconclusive (89).

1.2.5 Effects of Vitamin D on pulmonary inflammation and infection

Increased knowledge of the effects of Vitamin D on innate and adaptive immunity naturally lead to consideration of the effects of Vitamin D on pulmonary disease. Bronchial epithelial and resident immune cells convert circulating 25-OH Vitamin D to 1,25-$(OH)_2$ Vitamin D which boosts innate immunity to pathogens and regulates adaptive immune responses. (90) High vitamin D levels are associated with improved responses to glucocorticosteroids, a mainstay of treatment in many diseases affecting the lungs (91). Additionally, Vitamin D binding protein has been linked with a role in the pathogenesis in COPD, with high levels associated with lower FEV1 and higher macrophage activation (92),

A number of studies have investigated a role for Vitamin D deficiency in acute and chronic lung disease. The third national health and nutrition examination survey demonstrated an association between serum 25-hydroxyvitamin d and pulmonary function which persisted after adjustment for age, gender, height, body mass index, ethnicity, and smoking history (93). Epidemiological and observational studies have demonstrated a link between Vitamin D deficiency and risk of developing respiratory diseases including including viral respiratory infections, COPD, asthma, and TB. (94-98) Exposure to the sun and UV light was

utilised as a treatment for TB in the early 20th Century, with patients admitted to heliotherapy wards and moved outside in order to maximise sunlight exposure prior to the development of antibiotic therapy. (99) Trials of Vitamin D supplementation in patients with tuberculosis have not demonstrated a significant overall benefit in recovery (100, 101), although a meta-analysis of 6 studies of Vitamin D supplementation in patients with TB demonstrated a non-significant reduction of TB infections patients supplemented with vitamin D (99). Trials of Vitamin D supplementation to prevent other respiratory tract infections have also been undertaken, and while the results of individual trials have been inconclusive, a meta-analysis in 2013 demonstrated a protective effect of supplementation (102). In asthma, a meta-analysis of nine trials reported that in people with predominantly mild to moderate disease, vitamin D supplementation is likely to reduce both the risk of severe asthma exacerbation and healthcare use (103), which may again point to an effect on the risk of developing respiratory tract infections.

1.2.6 Vitamin D and ARDS

The known effects of Vitamin D on human immunity point to potential pathogenic links between vitamin D deficiency and ARDS. The effect of cathelicidins, released by resident alveolar macrophages, on wound healing, angiogenesis and modulating apoptosis is highly relevant to ARDS pathogenesis, in addition to the broad antimicrobial peptide which may play a role in reducing infection and therefore preventing lung Injury from occurring. Effects on the

adaptive immune system, promoting towards regulatory (Treg) cells and away from inflammatory Th17 cells may promote the resolution of Lung Injury.

There have been a few studies of the role of Vitamin D in animal models of Lung Injury. A study using the LPS inhalation model in hamsters demonstrated that 1,25-$(OH)_2$ Vitamin D inhibited neutrophil recruitment in the lung by approximately 40% without increasing plasma calcium concentration. There were no effects on monocyte recruitment (104). A study investigating effects of Vitamin D on lung injury induced by reperfusion of femoral vessels in rats found that pre-treatment with cholecalciferol ameliorated Lung injury and systemic IL6 levels (105). However, a study using the LPS challenge model in mice found no relationship between serum vitamin D concentration and degree of lung injury, nor an effect on resolution of lung injury (106).

Several studies have reported high prevalence of Vitamin D deficiency in patients admitted to critical care, with one study reporting that 17% of patients had undetectable levels (107). In 2009 Jeng et al published a study showing that critically ill patients have lower plasma concentrations of 25-OH vitamin D than healthy controls and that this correlates with plasma concentrations of cathelicidin (63). Vitamin D deficiency prior to hospital admission has been found to be a predictor for short and long term all-cause mortality and for blood culture positivity in critically ill patients (108). A 2014 meta-analysis found that vitamin D deficiency significantly increases susceptibility for severe infections and mortality in critically ill patients (109).

1.2.7 Optimal Vitamin D Plasma concentrations

Vitamin D status is generally defined by circulating plasma concentrations of 25-OH Vitamin D. There is no consensus as to what plasma concentration is optimal. The Institute of Medicine in 2010 recommended that plasma concentrations less than 30nmol/L carry an increased risk of osteomalacia and should be considered deficient and plasma concentrations less than 50nmol/L carry an increased risk of secondary hyperparathyroidism and should be considered inadequate (110). Most recommendations on supplementation aim to achieve plasma concentrations greater than 50nmol/L. However, a number of clinical trials of Vitamin D supplementation have shown health benefits in increasing circulating 25-OH Vitamin D plasma concentrations higher than 50nmol/L including improvements in bone density, pain, well-being and mood and new cancer diagnoses (111). The 2011 Endocrine Society Guidelines recommend that 25-OH Vitamin D plasma concentrations less than 50nmol/L should be considered deficient and plasma concentrations between 50 and 75nmol/L should be considered insufficient (112). There is even less consensus on upper limits for Vitamin D. Rarely, prolonged ingestion of high doses of Vitamin D can result in hypercalcaemia although no plasma concentration of 25-OH Vitamin D which inevitably results in hypercalcaemia has been identified (113). A study in people living a traditional lifestyle in Tanzania found that mean plasma concentrations were 115nmol/L and plasma concentrations ranged from 58 to 171nmol/L in healthy individuals (114). A number of studies have found that patients with high 25-OH Vitamin D plasma concentrations have increased mortality, with the odds

of death increasing at plasma concentrations greater than 98nmol/L, 140nmol/L and 150nmol/L. However, all these studies were observational (115-117).

The normal range for circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D has recently been evaluated in 96 healthy volunteers using liquid chromatography tandem mass spectrometry and was found to be 59-159pmol/L This new method of measuring 1,25-$(OH)_2$ Vitamin D was compared with results of the vitamin D External Quality Assessment Scheme (DEQAS) as well as radioimmunoassays which is the more common method of analysis (118). Reference ranges for radioimmunoassays often vary according to the laboratory carrying out the measurements, but are normally in the region of 50-150pmol/L (119). Plasma concentrations of 1,25-$(OH)_2$ Vitamin D may vary diurnally; a study in post-menopausal women found plasma concentrations were lower in the morning than later in the day (120) but studies in younger people did not find any evidence of variation (121, 122).

1.2.8 Vitamin D Supplementation

Recommendations on dosing strategies to increase Vitamin D plasma concentrations in deficient patients vary from 800IU/day to intermittent intramuscular mega doses (300,000 or 600,000IU) in patients with malabsorption or who are unable to take oral therapy. (123, 124) The most common dose used in clinical practice is 800IU orally per day, often in conjunction with oral calcium. (125)

Trials of Vitamin D supplementation have used a wide range of doses, mainly consisting of daily doses over a period of weeks or months. A trial of 4000IU/day for 6 months in insulin resistant Asian women found that median 25-OH Vitamin D plasma concentrations increased from 21nmol/L to 80nmol/L in the treatment arm compared with an increase from 19nmol/L to 29nmol/L in the placebo arm (likely secondary to increasing sun exposure) (126). Another trial of 5000IU/day in nursing home residents resulted in mean 25-OH Vitamin D plasma concentrations increasing from 29nmol/L to 126nmol/L after 12 months. Plasma concentrations exceeded 74nmol/L in 92% of the patients at the end of this trial (127). Other trials have using intermittent higher doses. A trial of fortnightly doses of 120,000IU cholecalciferol over 6 weeks resulted in mean increase in 25-OH Vitamin D plasma concentrations of 35nmol/L (128). A trial in patients with asthma of 6 2-monthly doses of 120,000IU cholecalciferol resulted in significant increases in 25-OH Vitamin D plasma concentrations after 2 and 12 months (129). A trial of annual oral doses of 500,000IU cholecalciferol in older women at risk of falls showed that patients in the treatment arm had 25-OH Vitamin D plasma concentrations in excess of 120nmol/L on average compared to average plasma concentrations less than 50nmol/L in the placebo arm. However, it is of note that patients in the treatment arm of this trial were more at risk of falls than patients recruited to the placebo arm (130).

1.3 Conclusion

ARDS is an immune mediated form of acute respiratory failure characterised by neutrophilic alveolar inflammation, breakdown of the alveolar capillary barrier and inflammatory alveolar oedema. Vitamin D has been shown to play a key role in regulation of human immunity with wide ranging effects on cells of the immune system. Vitamin D deficiency has been implicated as a causative factor in a number of autoimmune diseases. Overall, the immunomodulatory effects of Vitamin D are complex and yet to be fully defined. While Vitamin D supplementation has been suggested as a possible treatment for multiple autoimmune diseases, the potential for both positive and negative effects on immune function mean that randomised placebo controlled trials are essential to establish whether supplementation can be of benefit.

CHAPTER 2

AIMS AND HYPOTHESIS

2.1 Aims

The aims of this thesis are to:

1. Determine the prevalence of Vitamin D deficiency in patients at risk of ARDS due to undergoing oesophagectomy. How do Vitamin D plasma concentrations relate to clinical and biological markers of ARDS post oesophagectomy?

2. Investigate the short term efficacy of a one-off high dose supplement of cholecalciferol in increasing Vitamin D plasma concentrations prior to oesophagectomy.

3. Examine the effects of a one off high dose supplement of cholecalciferol on biomarkers of alveolar oedema and systemic inflammation.

2.2 Hypothesis

Patients with Vitamin D deficiency are at greater risk of developing ARDS post oesophagectomy. Preoperative supplementation with cholecalciferol reduces post-operative alveolar oedema and systemic inflammation.

CHAPTER 3

GENERAL METHODS

3.1 Historical Cohorts

3.1.1 BALTI-prevention

The preliminary work in this thesis was carried out on stored samples from patients recruited to the translational sub study of the BALTI-prevention trial (EudraCT 2007-004096-19, ISRCTN 47481946). This was a trial of long acting beta-2 agonist (salmeterol) vs placebo for prevention of ARDS in patients undergoing transthoracic oesophagectomy. 55 stored plasma samples taken immediately prior to the operation were available for Vitamin D analysis and results were entered into a database of clinical and biochemical data. Plasma samples and data were collected and archived by Dr Daniel Park. The translational study was approved alongside the main trial by the South Birmingham Local Research Ethics Committee. (REC reference 07/H1207/233.). Recruitment to the study took place between March 2008 and September 2009. Samples were stored in aliquots of 0.5 millilitres at -80^{o}C until analysis was performed.

3.1.2 BALTI and BALTI2

Vitamin D analysis was also carried out on 52 stored plasma samples taken on the day of enrolment from patients recruited to the BALTI (35 samples) and BALTI2 (17 samples) trials which were trials of beta-2 agonists in patients diagnosed with ARDS. The trials were both double blind placebo controlled trials of intravenous salbutamol in the treatment of ARDS. Samples from the BALTI trial were collected and archived by Prof Gavin Perkins. The trial was approved by

East Birmingham Local Research Ethics Committee. 40 patients were recruited to the BALTI trial between January 2001 and December 2003 at Birmingham Heartlands hospital. Samples were stored in aliquots of 0.5 millilitres at -80^{o}C until analysis was performed. There was insufficient plasma for analysis from 5 patients. Samples from the BALTI-2 trial (EudraCT Number: 2006-002647-86, ISRCTN38366450) were collected and archived by Dr Christopher Bassford. The trial was approved by the Oxfordshire Local Research Ethics Committee (REC reference 06/Q1604/123). The 17 samples used in this work were recruited to a bronchoscopic substudy of the trial. Samples were stored in aliquots of 0.5 millilitres at -80^{o}C until analysis was performed. The overall trial was suspended on safety grounds by the trial steering committee on 26th March 2010. 326 patients had been recruited at this point, and an interim analysis was undertaken. This showed an increased mortality rate in those patients given the active drug compared to those treated with placebo.

3.2 Patient recruitment

3.2.1 Open label dosing study

18 patients were recruited to an open label dosing study of cholecalciferol to help determine what dose of cholecalciferol to use in the placebo controlled trial (EudraCT 2011-004199-12, ISRCTN 66719785). Patients were recruited between April and September 2012. Patients were eligible for inclusion in the trial if they met the criteria detailed in table 3.1.

Table 3.1 Inclusion and exclusion criteria for open label dosing study and Vindaloo trial

Inclusion Criteria	Exclusion Criteria
Planned transthoracic oesophagectomy at a participating centre	known intolerance of vitamin D
aged over 18 years on day of first dose of IMP	known sarcoidosis, hyperparathyroidism, or nephrolithiasis
Ability to give written informed consent to participate in the study	taking more than 1000iu/day vitamin D supplementation in the month preceding enrolment
	known baseline serum corrected calcium >2.65 mmol/L
	undergoing haemodialysis
	Pregnant or breastfeeding
	Taking cardiac glycoside, carbamazepine, phenobarbital, phenytoin, primidone or long-term immunosuppressant therapy
	Taking oral preparation containing > 10 micrograms vitamin D/day up to 2 months before first dose of IMP
	Diagnosis of COPD with an FEV1 less than 50% predicted or resting oxygen saturations of less 92%

3 doses of cholecalciferol were trialled sequentially – patients 1-6 received 100,000IU cholecalciferol, patients 7-12 received 200,000IU cholecalciferol and patients 13-18 received 300,000IU. These doses were chosen because a review of the available literature at the time showed that a single oral dose of 100,000IU of Vitamin D_2 had been shown to increase serum total 25(OH)D levels by 110

nmol/l in TB patients with all volunteers achieving serum levels above 75 nmol/l at one week post dose, with no patient developing hypercalcaemia (131). Higher single oral doses had been successfully used in elderly patients with 300,000 IU of Vitamin D_3 achieving greater serum levels than regular oral dosing without major toxicity (132). Patients were administered the dose of cholecalciferol after consenting to inclusion in the trial at the pre-operative assessment clinic appointment which was 3-14 days prior to the planned date of the operation. Plasma concentrations of 25-OH Vitamin D peak at 7 days post oral dosing and decrease linearly thereafter, returning to baseline around day 84 on average (133). This window was therefore pragmatically chosen in order for the operation to coincide with peak plasma 25-OH Vitamin D concentrations while maximising recruitment of patients. The drug used was Vigantol® 812 oil (Merck Darmstadt Germany), an oily solution containing 20,000IU/ml cholecalciferol. Patients unable to swallow due to the severity of their oesophageal disease are routinely given a nasogastric or percutaneous endoscopic gastrostomy feeding tube and the drug was administered via that if required.

The primary outcome for the trial was 25-OH Vitamin D_3 plasma concentration on the day of oesophagectomy.

The trial was approved by the South Birmingham local research ethics committee (REC reference number: 11/WM/0330)

3.2.2 VINDALOO Trial

79 patients were recruited to the Vindaloo (VItamiN D to prevent Acute Lung injury following OesOphagectomy), a placebo controlled, double blind, randomised controlled trial between September 2012 and March 2015 (EudraCT 2012-000332-25, ISRCTN 27673620). The trial was approved by the South Birmingham local Research Ethics Committee (REC reference 12/WM/0092). The inclusion and exclusion criteria were the same as for the open label study described in table 3.1.

Following analysis of the results of the open label study (see chapter 5) patients were allocated to 15ml (containing 300,000IU cholecalciferol) Vigantol® oil or 15ml placebo Miglyol 812 oil using sequential patient study number as predetermined randomisation by the supplier, Nova Labs. Neither investigators nor patients were aware of the subject's allocation while they were participating in the trial.

The primary endpoint for the Vindaloo trial was extravascular lung water at the end of the operation and the trial was powered to detect a change of 20% in EVLWI with a power of 80% (p=0.05). This study was conceived as a phase 2, proof of efficacy study and therefore the endpoint chosen was not intended to be clinically meaningful. As a group, patients undergoing oesophagectomy have a mean post-operative EVLWI of 10.1ml/kg and standard deviation of 3.0ml/kg and the power calculation estimated 34 patients were required in each arm to reach the primary endpoint. In order to allow for dropouts such as open/close cases,

changes to operative plans and other difficulties with data collection, it was intended to recruit 40 patients in each arm of the study.

3.3 Transpulmonary Thermodilution Analysis ($PICCO_2$)

$Picco_2$ measurements were collected at the beginning of the operation (after induction of anaesthesia but before the operation commenced), at the end of the operation and on the first morning after the operation. The theory and calculations behind $PICCO_2$ measurements is explained in detail in section 1.1.9.

All patients undergoing oesophagectomy have a central venous catheter inserted as a routine part of clinical care. Patients agreed to femoral artery catheter insertion as part of the trial consent process. The $PICCO_2$ catheter was inserted into the right femoral artery by a member of the trial team under aseptic conditions using ultrasound guidance where required – ultrasound guidance was required in order to site the line one case, and in one further case the line could not be sited despite ultrasound guidance. Central Venous Pressure (CVP) was measured using the central venous catheter and manually entered into the $PICCO_2$ machine prior to obtaining $PICCO_2$ measurements.

$PICCO_2$ readings were obtained by connecting an injectate sensor to the central venous catheter and the $PICCO_2$ machine. At least 15ml of cold saline was injected into the superior vena cava via the central venous catheter and the $PICCO_2$ machine calculated measures of haemodynamics and transpulmonary volumes by analysing the thermodilution curve. Three injections were carried out and the $PiCCO_2$ measures for these studies were calculated from the mean of the

three readings. Where the individual $PiCCO_2$ measures of cardiac output varied by more than 20%, a fourth injection was performed and the deviant measurement was discarded, as per the guidance in the supplied manual. Studies have shown that using 3 injections results in a %CV <10% for Cardiac Index (CI), Global End Diastolic Volume Index (GEDI) and Extravascular Lung Water Index (EVLWI) (134, 135).

3.4 Blood Collection and Processing

Blood samples were collected on the day of drug administration (baseline), on the morning of the day of the operation (preop), at the end of the operation (post op), on the morning of the first day after the operation (day 1) and on the morning of the third day after the operation (day 3). Where possible, blood was collected from pre-sited arterial or central lines, or alongside clinical samples, to avoid subjecting the patients to extra venepuncture. All baseline blood samples were venous and all pre- and post op samples were arterial, subsequently samples were collected from arterial lines were available or from the venous circulation if arterial lines had been removed. Blood was collected into 7ml Lithium Heparin and 4.5ml Ethylenediaminetetraacetic acid (EDTA) Vacutainer® tubes (Becton Dickinson Ltd, Oxford, UK).

In the laboratory, blood in vacutainer tubes was placed in a centrifuge and spun at 560g for 10 minutes. The supernatant (plasma) was aspirated immediately and divided into 0.5ml aliquots, then stored at -80C in cryovials for future analysis.

For analysis of peripheral blood mononuclear cells, after plasma was removed the blood cells were diluted 4:1 with Phosphate Buffered Saline (PBS). 15mL of this cell solution was over-layered on 10mL of lymphoprep™ (Axis-Shield, Dundee, UK) and spun in a centrifuge at 800g for 20 minutes with minimal acceleration and no brake to avoid disruption of the layers. After centrifugation an interphase buffy layer was visible (figure 3.1) which was aspirated using a Pasteur pipette and washed using PBS.

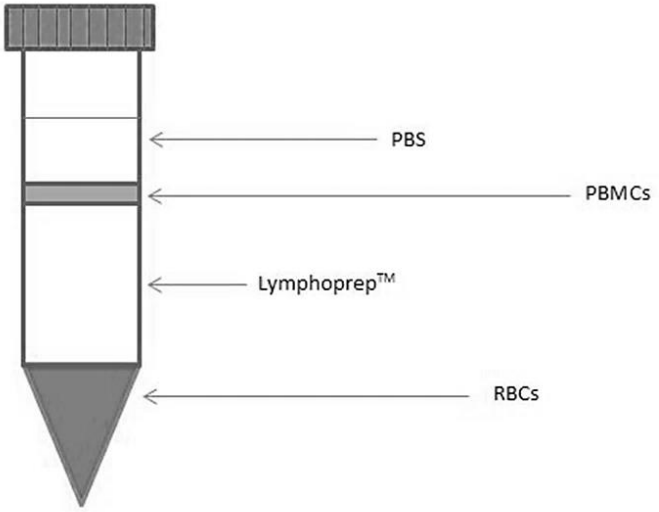


Figure 3.1 Collection of PBMCs from blood samples using Lymphoprep™

Cell count and viability was then assessed using a haemocytometer and the trypan blue exclusion test: 10µl of cell solution was mixed with 10µl of filtered 0.4% trypan blue solution, and left to incubate for 5 minutes. 10µl of this mixture was then placed on a haemocytometer with an improved Neubaur grid. Non-viable cells take up the trypan blue dye and therefore appear blue in colour, while viable cells exclude the dye and are paler than the non-viable cells. The

total number of viable cells was calculated by doubling the number of viable cells on the haemocytometer grid to account for trypan blue solution dilution, and then multiplying by 10^4 to give the number of cells per ml in the cell suspension. Viability was greater than 95% in all cases (Mean=98.9 SD=0.57, n=117). Purity was assessed using by fixing and staining the cells and visual inspection under the microscope. 50µL of the re-suspended cell pellet was placed in the cytospin apparatus and spun at 450g for 3 minutes in a Shandon Mk II cytocentrifuge (Thermo electron corporation Basingstoke, UK). The slides were air dried quickly and then stained with Diff-Quick (Baxter Incorp., UK). The differential cell count was performed by counting numbers of different cells until a total of greater than 100 cells had been seen. The percentage of each cell type seen was then calculated. Mean purity was 95.1% (n=117, SD 3.04).

3.5 Analysis of Plasma

3.5.1 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme Linked Immunosorbent Assays (ELISAs) use a sandwich technique to determine concentration of analytes in samples. It uses a 96 well plate with capture antibody coated to the walls of the wells. When a solution containing the analyte of interest is added to the wells it binds to the capture antibody. Any unbound substance is then washed off and a detection antibody added which binds to the combined capture antibody and analyte. Unbound detection antibody is then washed off and a solution containing hydrogen peroxide and tetramethylbenzidine (TMB) added which turns the solution a blue colour as a

result of interaction with the detection antibody. The intensity of the blue colour is proportional to the amount of analyte bound in the conjugate. The reaction is stopped by addition of sulphuric acid which turns the solution yellow and allows results to be read using a plate reader. Comparison of the optical density in the investigation wells with optical density from wells with a known concentration of the analyte allows concentration of analyte in the investigation sample to be calculated.

3.5.1.1 Vitamin D Binding Protein

Vitamin D Binding Protein (VDBP) concentrations were measured using a commercially available kit (Immundiagnostik, Bensheim, Germany). The kit contains a 96-well plate precoated with polyclonal anti-VDBP antibodies. This plate was washed with the supplied wash buffer, then 100µL standard, control or prediluted sample was added. The standard range of concentrations was supplied in the kit and comprised 60ng/ml, 20ng/ml, 6.6 ng/ml, 2.2ng/ml and 0ng/ml. Two quality controls were provided in the kit and a biological control was also included to assess inter-plate variation. Plasma samples from blood collected in lithium heparin vacutainer tubes were diluted 1:40,000 prior to use. Duplicates of each standard, control and sample were used. The plate was covered with a self-adhesive plastic film and placed on a horizontal mixer for 1 hour at room temperature. The contents of the plate were discarded and the plate washed using the provided wash buffer. 100µL conjugate was added to the wells and the plate incubated for 1 hour on a horizontal mixer at room

temperature. The contents of the wells were again discarded and the plate washed with wash buffer. 100µL TMB substrate was added to the wells and the plate incubated for 10-20 minutes at room temperature (the time of incubation was determined by visual inspection of the colour change as recommended by the manufacturer). When good differentiation of the colour was observed 100µL Stop solution was added to the wells and mixed well. Absorption was immediately determined using a Synergy-2 microplate spectrophotometer (Biotek, Winooski, Vermont, USA) reading at 450nm against 620nm as a reference. Mean absorbance of the 0ng/ml standard was subtracted from the absorbance of standards, controls and standards. Regression analysis of the standard absorbance curve allowed calculation of the VDBP concentration in each sample.

3.5.1.1.1 Quality Control of the VDBP Assay

The test was carried out using 11 separate kits. R^2 of the standard curve was greater than 0.95 in all cases (minimum R^2=0.997). An example of the standard curve is shown in figure 3.2.

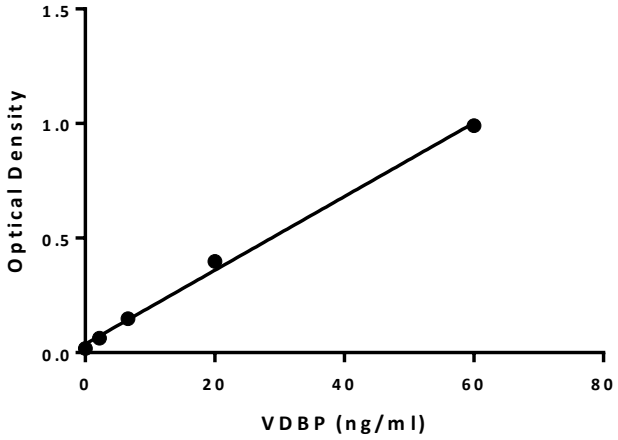


Figure 3.2 Sample Standard Curve for Vitamin D Binding Protein (VDBP)

Spiking experiments were undertaken in which pooled healthy control plasma was measured with and without supplementation with a known concentration of VDBP. Expected values were calculated using the mean value obtained from pooled unspiked replicates and the concentration of the spike added. Mean percentage recovery was 104.7% expected, with a coefficient of variance (CV) of 2.4%. These are comparable with the expected variance provided by the manufacturer. A biological control sample was included in each plate to determine interpolate variation. The CV was 28.9% which is below the range of acceptable variation of 30%. The range of values assessed varied quite significantly with some values at the limit of the assay. Inevitable human errors and the large dilution factor involved compound these issues to produce a broader cv. The manufacturer provided two quality controls consisting of predetermined concentrations of VDBP. All values for the controls were within the acceptable range set by the manufacturer with the exception of the higher control on one plate. This control exceeded the upper limit of the acceptable

range by 2.5ng/ml. As this variation was small, and the biological sample from the plate was within 1 standard deviation of the mean concentration, results from this plate were included in the analysis. The limit of detection was 6.09 ng/ml.

3.5.1.2 LL-37

Total LL-37 plasma concentrations were measured by Dr Aaron Scott using indirect ELISA as described by Chakraborty et al (136), with minor modifications. This method has previously been validated by Dr Scott (137). The standard range of concentrations comprised 125ng/ml, 62.5ng/ml, 31.25ng/ml, 15.63ng/ml, 7.81ng/ml and 0ng/ml. LL-37 standards and samples were made up to 50 µl in serum free media and incubated in Greiner high binding plates (Sigma-Aldrich, St Louis, MO, USA) overnight at 37°C in an unsealed plate to allow adherence. Plates were washed three times with phosphate buffered saline with 0.05% Tween 20 (PBST) and blocked using PBST containing 1% Bovine Serum Albumin (BSA) for 1 hour at room temperature with gentle agitation. Polyclonal rabbit anti-human antibody directed against aa 134-170 of hCAP18 (Innovagen, Lund, Sweden) was diluted 1:10000 in PBST and 100 µl added to each well. Following incubation for 2 hours at room temperature, plates were again washed three times with PBST and 100 µl of goat anti-rabbit horseradish peroxidase (HRP) antibody (1:1000) added per well. Plates were incubated for 1 hour at room temperature then washed with PBST. HRP activity was measured by the addition of 100 µl TMB per well, followed by 15min incubation in the dark at room

temperature. The reaction was stopped by addition of 100µl 1M Hydrochloric Acid followed by reading at 450 nm. A standard curve was generated for each assay by plotting optical density from the standards versus standard concentrations. Regression analysis of the standard absorbance curve allowed calculation of the LL37 concentration in each sample. R^2 of the standard curve was greater than 0.95 in all cases. An example of the standard curve is demonstrated in figure 3.3. The Limit of Detection was 62.52pg/ml, All values stated were in ng/ml, well within the detection limit. CV recovery with recombinant LL-37 spike was <10%.

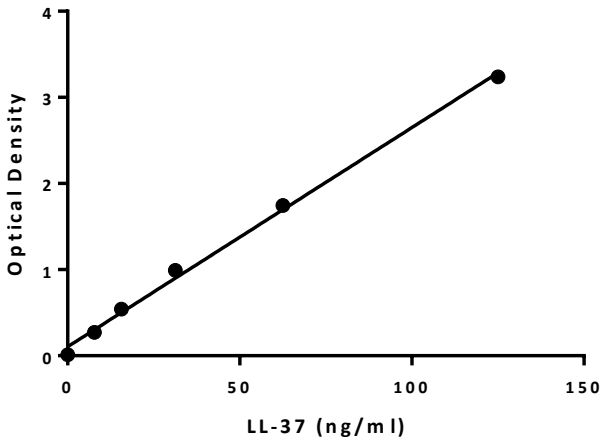


Figure 3.3 Sample Standard Curve for LL37

3.5.2 Luminex

Two different commercially available Luminex multiplex kits were used to measure the inflammatory cytokines, IL-6, IL-8, IL-10, TNFa, IL2, IL1ra, Il1β and IL17 (R&D Systems Ltd, Abingdon, UK) and the soluble cytokine receptors TNFRI, TNFRII and sRAGE (Millipore, Billerica, Massachusetts). These analytes were

chosen because they were identified during analysis of the BALTI-prevention trial as significantly changing over the course of oesophagectomy, or because they have previously been shown to be affected by Vitamin D. Luminex uses colour coded magnetic microbeads which are precoated with antibodies which specifically bind to the analytes of interest. A biotinylated antibody is then added followed by streptavidin-phycoerythin (Strep-PE) conjugate, which binds to the biotinylated antibody. The microbeads are then suspended in buffer and analysed using a Luminex 200. Two lasers are used – one identifies the analyte that is being detected and one determines the magnitude of the Strep-PE signal which is in proportion to the amount of analyte bound to the bead.

The procedure was similar for both kits. Seven doubling solutions of the supplied standard were used. The standard contained a known concentration of all of the analytes being investigated. The microbead solution was resuspended by vortexing and 50µL of microbead solution added to each well of a 96 well microplate. 50µL of standard, diluted plasma sample or control was added to each well and the plate was sealed and incubated for 3 hours at room temperature on a horizontal plate shaker. The plate was washed by applying a magnetic holder to the bottom of the plate to hold the magnetic beads in place, removing the liquid, then applying wash buffer 3 times. Next, 50µL biotin antibody cocktail was added to the wells and the plate sealed and left to incubate for 1 hour at room temperature. The plate was washed as previously, and 50µL Strep-PE added to each well. The plate was covered and incubated for

30 minutes at room temperature and then the plate was washed again. The microbeads were resuspended in buffer and read using the luminex instrument.

3.5.2.1 Quality Control of Luminex Assay

The standard curves were generated by the luminex software which uses a 5PL logistic regression to calculate analyte concentrations. The inter assay and intra assay %CVs for the different analytes are shown in table 3.2, along with the limits of detection. None of the analytes measured were below the lower limit of detection. The sensitivity of the Magplex kits was sufficiently to accurately measure all analytes at the dilutions used. These panels have been by the manufacturers against quantikine/high sensitivity ELISA kits. Data from theme Magplex panels correlates with the ELISA results. Whilst the acceptable variability with the Millipore kits was especially high, we are happy the values measured lie within the limits determined during validation.

Table 3.2 Assay characteristics for Luminex Analytes

Analyte	Inter assay %CV	Intra assay %CV	Limit of Detection
IL6	13.1	5.7	1.7
IL8	13.8	6.7	1.8
IL10	24.1	5.9	1.6
TNF-a	20.9	4.0	1.2
s-RAGE	33.7	9.2	7.2
s-TNF-RI	34.3	16.0	41
s-TNF-RII	36.3	15.9	0.4
IL2	18.2	4.5	1.8
IL1ra	20.9	8.9	18
IL1β	28.2	6.7	0.8
IL17	14.6	2.6	1.8

3.5.3 Measurement of plasma 25-OH and 1,25-$(OH)_2$ Vitamin D

Plasma 25-OH Vitamin D_2 25-OH Vitamin D_3 and total 1,25-$(OH)_2$ Vitamin D plasma concentrations were measured by the bioanalytical facility at the University of East Anglia, Norwich. The professionals at this laboratory are trained to meet the standards of good laboratory practice (GLP) and the facility participates in the vitamin D External Quality Assessment Scheme (DEQAS). 25-OH Vitamin D plasma concentrations were measured by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and 1,25-$(OH)_2$ Vitamin D plasma concentrations were measured by enzyme immunoassay (EIA). The lower limit of detection for 25-OH Vitamin D (either isoform) were 2.5nmol/L and for 1,25-

$(OH)_2$ Vitamin D was 20pmol/L. The normal range for 1,25-$(OH)_2$ Vitamin D using this technique is 43-144pmol/L. The assay characteristics for both isoforms of 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D are shown in table 3.3. For the 25-OH Vitamin D measurements the intra-assay %CV was marginally higher than the inter-assay %CV, but the results obtained were validated by the facility in Norwich and we are satisfied the values measured lie within acceptable limits.

Table 3.3 Assay characteristics for 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D

	Inter assay %CV	Intra assay %CV	% Recovery
25-OH Vitamin D_2	10.0	10.8	95.8
25-OH Vitamin D_3	9.2	9.8	96.1
1,25-$(OH)_2$ Vitamin D	16.9	13.2	n/a

3.6 Analysis of PBMCs

PBMCs were analysed for numbers of natural killer cells using flow cytometry. After extraction from blood collected with lithium heparin (section 3.4), PBMCs were pelleted by centrifugation then resuspended in RPMI media at a concentration of 1 million/ml. 100µL of cell solution per well was transferred into Serocluster™ 96 well flexible plate (Costar, Corning, Amsterdam, The Netherlands) and stained for the cell surface antibodies CD3, CD56 and CD16. Cells were stained using 1µL each of Monoclonal Mouse anti-human CD3-APC (Clone BW264/56), Monoclonal mouse anti-human CD56-PE (clone AF12-7H3) and monoclonal mouse anti-human CD16-FITC (clone VEP13) obtained from Miltenyi Biotec (Gladback, Germany). One well did not have any antibody added,

three contained single colour controls and one contained all three antibodies to allow cell identification. A further well was stained using CD3-APC, CD56-PEand IgG1-FitC to help gate set-up and to reduce false positives as a result of non-specific staining of the CD16 antibody. Isotype controls were not used for CD3 or CD56 as the differentiation between positive and negative cells for these markers was bimodal, and therefore the use of isotype controls to correct for non-specific binding of the antibody was not felt to offer a more accurate result than using the internal control of unstained cells. The plate was covered using aluminium foil and incubated at 4C for 20 minutes before being washed using phosphate buffered solution (PBS).

Cells were then analysed using flow cytometry using a Dako CyAn ADP™ flow cytometer and 'Summit' version 4.3 (Dako, Fort Collins, CO, USA). The same machine was used to assess all samples in this study to minimise experimental variables. The machine was cleaned prior to use to minimise the risk of contamination. Cell solutions were briefly vortexed before being loaded into the analyser to ensure a uniform distribution of cells. The cell solution with no antibody staining was loaded into the analyser first. At least 10,000 events were recorded. Lymphocytes were gated using a forward scatter vs side scatter plot. This allowed identification of cells based on size and granularity (Figure 3.4A). Next, single cells were selected by excluding events with a larger than expected forward scatter area. This removes any clusters of cells or particles from the analysis (Figure 3.4B). The population of unstained single cells was examined to ensure there was no auto-fluorescence to complicate analysis of antibody

stained cells. Next, the single colour controls were analysed to ensure that there any overlap between emission wavelengths of different fluorochromes was minimised. Next the cell solution that had been stained with CD3, CD56 and CD16 was analysed. Cells which were CD3 negative were selected (Figure 3.4C) and assessed for CD56 and CD16 binding (Figure 3.4D and E).

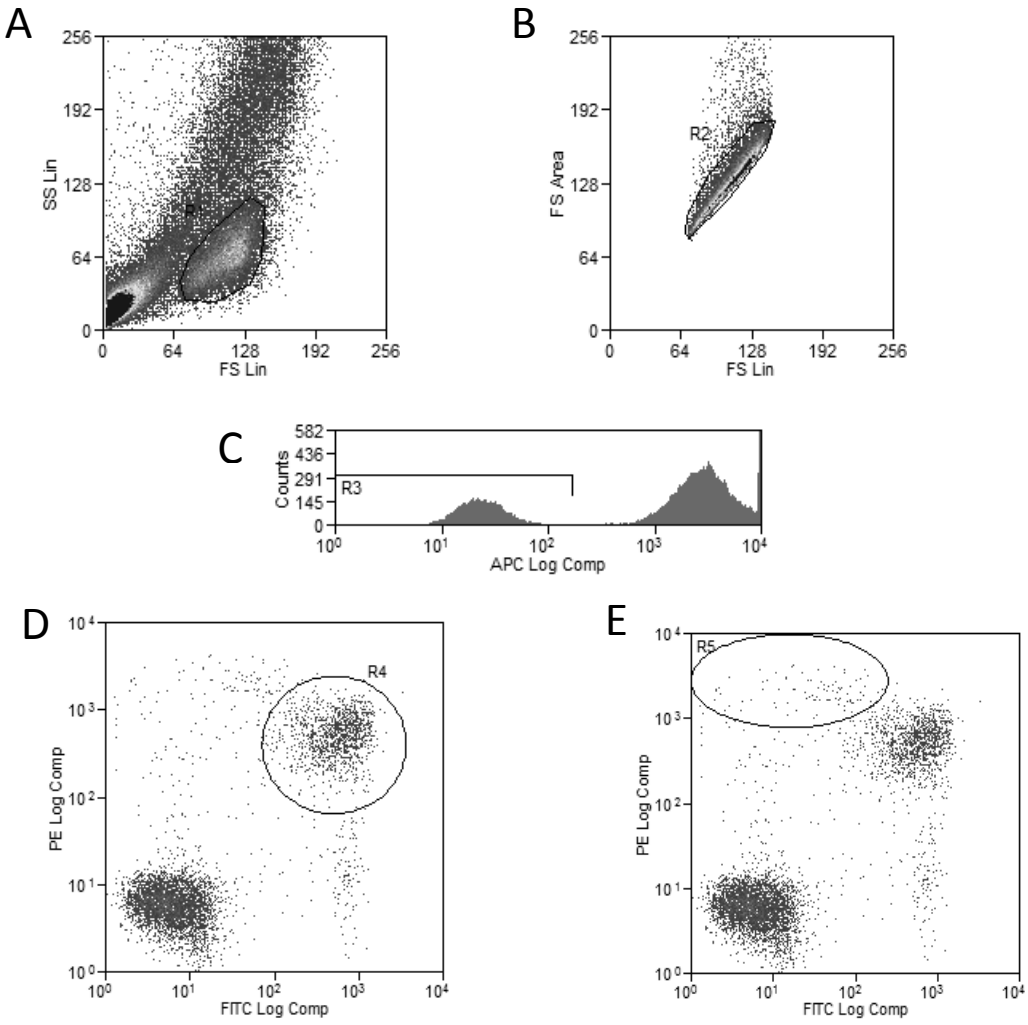


Figure 3.4 Gating strategy for identification of NK cells

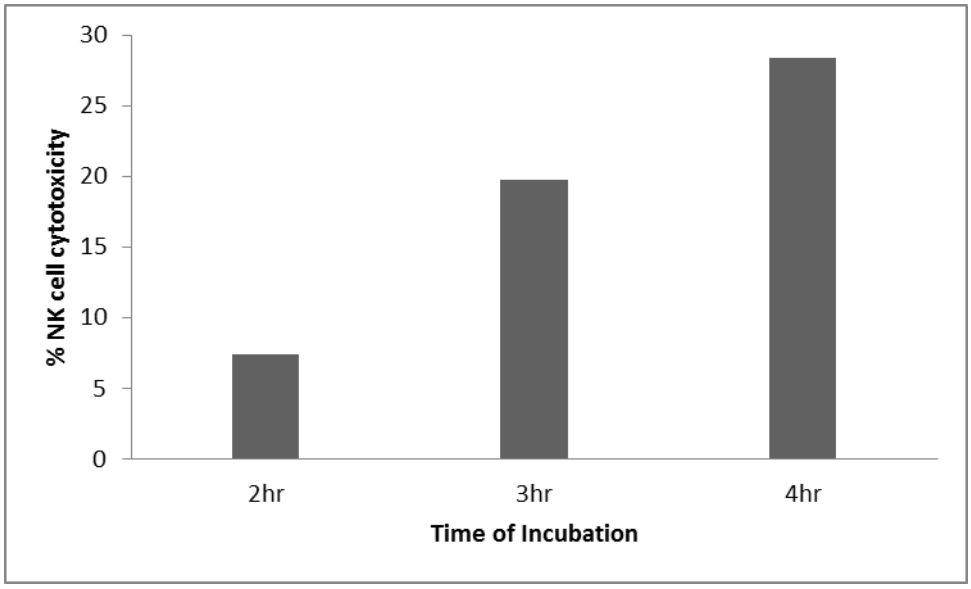
R1 indicates cells within identified as lymphocytes, R2 identifies the single cell population, R3 identifies CD3- cells, R4 identifies CD56^{dim}, CD16+NK cells and R5 identifies CD56^{bright} CD16- NK cells. Data shown in graphs C, D and E represents log mean fluorescence, which has been compensated using single stained samples to reduce overlap between fluorochromes

3.7 In Vitro Assays

3.7.1 Natural Killer Cell Cytotoxicity Assay

The effect of Vitamin D on the cytotoxic capacity of Natural Killer cells was investigated using a cytotoxicity assay. The method used was similar to that described by Gilman Sachs et al (138). The human erythromyelocytic leukemia cell line K562 (ATTC, Manassas, VA, USA) was used as the target cell population as it is known to be sensitive to NK cell cytotoxicity (138). K562 cells were suspended in RPMI media supplemented with 10%FCS, and CellTracker™ Green added to the suspension at a concentration of 1nM (Molecular Probes, Life-Technologies, Paisley, UK). Cells were incubated for 45 minutes at 37°C in a humidified 5% CO_2 atmosphere, then centrifuged and resuspended at a concentration of 10^5cells/ml in fresh culture media. PBMCs, isolated from whole blood as described in section 3.4, were suspended in culture media at a concentration of 5 million cells/ml. The assay was carried out in duplicate using a round bottomed 96 well tissue culture plate. 100µL PBMC solution and 100µL K562 cell solution were placed in each well so that the PBMC:K562 cell ratio was 50:1. Control wells consisting of PBMCs only and K562 cells only were also created. Where applicable, 100nM 25-OH Vitamin D_3, 100nM 1,25-$(OH)_2$ Vitamin D and/or 20ng/ml IL-2, along with appropriate vehicle controls were added to the wells. 100nM 25-OH Vitamin D_3 is a similar concentration to physiological plasma concentrations in patients who are Vitamin D sufficient. 100nM 1,25-$(OH)_2$ Vitamin D is a supraphysiological dose, but is the dose used in previous

studies which reported finding an effect (73). The tissue culture plate was gently centrifuged (50g for 1 minute at room temperature) to ensure contact between the cells and then incubated for 3 hours. This ratio was chosen after measuring NK cell cytotoxicity at PBMC:K562 cell ratios of 25:1, 50:1 and 100:1 after 2, 3 and 4 hours(figure 3.5). While %NK cell cytotoxicity was greatest after 4 hours and at ratios of 100:1, incubations at ratios of 50:1 and for 3 hours was felt to be more pragmatic in view of the need to complete experiments in a timely manner.



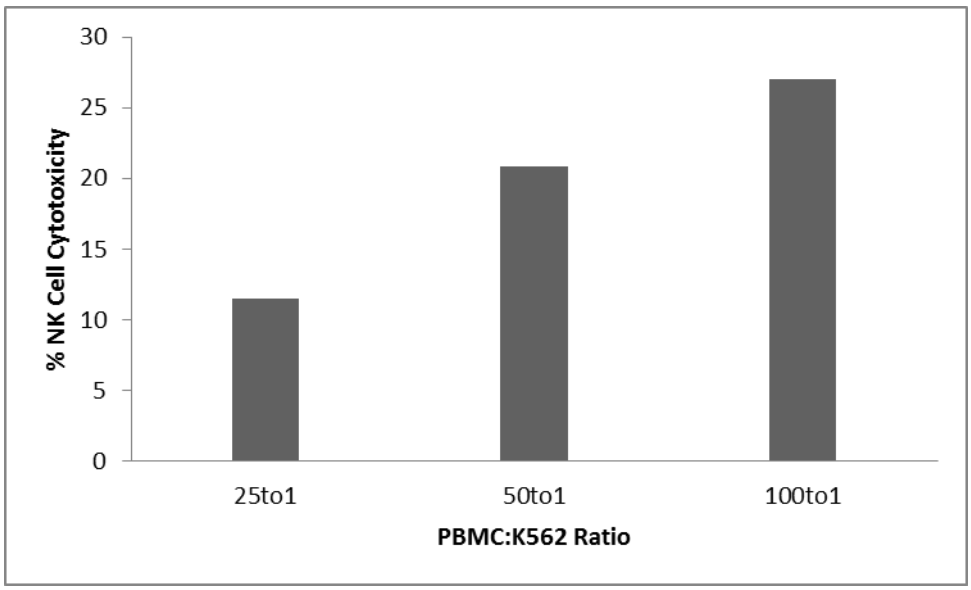


Figure 3.5 Effects of different PBMC:K562 cell ratios and assay durations on %NK Cytotoxicity.

After incubation, the tissue culture plate was centrifuged to pellet cells at the bottom of the wells, supernatant was removed and the cells resuspended in 200µL PBS and transferred to polypropylene tubes. 22µL SYTOX® Blue dead cell stain (pre-diluted 1:800 in PBS) was added to each tube and mixed 1 minute prior to analysis on the flow cytometer.

The K562 cells, having been stained with CellTracker™ Green, are FitC positive. The FitC positive population were selected and cells assessed for SYTOX® Blue positivity (Violet-1 Channel). The K562 only control cells were assessed first to evaluate spontaneous death, then the PBMC and K562 cell combination assessed to evaluate total cell death. The %NK cytotoxicity was calculated as the difference between total cell death and spontaneous cell death. Figure 3.6 shows an example of the cell staining.

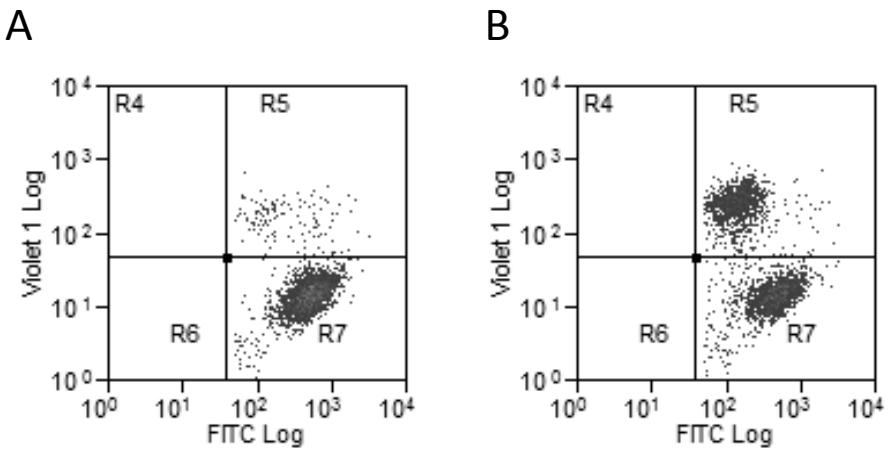


Figure 3.6 Natural Killer Cell Cytotoxicity Assay Example

Cells in R5 are positive for SYTOX® Blue staining A - K562 cells only, B - PBMCs and K562 Cells.

3.7.2 Separation of Natural Killer cells

Natural Killer cells were purified from PBMCs by negative selection using a commercially available isolation kit (Miltenyi Biotec, Gladbach, Germany). The specific antibodies used in this kit are not released by Miltenyi as it is commercially sensitive information. Following PBMC extraction from whole blood using the method described in section 3.4, 10 million cells were

centrifuged to form a pellet, then resuspended in 40µL of autoMACS™ running buffer (Miltenyi Biotec, Gladbach, Germany). 10µL of NK cell biotin conjugated antibody cocktail (targeted against non-NK cell antigens) was added to the cell solution then the mixture was incubated at 4°C for 15 minutes, with gentle flicking every 5 minutes to prevent cells settling at the bottom of the tube. Next, 20µL of magnetic microbead conjugated antibody cocktail, targeted against biotin was added to the cell solution and the mixture was incubated at 4°C for 15 minutes, with gentle flicking every 5 minutes. The cell solution was then washed and resuspended in autoMACS™ running buffer. The NK cells were isolated using a QuadroMACS™ (midi) magnetic separator and a LS column (Miltenyi Biotec, Gladbach, Germany). The column was prewashed with 3 mL autoMACS™ running buffer which were discarded and a new collection tube placed under the column. Next, the sample was loaded and washed through the column with a further 7 mL autoMACS™ running buffer. The purified NK cells pass through the column while other cells are bound to magnetic microbeads and therefore stick to the walls of the column. The elute was pelleted by centrifugation then resuspended in culture media (RPMI). Purity of the NK cell population was assessed by flow cytometry, using the method for identifying NK cells described in section 3.6. An example is shown in figure 3.7. Purity >95% was predetermined to be acceptable and was achieved on each occasion.

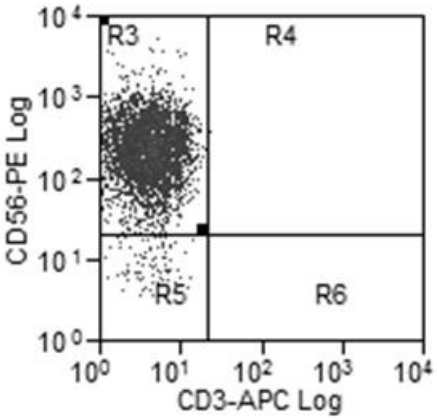


Figure 3.7 Example of cell population obtained using negative isolation kit

3.7.3 Collection of RNA

RNA was extracted from purified natural killer cells, type 2 alveolar epithelial cells, monocytes and alveolar macrophages using a commercially available kit (NucleoSpin® RNA Extraction Kit, Machey Nagel, Duren, Germany). Type 2 alveolar epithelial cells and alveolar macrophages were extracted from human lung resection tissue provided by the Midlands Lung Tissue Collective (MLTC). The MLTC is a regional scheme involving The University of Birmingham, Glenfield Hospital Leicester and Birmingham Heartlands Hospital that allows the use of lung resection specimens obtained from patients undergoing either a lobectomy or pneumonectomy for research into lung disease. The surgery is conducted at Birmingham Heartlands Hospital Thoracic Unit as part of therapy for lung cancer. Patients recruited had provided consent for any tissue removed as a part of their surgery not required for diagnostic or clinical purposes to be used for research

(North West REC Reference 07/MRE08/42; UKCRN ID 6664). Monocytes were extracted from PBMCs and isolated by plastic adhesion.

In order to extract alveolar macrophages from lung tissue, 2000ml of 0.9% saline was flushed through the sample by injection through a 14 gauge hollow needle moved through the sample from multiple sites. The washed through fluid that was produced during this process was collected, decanted into 50ml centrifuge tubes and spun in a pre-chilled centrifuge at 4^{o}C and 500g for 5 minutes. The supernatant was then discarded and the cell pellets pooled and re-suspended in 15ml of RPMI. The cell solution was over-layered on 10ml of lymphoprepTM (Axis-Shield, Dundee, UK) and spun in a centrifuge at ambient temperature for 20 minutes at 800g with the centrifuge brake turned off. The resultant buffy inter-phase was aspirated with a Pasteur pipette into a 15ml centrifuge tube. A cell count and viability assay was then performed on this suspension using a haemocytometer and the trypan blue exclusion test as described above. The original cell suspension was then diluted to give a concentration of 1 million cells per ml. Cells were cultured in 24-well cell culture plates at 37oc in a 5% CO_2 atmosphere. Alveolar macrophages were isolated from the cell solution by plastic adhesion.

The lung sample was then further processed to extract type II alveolar epithelial cells. Trypsin solution was instilled into the lung tissue in exactly the same way as the saline lavage, until it overflowed into the Petri dish(10-15 ml/5cm3 piece). The covered Petri dish was placed into a 37^{o}C, 5% CO_2 incubator for 15 min. This

process was repeated twice to give a total trypsinisation period of 45 min. Next, the tissue was chopped finely into 1-2mm^3 pieces in the presence of FCS (10ml/5cm3 piece) and DNase I (250 –µg/ml HBSS; 7ml/5cm3 piece). FCS inhibits trypsin activity and DNase I was added to degrade glutinous DNA released from ruptured cells during the mincing process. The minced tissue suspension was shaken vigorously by hand for 5 min; this stage enhances type II cell recovery. The tissue suspension was filtered through a large gauge mesh (400-500 µm) and then a 40 µm cell filter to remove undigested tissues and debris from the enzymatically-released epithelial cells, which pass through the filter. The filtrate was centrifuged at 300g at 12^0C for 7 min. The cell pellet was suspended in 50-100 ml 50% DCCM-1 and 50% HBSS containing 100 –µg/ml DNase I (10 ml of HBSS containing 250 –µg/ml DNase I, 15 ml of HBSS and 25 ml of DCCM-1 without 10% FCS). The resuspended cell suspension were plated into either T-75 culture flasks and incubated at 37^0C for ninety minutes to enable any contaminating macrophages to adhere. The media containing the nonadherent type II cell-enriched cell population was removed and centrifuged at 300g, 12^0C for 7 minutes. The cell pellet was resuspended in 3 ml of red cell lysis buffer and incubated for 3 min. 10ml HBSS was added and the solution centrifuged at 300g, 12^0C for 7 minutes. The cell pellet was resuspended in 5mL 10% DCCM. Cells were counted using a haemocytometer. Cells were plated onto collagen-coated plates and put on plate shaker for three minutes to spread cells evenly. Cells were incubated in a humidified atmosphere in 5% CO_2 at 37^0C for 24 hours. After 24 hours the media and non-adherent cells were gently removed, leaving

remaining loosely attached cells and fresh 10% DCCM-1 medium was applied. After another 16-24 h, the media was removed and remaining loose cells washed off with HBSS. Fresh DCCM-1 medium was added. The cells formed a confluent monolayer within 3 days of plating which was used for RNA extraction.

Cells were pelleted by centrifugation, then lysed using 3.5µL β-mercaptoethanol and 350µL buffer RA1. The lysate was filtered by centrifuging for 1 minute at 11,000g. 350µL 70% Ethanol was added to the homogenized lysate and mixed by pipetting, and then the RNA was captured by being passed through the supplied column. After further centrifugation to remove unbound lysate, membrane desalting buffer (MDB) was passed through the RNA column and the column was once again centrifuged. DNase reaction mixture was added to the column to digest deoxyribose nucleic acid (DNA) and left to incubate for 15 minutes at room temperature. Then the column was washed and dried using first RAW2 buffer to inactivate the DNase, then RA3 buffer to remove the digested DNA and other residual chemicals. Finally, the purified RNA was removed from the column using RNase-free water. Extracted RNA concentration and purity was checked using a NanoDrop 2000 Spectrophotometer (NanoDrop Products, Wilmington, DE, USA) routinely revealing a 260/280 ratio of ~2.0-2.1 confirming minimal impurity. Mean RNA concentration was 62.8 ng/µl (SD 26.6 ng/µl).

3.7.4 Real Time Polymerase Chain Reaction

Real-time polymerase chain reaction or quantitative PCR (qPCR) is used to determine the relative quantities of specific sequences of nucleic acids present in

biological samples. A fluorescent DNA probe is used to identify the target sequences which are repeatedly exposed to optimal replication conditions in order to increase their quantity. The signal generated by the fluorescent DNA probe increases with every cycle as the quantity of DNA increases and the number of cycles before the signal from the probe crosses a pre-set threshold can be used to calculate the quantity of DNA in the original sample, compared to a control.

In order to carry out qPCR, the extracted RNA needed to be converted into complimentary DNA (cDNA). This was carried out using a commercially available high capacity RNA-to-cDNA™ kit (Applied Biosystems, Carlsbad, CA, USA) according to manufacturer instructions. cDNA concentration was assessed by spectrophotometry and diluted to 10 ng/mL. qPCR was performed in duplicate using the Light Cycler® 480 instrument (Roche Diagnostics GmbH, Manheim, Germany). Added to each well was a mixture of 10 µL Master Mix (Roche), 9 µL cDNA and 1 µL primer (table3.4). The reaction protocol was 10 minutes pre-incubation followed by 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second, then 30 seconds of cooling.

Table 3.4 Primers used for qPCR

Target	Reference Sequence	Forward Sequence	Reverse Sequence
Vitamin D Receptor (VDR)	NM_000376.2 NM_001017535.1 NM_001017536.1	5'- GCTTGTCAAA AGGCGGCAG-3' 5'-AGCCTCAATGAG GAGCACTCCAAG-3' 5'-GGGGGTCTCA GGATAGGGAC -3'	5'-CCCAAAGGC TTCTGGTCCG -3' 5'-CGGGTGAGGAG GGCTGCTGAGTA-3' 5'-ACGTTCCGGT CAAAGTCTCC -3'
GAPDH	NM_002046.3	GACCCCTTCATTG ACCTCAACTAC	TGGAATTTGCC ATGGGTGGAAT

Data was outputted as a Cp value per well. Each sample/gene combination was performed in duplicate and a mean Cp value calculated. ΔCp was calculated by subtracting the mean Cp of the gene of interest from the mean Cp of the stably expressed housekeeping gene GAPDH. This acts as an internal comparison, accounting for sample differences. Because the ΔCp value is the inverse of the degree of gene transcription, it was multiplied by -1 to aid data presentation. The use of GAPDH as a single housekeeping gene is our laboratory's standard protocol which has been used for multiple similar experiments by members of our group. The expression of GAPDH by the different cell types is shown in figure 3.8 – there was no significant difference in GAPDH expression across the different cell types.

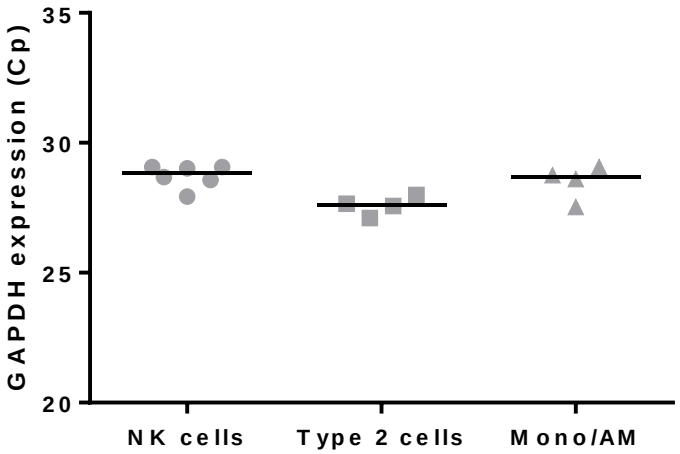


Figure 3.8 Expression of GAPDH by the different cell types examined

n=6 for NK cells and n=4 for Type 2 cells and Monocytes/AM. Kruskall Wallis test overall was not significant (p=0.0587) and Dunn's multiple comparsions test showed no significant differences between individual data sets.

3.8 Severity Scores

3.8.1 APACHE-II

The Acute Physiology and Chronic Health Evaluation II (APACHE-II) score is tool which can help to quantify severity of illness and predict mortality on admission to ITU. It was developed using records from over 5000 individuals admitted to ICUs in 13 different US hospitals. It uses a point score based on admission values of 12 physiological measures (core temperature, Glasgow coma score, mean arterial pressure, heart rate, respiratory rate, oxygenation, arterial pH, haematocrit, white cell count, serum sodium, potassium and creatinine) age and

previous health status. Possible scores range from 0 to 71 with more severe illness scoring higher.

3.8.2 SOFA

The Sequential Organ Failure Assessment (SOFA) was designed to focus on organ dysfunction and morbidity following ITU admission. The score is made up of 6 organ systems, each of which is allocated a score ranging from 0 to 4. The scoring system is detailed in table 3.2.

Table 3.5 SOFA scoring system (139)

Parameter		Score				
		0	1	2	3	4
Respiratory	P/F Ratio (mmHg) and Mechanically ventilated	>400 No	<400 No	<300 No	<200 Yes	<100 Yes
Nervous	GCS	15	13-14	10-12	6-9	<6
Cardio-vascular	MAP (mmHg) Inotropes*	>70 None	<70 None	n/a Dop≤5 or Dob(any)	n/a Dop>5 or Epi≤0.1 or Nor≤0.1	n/a Dop>15 or Epi>0.1 or Nor>0.1
Liver	Bilirubin (µmol/L)	<20	20-32	33-101	102-204	>204
Coagulation	Platelets ($x10^9$/L)	>150	<150	<100	<50	<20
Renal	Creatinine (µmol/L) or Urine output (ml/d)	<110	110-170	171-299	300-440 <500	>440 <200

**Inotrope drug doses are in µg/kg/min. Dop = dopamine, Dob=Dobutamine, Epi=Epinephrine, Nor=Norepinephrine*

3.9 Diagnosis of post-operative ARDS

In order to determine which patients developed ARDS post oesophagectomy, all chest radiographs and CT scans performed on study patients as part of their routine clinical care were examined by at least 2 doctors with experience in

diagnosing ARDS. Imaging from the institutions involved in the study were stored in digital form on the hospital Picture Archiving and Communications Systems (PACS) and viewed on a single workstation. The clinicians examining the imaging decided whether there was radiological evidence of ARDS (ie bilateral opacities not fully explained by effusions, lobar/lung collapse or nodules). This was then entered into a database alongside oxygenation and ventilation data in order to determine whether all criteria for ARDS had been met. Diagnosis of ARDS was made according to the Berlin criteria (table 1.1). Chest Radiograph criteria was judged to have been met if the clinicians examining the radiographs agreed that the radiographs were consistent with ARDS. Oxygenation criteria were judged to have been met if the P/F ratio was consistent with ARDS on at least one occasion and radiograph criteria had been met.

3.10 Data Handling and Analysis

3.10.1 Data Collection, Storage and Validation

Data for the trials was prospectively collected using a bespoke case report form (CRF) (Appendix 1). Baseline data was collected from the medical records or by speaking with the patient. PICCO data was collected at the time of measurement by writing the obtained measurements directly into the CRF. As there was no source data for this important aspect of the investigation, figures obtained were checked by 2 people at the time of acquisition and indexed values obtained from the machine were checked by manually recalculation. No differences were found between the transcribed indexed data and the calculated index data.

Patients were allocated a trial ID to ensure patient anonymity. The CRFs were securely stored in a locked filing cabinet in a pass-card protected room at the Centre for Translational Inflammation Research (CTIR), University of Birmingham. Once collected, data was transferred to electronic storage for analysis using Microsoft Excel 2010. Results of laboratory analysis were also stored electronically in anonymised form. All electronic data was stored in a password protected, encrypted format and backed up regularly using the University servers. Data integrity was regularly checked against the source data.

3.10.2 Data Analysis

Data was analysed using Prism v6.05 (GraphPad, La Jolla, CA, USA) except for linear regression which was analysed using SPSS v21 (IBM, Somers, New York, USA). Where missing data was required for analysis (for example for paired data), the most recent known value was carried forward where less than 20% of values were missing. Where more than 20% of data was not available, data was analysed using non-paired tests. Continuous data was assessed for normality using D'Agostino and Pearson Omnibus test and the appropriate parametric or non-parametric test applied. Statistical significance was pre-defined as $p<0.05$.

To assess the significance of differences between two sets of data the unpaired T test was used for parametric data, while the Mann Whitney U Test was used for non-parametric data, except paired data which was assessed using paired T test (parametric) or Wilcoxon signed rank test (non-parametric). For comparison of three or more datasets, ANOVA was used for parametric data, Kruskal Wallis test

was used for non-parametric data, Repeated Measures one way ANOVA was used for parametric paired data and Friedman's test was used for non-parametric paired data with Holm-Sidak (parametric) or Dunn's (non-parametric) multiple comparison test to assess differences between individual sets. No adjustment for multiple comparisons was made when assessing the cytokine data as this work was intended to be hypothesis generating and in order to power a study where multiplicity could be adjusted for, greater numbers would be required. Correlations were tested using Pearson for parametric data and Spearman rank correlation for non-parametric data. Nominative data was assessed using Fisher's exact test where the expected frequency of one or more cells was less than 5 and Chi squared test for larger samples.

CHAPTER 4

VITAMIN D DEFICIENCY IN PATIENTS UNDERGOING OESOPHAGECTOMY

The contents of this chapter have been published: Vitamin D deficiency contributes directly to acute lung injury. RCA Dancer, D Parekh, S Lax, V D'Souza, S Zheng, CR Bassford, D Park, D Bartis, R Mahida , AM Turner, E Sapey, W Wei, B Naidu, PM Stewart, WM Fraser, KB Christopher, MS Cooper, F Gao, DM Sansom, AR Martineau, GD Perkins, DR Thickett. **Thorax. 2015;70(7):617-24**.

4.1 Introduction

Interest in the immunomodulatory effects of vitamin D has prompted investigators to consider whether vitamin D deficiency may be associated with the development of critical illness. In 2009 Jeng et al published a study showing that critically ill patients have lower plasma concentrations of 25-OH vitamin D than healthy controls and that this correlates with plasma concentrations of cathelicidin (63). Multiple subsequent studies have shown high prevalence of vitamin D deficiency in ICU populations and that 30 day mortality is increased in patients admitted to the ICU with vitamin D deficiency (108, 140). In one study, blood culture positivity was higher in patients with vitamin D deficiency (108), while a further study showed that patients with vitamin D deficiency in the first 30 days after admission with severe sepsis or septic shock were more likely to die that patients with sufficient plasma concentrations (141). No previous studies have investigated the prevalence of Vitamin D deficiency specifically in humans with ARDS, but studies in animal models have suggested that vitamin D may ameliorate lung injury, reducing systemic IL6 concentrations and neutrophil recruitment to the lung (see section 1.2.6).

Oesophagectomy patients are at high risk of post-operative ARDS with preoperative factors such as chronic respiratory disease, smoking pack-years and abnormal lung function associated with increased risk (142). Patients awaiting oesophagectomy often have difficulty swallowing and are therefore at risk of malnutrition. 30% of patients had impaired nutritional status in one study (143).

The prevalence of Vitamin D deficiency in patients undergoing oesophagectomy is unknown but a study of preoperative 25-OH Vitamin D plasma concentrations in patients awaiting cardiac surgery found that 60% of patients were deficient in Vitamin D (<50nmol/L) and a further 25.5% had insufficient plasma concentrations (50-75nmol/L). The same study found that 29.5% of patients had preoperative 1,25-$(OH)_2$ Vitamin D plasma concentrations below 41pmol/L (144).

4.2 Hypothesis and Research Questions

4.2.1 Hypothesis

Patients undergoing oesophagectomy have high prevalence of vitamin D deficiency and those patients who are deficient have higher risk of post-operative ARDS. Patients with vitamin D deficiency have lower survival post-oesophagectomy then patients with higher plasma concentrations.

4.2.2 Research Questions

1. How do plasma concentrations of circulating Vitamin D in patients undergoing oesophagectomy compare with patients with ARDS and healthy controls?
2. Is Vitamin D deficiency a risk factor for ARDS post oesophagectomy?
3. How does Vitamin D deficiency relate to biomarkers of alveolar oedema and systemic inflammation?
4. Do circulating Vitamin D plasma concentrations predict post-operative mortality?

4.3 Methods

Initial investigations were carried out on stored samples taken on the day of recruitment from 52 patients with ARDS recruited to the BALTI and BALTI2 trials (41, 145) and preoperative samples from 55 patients at risk of ARDS due to undergoing oesophagectomy who were enrolled in the translational sub-study of BALTI-prevention (146). 25-OH Vitamin D_3 plasma concentrations were also measured in 18 healthy volunteers (10F:8M mean age 46.9, SD 14.5), for comparison with the patient groups. 25-OH Vitamin D_3 were measured by LC-MS/MS and 1,25-OH_2 Vitamin D plasma concentrations were measured by EIA in a DEQAS certified laboratory. Plasma Vitamin D Binding Protein plasma concentrations were measured using ELISA. Results from the oesophagectomy patients were analysed using a database of clinical, PICCO and biochemical parameters. In order to determine whether patients had developed ARDS or not post-operative chest radiographs and oxygenation were analysed by 2 respiratory physicians and 1 radiologist with experience of diagnosing ARDS.

Statistical significance was defined as $p<0.05$. Statistical tests used are detailed in section 3.10.2. Continuous data in tables is presented as mean (SD) for parametric data and median (IQR) for non-parametric data. Nominative data is expressed as n (%). Statistical significance in the graphs is denoted using * for $p\leq0.05$, ** for $p\leq0.01$, *** for $p\leq0.001$ and **** for $p\leq0.0001$. Non-significance is denoted using ns. For comparison of survival curves, significance was

calculated using Log Rank (Mantel Cox) test and hazard ratios using Mantel-Haenszel test using Prism v6.05 (GraphPad, La Jolla, CA, USA).

4.4 Results

4.4.1 Comparison of 25-OH Vitamin D_3 plasma concentrations in patients with and at risk of ARDS

All patients with ARDS and 52 patients in the at-risk group (95%) were Vitamin D deficient (25-OH Vitamin D_3 plasma concentrations <50nmol/L). 8 healthy volunteers were also Vitamin D deficient (44%). Plasma concentrations of circulating 25-OH Vitamin D_3 were significantly lower in at-risk patients than in healthy controls. Patients with ARDS had greater plasma concentrations of Vitamin D deficiency than patients at risk of ARDS due to undergoing oesophagectomy (figure 4.1)

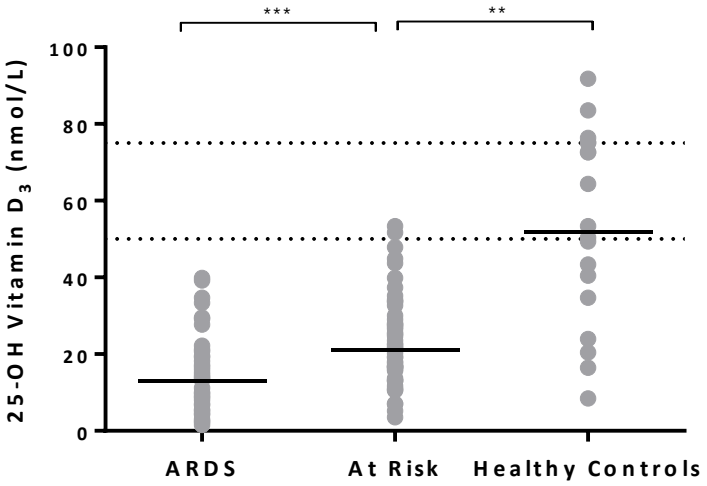


Figure 4.1 25-OH Vitamin D_3 in patients with and at-risk of ARDS

n=52 for ARDS, n=55 for At-Risk, n=18 for Healthy Controls. Black horizontal lines indicate median for each dataset. Dotted reference lines indicate 25-OH Vitamin D_3 50 and 75nmol/L. Overall Kruskal Wallis p<0.0001, significance shown between groups uses Dunns correction for mulitple comparisons.

Circulating plasma concentrations of Vitamin D Binding Protein (VDBP) were also measured in the majority of the patients with and at-risk of ARDS. Again, patients with ARDS had significantly lower plasma concentrations of VDBP compared with the at-risk cohort (p<0.0001), who had significantly lower plasma concentrations than healthy controls (p=0.0018, figure 4.2).

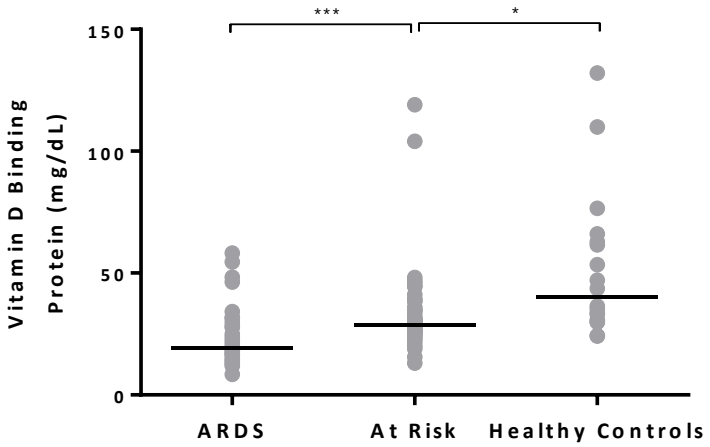


Figure 4.2 Vitamin D Binding Protein in patients with and at risk of ARDS

n=42 for ARDS, n-47 for at-risk and n=18 for healthy controls. Black horizontal lines indicate median for each dataset. Overall Kruskal Wallis p<0.0001 significance shown between groups uses Dunns correction for mulitple comparisons.

4.4.2 1,25-$(OH)_2$ Vitamin D in patients with and at-risk of ARDS

Preoperative circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D were normal in the majority of patients undergoing oesophagectomy. Patients with ARDS had lower circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D, and 17/52 (33%) had undetectable plasma concentrations. No patients in the at-risk group had undetectable plasma concentrations of 1,25-$(OH)_2$ Vitamin D (figure 4.3).

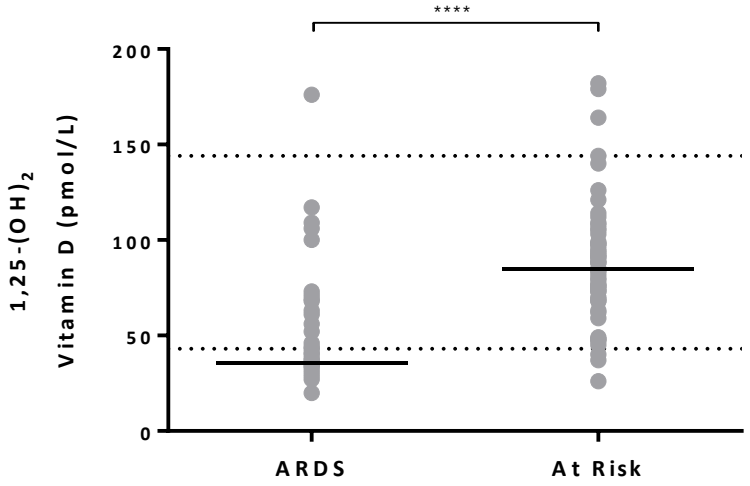


Figure 4.3 1,25-$(OH)_2$ Vitamin D in patients with and at-risk ARDS

n=52 for ARDS and n=55 for At-Risk. Dotted reference lines indicate normal range 43-144pmol/L. Where plasma concentrations were undetectable (<20pmol/L), a value of 19.9 has been assigned for analysis and illustrative purposes. P<0.0001 (Mann Whitney).

4.4.3 Correlation between 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients undergoing oesophagectomy

Spearman Rank correlation between plasma concentrations of 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D was significant, but did not show a strong relationship (Spearman ρ=0.2745, p=0.0425, r^2=0.0623, figure 4.4).

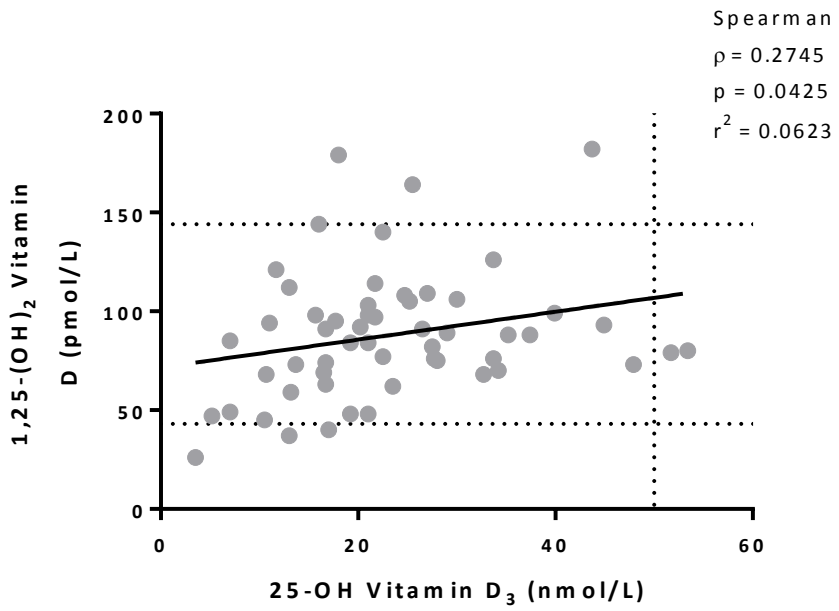


Figure 4.4 Correlation between 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients undergoing oesophagectomy

n=55. Dotted reference lines indicate 25-OH Vitamin D 50nmol/L and 1,25-$(OH)_2$ Vitamin D 43-144pmol/L.

4.4.4 Preoperative risk factors for development of ARDS post oesophagectomy

Preoperative median plasma concentrations of vitamin D were significantly lower in those patients who developed ARDS post-operatively. Vitamin D status was the only preoperative predictive marker of ARDS on univariate analysis (see table 4.1).

Table 4.1 Comparison of patients who did and did not develop ARDS post oesophagectomy

		Patients with Lung Injury (n=14)	Patients without Lung Injury (n=41)	P-value
Male – n (%)		13 (93)	34 (83)	0.6639
Age (years) mean (SD)		61 (12.3)	63 (11.1)	0.5144
BMI (kg/cm^2) mean (SD)		25.7(5.6)	24.7 (4.5)	0.4942
FEV1 (% pred) mean (SD)*		89.2 (5.8)	94.7 (3.82)	0.4486
Tumour Type = Adenocarcinoma n (%)		11 (79)	29 (71)	0.7342
Tumour Stage n(%)	1	2 (14)	3 (7)	0.5596
	2	3 (21)	14 (34)	
	3	9 (64)	24 (59)	
Smoker n(%)	Current	6 (42)	8 (20)	0.1568
	Former	6 (42)	29 (71)	
	Never	2 (14)	4 (10)	
Pack Years median (IQR)		33 (20-40)	30 (15-45)	0.7335
Plasma 25-OH Vitamin D_3 (nmol/L) mean (SD)		**17.3 (7.65)**	**25.4 (11.9)**	**0.0207**
Plasma 1,25-$(OH)_2$ Vitamin D (pmol/L) median (IQR)		**66 (47-92)**	**89 (76-107)**	**0.0098**

*Data for FEV1 was not available for 4 patients

Univariate p values were not adjusted for multiple comparisons as the data was intended to be hypothesis generating and the n numbers were not sufficient to allow for this. Regression analysis of the above factors was also hampered by the small sample size. The analysis was carried out with the assistance of our statisician, Dr Peter Nightingale. The data was checked for collinearity (defined as VIF > 2.0) which, unsurprisingly, showed that smoking status and pack years were related. In view of the sample size and collinearity, the data was examined using a forward stepwise model. This model required two steps before no further significant variables were found and identified 1,25-$(OH)_2$ Vitamin D (p=0.006) and tumour stage (p=0.096) as predictors of lung injury in this group. While tumour stage was included in the model, it was not significant at the 5% level. The two variable model was significant (χ^2=12.593 p=0.006, Nagelkerke R square = 0.317). As there were 4 missing values for FEV1 and this parameter was not a significant predictor of lung injury, the analysis was run again without including FEV1. Using this analysis, 1,25-$(OH)_2$ Vitamin D was the only significant predictor of lung injury (χ^2=6.455, p=0.011, Nagelkerke R square 0.163) with an Odds Ratio of 0.970 (95%CI 0.944-0.996).

4.4.5 Identification of cut off values for 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D

In order to further investigate the relationship between Vitamin D and development of ARDS, a receiver operating characteristic (ROC) curve was used to identify values for 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D below which

risk of development of ARDS was increased. For 25-OH Vitamin D_3 the model was significant with an area under the curve (AUC) of 0.71 (95% CI 0.57-0.86 p=0.0189). A cut off of 17.85nmol/L has specificity 73.2% and sensitivity 64.3% with a likelihood ratio of 2.40 for development of ARDS (figure 4.5).

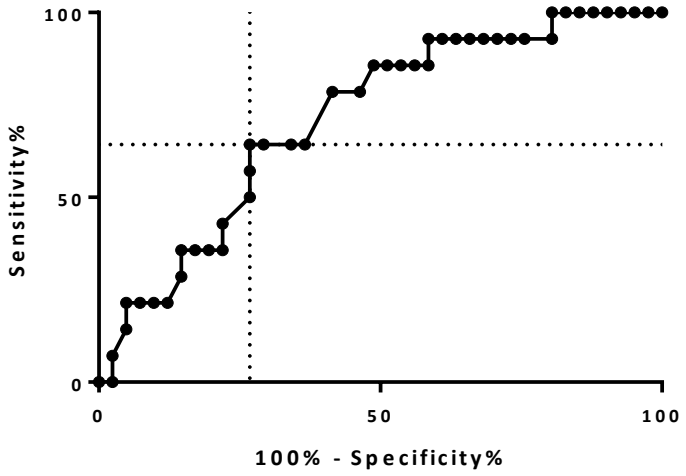


Figure 4.5 ROC curve to identify a value of 25-OH Vitamin D_3 below which development of ARDS is more likely

AUC 0.71 (95% CI 0.57-0.86 p=0.0189). Dotted reference lines indicate specificity of 73.2% and sensitivity 64.3%

For 1,25-$(OH)_2$ Vitamin D the model was also significant with AUC=0.73 (95% CI 0.56-0.90, p=0.0108). This showed that a cut-off of 1,25-$(OH)_2$ Vitamin D <71.50pmol/L has specificity 85.7% and sensitivity 64.3% with a likelihood ratio of 4.39 for prediction of development of ARDS (figure 4.6).

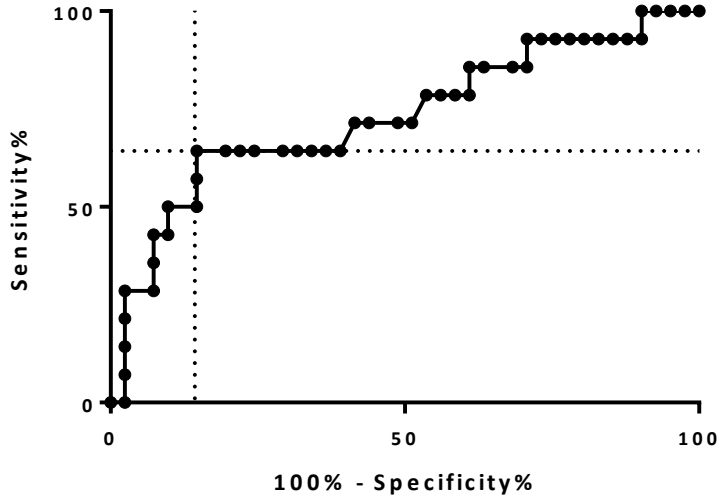


Figure 4.6 ROC curve to identify a value of 1,25-$(OH)_2$ Vitamin D below which development of ARDS is more likely

AUC 0.73 (95% CI 0.56-0.90, p=0.0108). Dotted reference lines indicate specificity of 85.7% and sensitivity 64.3%

4.4.6 Effects of Vitamin D status on cytokine plasma concentrations

Patients were divided into two groups according to 25-OH Vitamin D_3 plasma concentrations as suggested by the ROC analysis. Differences in cytokine plasma concentrations between the groups were analysed. The full data is shown in tables A1-A6 in the appendix. Figure 4.7 shows the change over time in plasma concentrations of IL6. Patients with higher plasma concentrations of 25-OH Vitamin D had marginally higher levels of IL6 post op, but did not have as great increases in IL6 during the perioperative period.

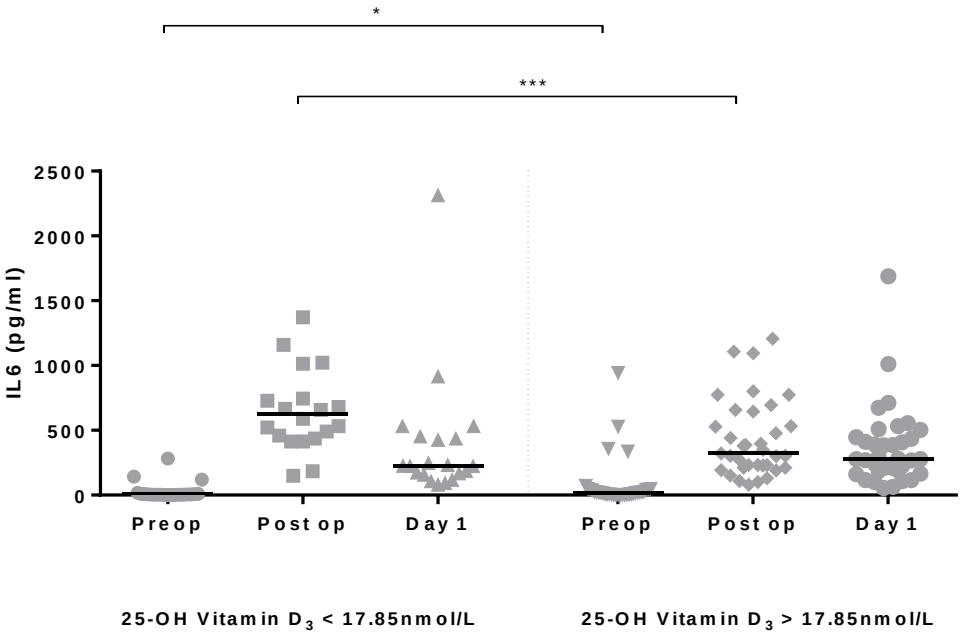


Figure 4.7 Comparison of perioperative changes in plasma concentration of IL6 between 25-OH Vitamin D_3 groups

n=20 (each timepoint) for 25-OH Vitamin D_3 < 17.85nmol/L, n=35 (each timepoint) for 25-OH Vitamin D_3 >17.85nmol/L. Overall Friedman test p<0.0001 for both groups. Difference between preop samples p=0.040, Post-op samples p=0.005 (both Mann Whitney).

Similar preoperative differences in IL8 were seen between the groups, but while plasma concentrations in patients who were most deficient in 25-OH Vitamin D_3 tended to fall by Day 1 post op, in the patients with higher plasma concentrations IL8 tended to continue to increase (figure 4.8).

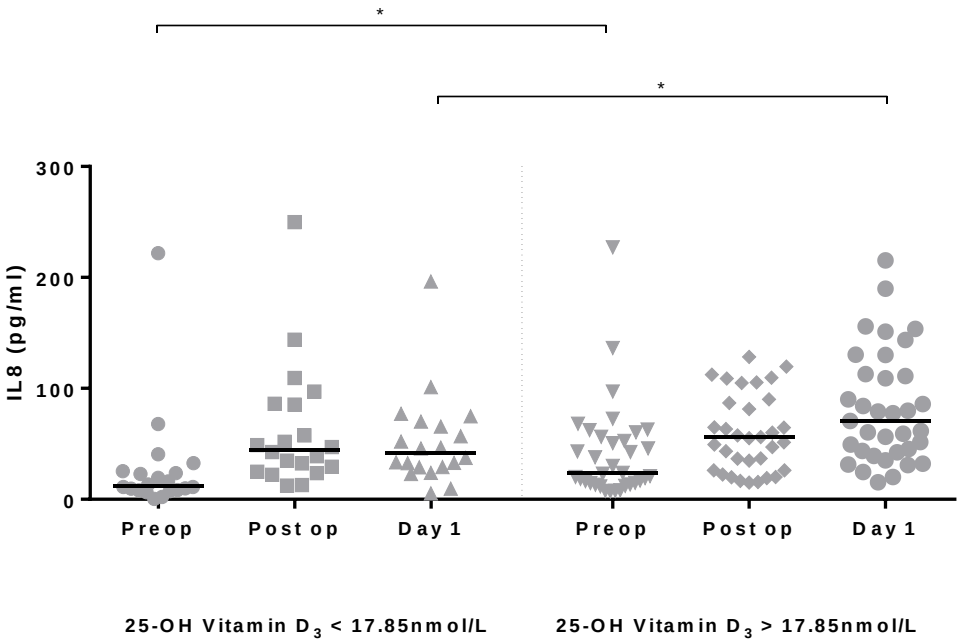


Figure 4.8 Comparison of perioperative changes in plasma concentration of IL8 between 25-OH Vitamin D_3 groups

n=20 (each timepoint) for 25-OH Vitamin D_3 < 17.85nmol/L, n=35 (each timepoint) for 25-OH Vitamin D_3 >17.85nmol/L. Overall Friedman test p<0.0001 for both groups. Difference between preop samples p=0.012, Day 1 samples p=0.015 (both Mann Whitney).

Similarly, plasma concentrations of IL17 were higher pre- and post-operatively in patients with greater plasma concentrations of 25-OH Vitamin D_3 (figure 4.9).

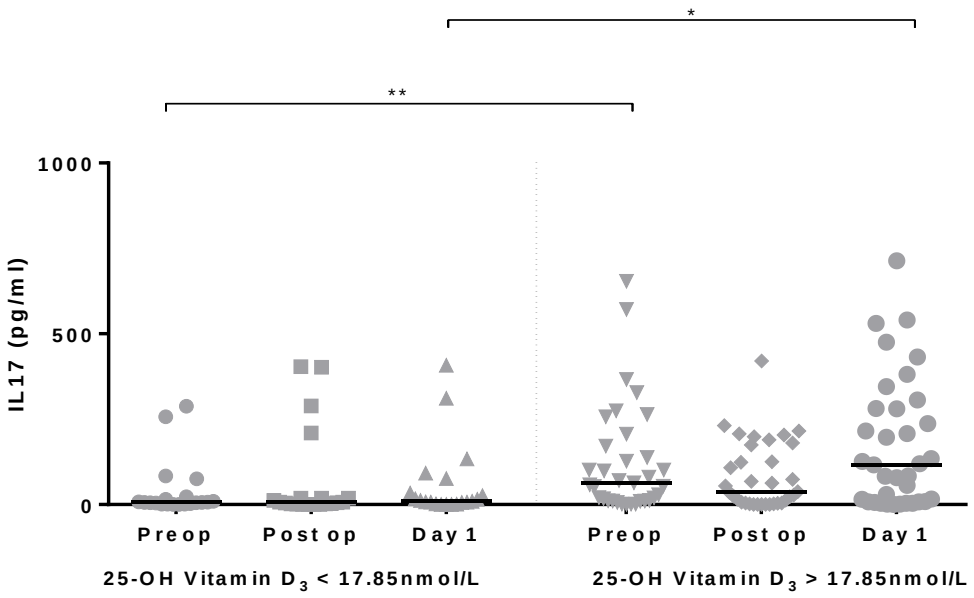


Figure 4.9 Comparison of perioperative changes in plasma concentration of IL17 between 25-OH Vitamin D_3 groups

n=20 (each timepoint) for 25-OH Vitamin D < 17.85nmol/L, n=35 (each timepoint) for 25-OH Vitamin D >17.85nmol/L. Overall Friedman test p=0.0045 for 25-OH Vitamin D < 17.85nmol/L and p<0.0001 for 25-OH Vitamin D > 17.85nmol/L. Difference between preop samples p=0.001, Post-op samples p=0.016 (both Mann Whitney).

When the cohort was divided according to 1,25 Vitamin D the differences generally were not replicated (tables A1-A6).

4.4.7 Effects of Vitamin D status on perioperative changes in alveolar oedema

Perioperative changes in alveolar oedema, as measured by PICCO catheter, were compared between groups. EVLWI significantly increased perioperatively in both 25-OH Vitamin D_3 groups, but while in patients with 25-OH Vitamin D_3 plasma concentrations greater than 17.85 EVLWI returned to baseline by D1, in patients with more severe deficiency day 1 EVLWI was significantly higher than preop plasma concentrations (figure 4.7). As baseline values varied, fold changes were analysed. Patients with 25-OH Vitamin D_3 less than 17.85 had greater increases in extravascular lung water index (EVLWI) perioperatively compared with patients with less severe deficiency ($p=0.0113$) but there was no significant difference in fold change between the post op and day 1 measurements ($p=0.4782$, figure 4.8).

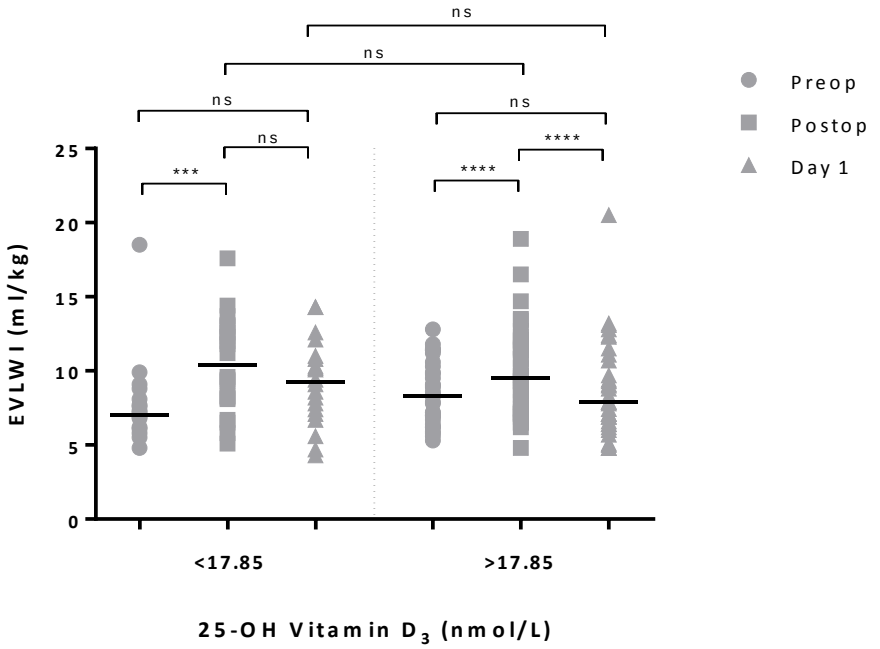


Figure 4.10 Differences between 25-OH Vitamin D_3 groups in perioperative EVLWI

n=20 for <17.85 datasets and n=35 for >17.85 datasets. Black horizontal lines indicate median for each dataset. Friedman test for 25-OH Vitamin D_3<17.85nmol/L p=0.0013, Friedman test for 25-OH Vitamin D_3>17.85nmol/L p<0.0001.Friedmans test with multiple comparisons used to test for differences within groups, Mann Whitney test used to test for differences between individual timepoints

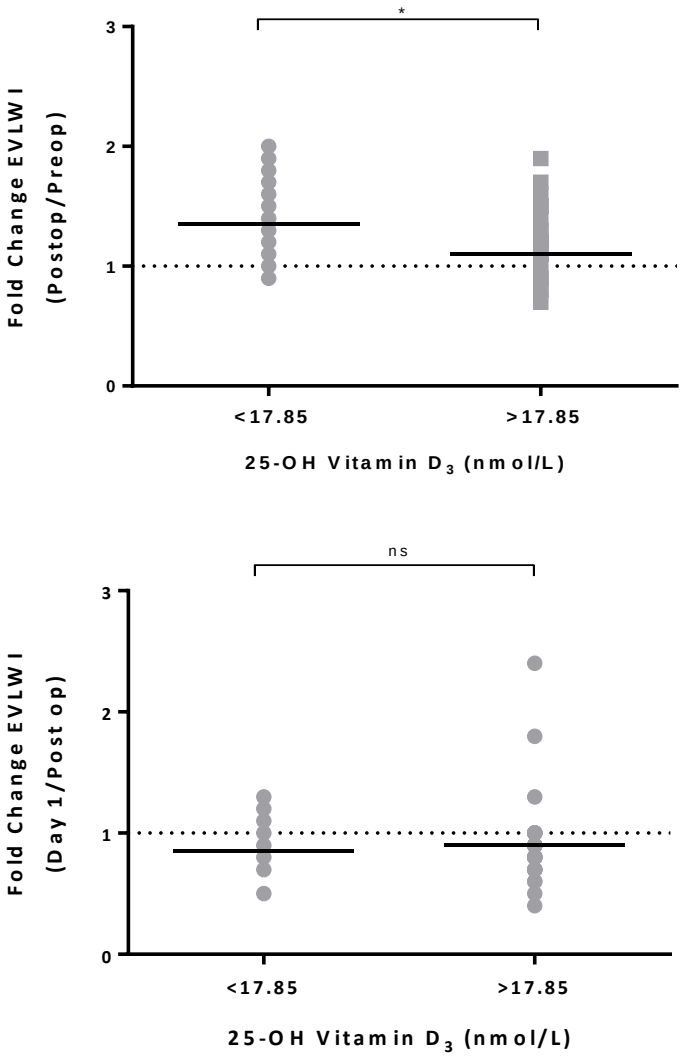


Figure 4.11 Differences between 25-OH Vitamin D_3 groups in perioperative fold change in EVLWI

n=20 for 25-OH Vitamin D_3 <17.85 and n=35 for 25-OH Vitamin D_3 >17.85. Comparison made using unpaired T test for post-op and Mann Whitney Test for day 1. Dotted reference line indicates fold change=1; data points below the line decreased post op and data points above the line increased post op

For PVPI, there was a significant rise perioperatively, followed by a significant fall between post op and day 1 measurements which resulted in no significant difference between preop and day 1 PVPI (figure 4.9). The difference in fold change increases in PVPI between the preoperative and post-operative time

points was not significant at the 5% level (p=0.0619) and no difference in fold change between post op and day 1 (p=0.5818, figure 4.10).

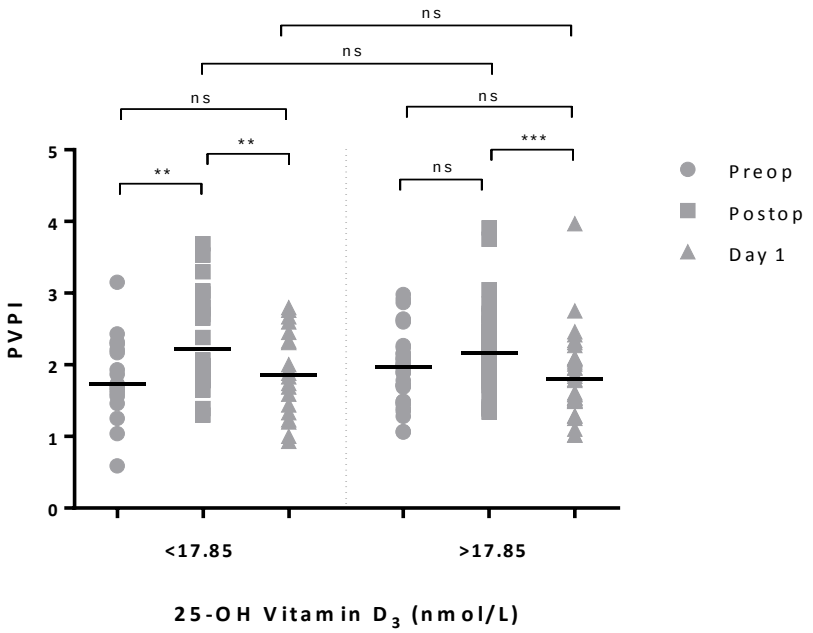


Figure 4.12 Differences between 25-OH Vitamin D_3 groups in perioperative PVPI

n=20 for <17.85 datasets and n=35 for >17.85 datasets. Black horizontal lines indicate median for each dataset. RM one-way ANOVA for 25-OH Vitamin D_3<17.85nmol/L p=0.0001, Friedman test for 25-OH Vitamin D_3>17.85nmol/L p=0.0002.

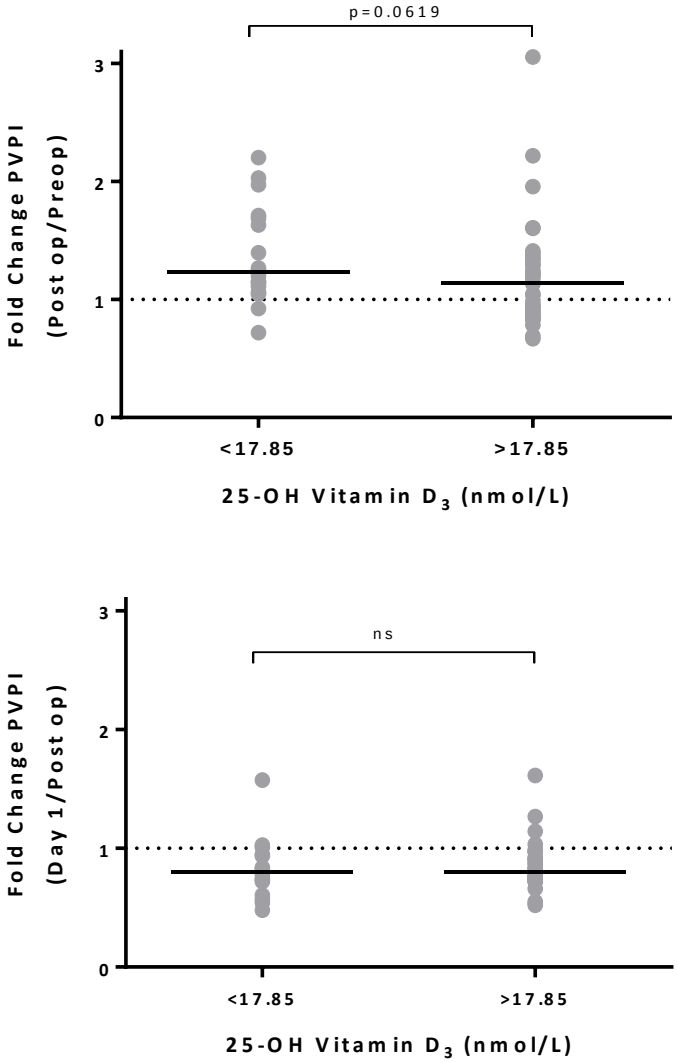


Figure 4.13 Differences between 25-OH Vitamin D_3 groups in perioperative fold change in PVPI

n=20 for 25-OH Vitamin D_3 <17.85 and n=35 for 25-OH Vitamin D_3 >17.85. Comparison between groups made using Mann Whitney test. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

When changes are considered by 1,25-$(OH)_2$ Vitamin D plasma concentration, similar changes in EVLWI and PVPI are seen in both groups (figures 4.11 and 4.12).

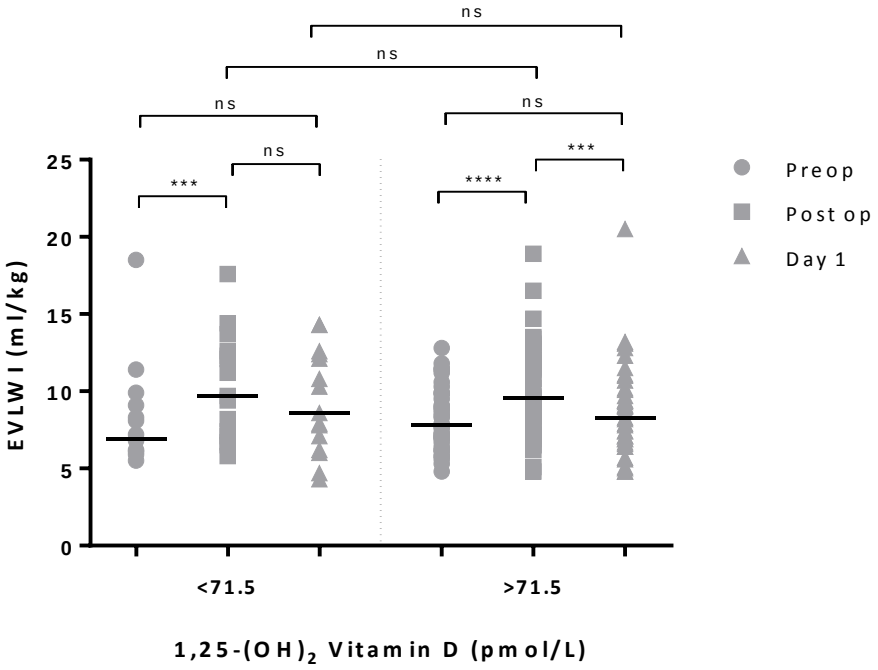


Figure 4.14 Differences between 1,25-$(OH)_2$ Vitamin D groups in perioperative EVLWI

n=15 for <71.5 datasets and n=40 for >71.5 datasets. Black horizontal lines indicate median for each dataset. Friedman test for 1,25-$(OH)_2$ Vitamin D<71.5pmol/L p=0.0007, Friedman test for 1,25-$(OH)_2$ Vitamin D>71.5pmol/L p<0.0001.

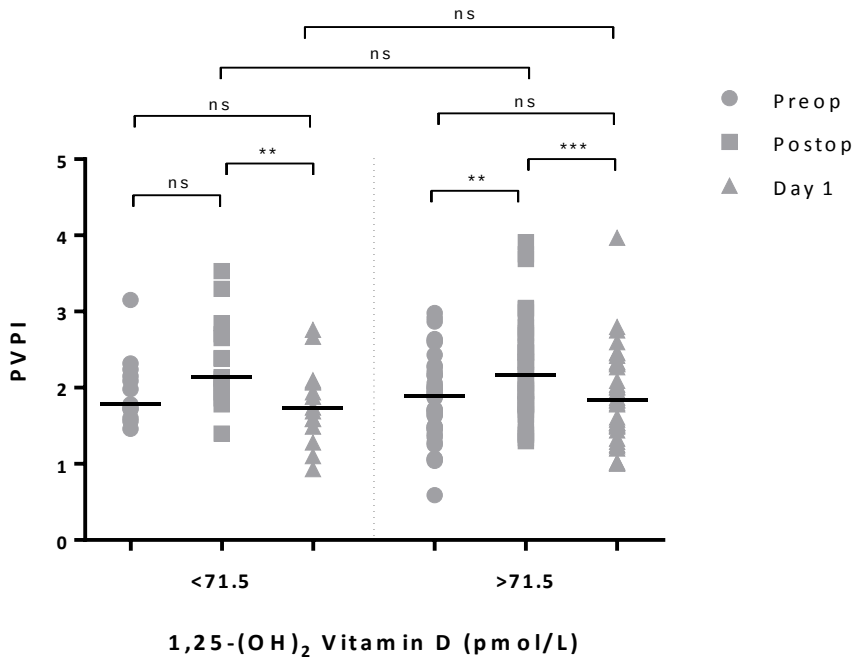


Figure 4.15 Differences between 1,25-$(OH)_2$ Vitamin D groups in perioperative PVPI

n=15 for <71.5 datasets and n=40 for >71.5 datasets. Black horizontal lines indicate median for each dataset. Friedman test for 1,25-$(OH)_2$ Vitamin D<71.5pmol/L p=0.0037, Friedman test for 1,25-$(OH)_2$ Vitamin D>71.5pmol/L p<0.0001.

There were no significant differences in fold change between groups when 1,25-$(OH)_2$ Vitamin D was used to define groups.

4.4.8 Association between Vitamin D and survival

There was no significant difference in survival between oesophagectomy patients with 25-OH Vitamin D_3 < 17.85nmol/L and patients with higher plasma concentrations (p=0.0988, figure 4.16). In ARDS patients recruited to the BALTI and BALTI2 studies there was no difference in plasma concentrations of 25-OH

Vitamin D between patients who died within 28 days of admission and those who survived (p=0.2712, figure 4.17).

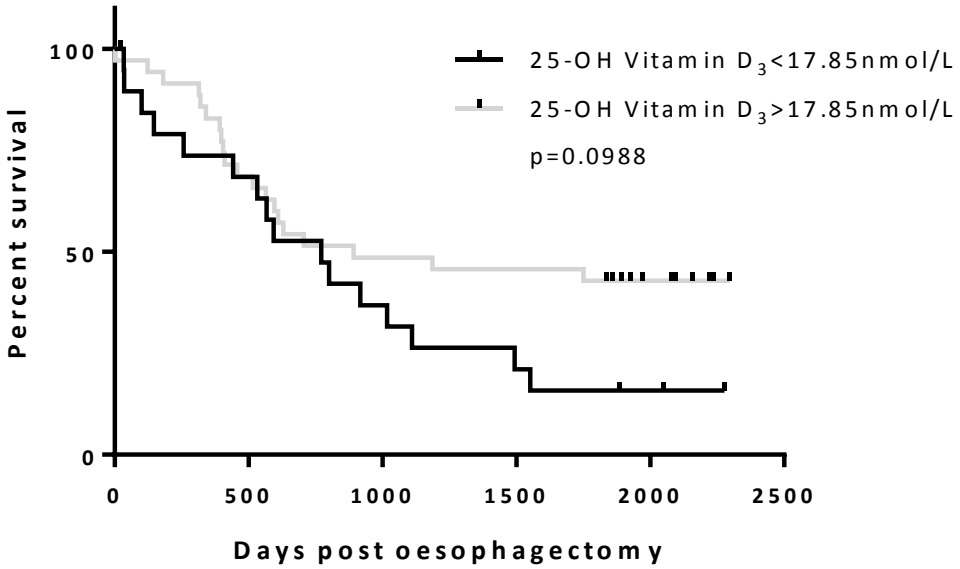


Figure 4.16 Kaplan Meier Survival Curve comparing differences in survival of patients post oesophagectomy by 25-OH Vitamin D_3 group

n=20 for 25-OH Vitamin D_3<17.85 and n=35 for 25-OH Vitamin D_3>17.85

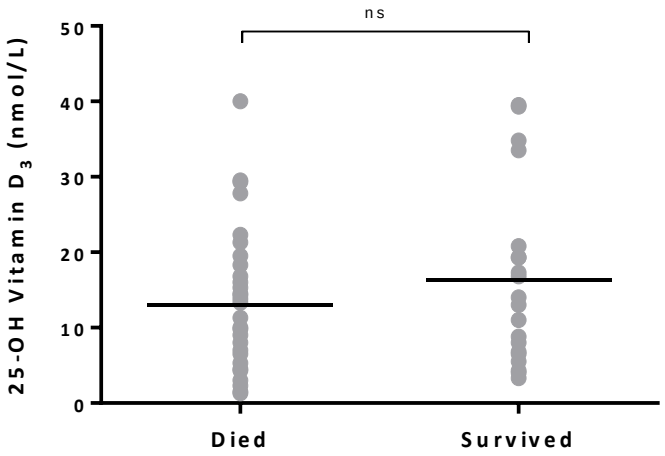


Figure 4.17 Comparison of 25-OH Vitamin D_3 plasma concentrations with 28-day mortality in patients with ARDS (BALTI and BALTI2 cohorts)

32 patients died within 28 days and 20 patients survived. Unpaired T test used for comparison between groups. Horizontal black lines indicate mean for each dataset

Median circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients from the BALTI and BALTI2 studies with ARDS who survive their hospital admission are higher than in patients who do not survive (p=0.0083 figure 4.18).

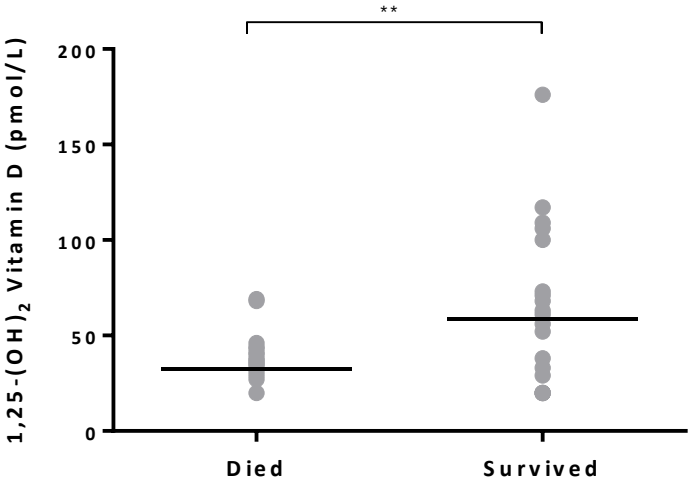


Figure 4.18 Comparison of 1,25-$(OH)_2$ Vitamin D plasma concentrations in ARDS patients with 28-day mortality

32 patients died within 28 days and 20 patients survived. Mann Whitney test used to compare groups. Horizontal black lines indicate median for each dataset

Median post-operative survival in patients with preoperative 1,25-$(OH)_2$ Vitamin D plasma concentrations less than 71.5nmol/L was 565days compared with 1187days in patients with greater plasma concentrations (Mantel-Haenszel hazard ratio 3.774, 95%CI 1.622-8.784, p=0.0021, figure 4.19).

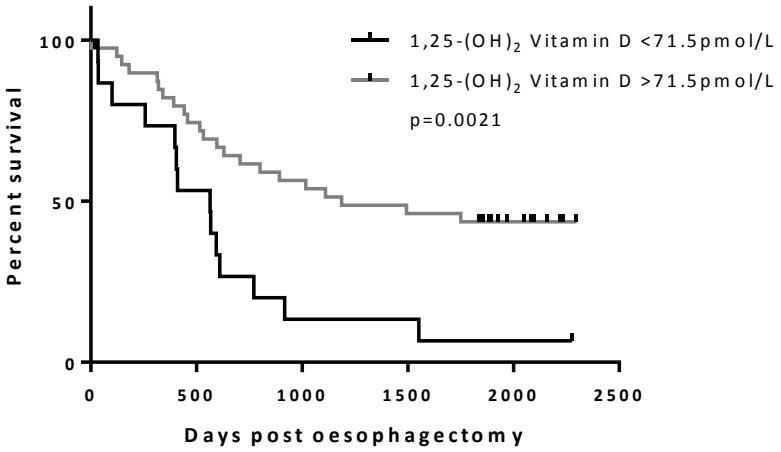


Figure 4.19 Kaplan Meier Survival Curve comparing differences in survival of patients post oesophagectomy by 1,25-$(OH)_2$ Vitamin D group

n=15 for 1,25-$(OH)_2$ Vitamin D < 71.5pmol/L, n=40 for 1,25-$(OH)_2$ Vitamin D > 71.5pmol/L

Data regarding cause of death was not available for all patients. However, examination of the notes for 31 of the patients who had died since their operation revealed that 19 (61%) had evidence of cancer recurrence at the time of their death.

4.5 Discussion

We have found that Vitamin D deficiency is common in patients undergoing oesophagectomy and that patients who develop ARDS post op have lower plasma concentrations of both 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D. This compares with patients on the intensive care unit who have ARDS, in whom we found vitamin D deficiency was ubiquitous. Our findings suggest that Vitamin D deficiency increases risk of development of ARDS.

Plasma concentrations of both 25-OH Vitamin D_3 and 1,25-$(OH)_2$ Vitamin D early in the course of the disease in patients with established ARDS were lower than preoperative plasma concentrations in the patients undergoing oesophagectomy who developed ARDS (25-OH Vitamin D3: established ARDS median plasma concentrations 13.1nmol/L, oesophagectomy ARDS median plasma concentrations 17.3nmol/L, 1,25-$(OH)_2$ Vitamin D: established ARDS median plasma concentrations 36pmol/L, oesophagectomy ARDS median plasma concentrations 66pmol/L). This suggests Vitamin D plasma concentrations may fall early in the course of the illness prior to development of ARDS (i.e. during the course of development of pneumonia, sepsis, pancreatitis etc). Potential mechanisms by which vitamin D deficiency could be established during the onset of lung injury include malabsorption from the gut, dysregulated hydroxylation of precursors in the liver, insufficient VDBP synthesis to bind, store and transport 25-OH vitamin D, ineffective renal uptake in the proximal tubule cell of VDBP-bound vitamin D and dysregulated 25-OH vitamin D metabolism in the kidneys. High metabolism of Vitamin D during severe illness may leave patients with pre existing Vitamin D defiency unable to replenish stores of Vitamin D and at risk of dysregulated immunity.

VDBP is a member of the albumin family and the regulation of its production is poorly understood. Our finding of reduced VDBP in ARDS versus at risk and normal individuals supports a role for either reduced production or increased losses as an explanation for some of the degree of deficiency seen. Equally the low observed plasma concentrations of circulating 1,25-$(OH)_2$ vitamin D in the

ARDS patients suggests reduced renal metabolism as this is the major source of circulating 1,25-$(OH)_2$ Vitamin D (48). Clearly, Vitamin D deficiency in the context of critical illness is likely to be multifactoral.

The prevalence of Vitamin D deficiency in the oesophagectomy cohort was higher than expected. A recent study of patients with colon, lung and breast cancer admitted to a general ward for active treatment found that 77% of patients had 25-OH Vitamin D plasma concentrations <50nmol/L and that median plasma concentrations across these three populations was 38.2nmol/L, compared with prevalence of 95% deficiency and median plasma concentrations of 21nmol/L in our cohort. The high plasma concentration of deficiency in patients undergoing oesophagectomy meant that it was not possible to use the lower limit of normal of 25-OH Vitamin D_3 (50nmol/L) to compare patients. Using the ROC curve analysis we identified a cut off for 25-OH Vitamin D_3 which represented increased risk of ARDS. A recently published study found that similar 25-OH Vitamin D plasma concentrations (17.5nmol/L) carry an increased risk of mortality in severe sepsis (147) which supports the significance of this plasma concentration of severe deficiency.

We found that preop inflammatory cytokines were slightly higher in patients with 25-OH Vitamin D_3 plasma concentrations >17.85nmol/L, but perioperative inflammatory response was lower in these patients. The greater absolute plasma concentrations and fold change in IL6 in patients with lower plasma concentrations of 25-OH Vitamin D_3 may be important as IL6 has been

highlighted as a cytokine which is associated with fewer ventilator free days and increased mortality in patients with ARDS (148, 149). The same studies suggested that IL8 may also be a factor in ARDS, but while patients with more severe Vitamin D deficiency had greater perioperative fold change in IL8, there was no difference in absolute plasma concentrations immediately post op and by day 1 post op the patients with greather plasma concentrations of 25-OH Vitamin D_3 had higher IL8. In addition, while IL8 plasma concentrations in patients with severe 25-OH Vitamin D_3 deficiency tended to fall, plasma concentrations in patients with higher plasma concentrations tended to rise. This finding was not replicated in the 1,25-$(OH)_2$ Vitamin D groups.

Patients with lower plasma concentrations of 25-OH Vitamin D_3 had greater perioperative increases in extravascular lung water, a biomarker of alveolar oedema which has been shown to be an independent risk factor for outcome in ARDS (150). There was also a trend towards greater increases in PVPI at the post op timepoint. As PVPI is the ratio between Extravascular Lung Water and Pulmonary Blood Volume, higher PVPI indicates that alveolar oedema is likely to be inflammatory rather than hydrostatic in nature. This finding, supports an inflammatory mechanism behind perioperative increases in EVLWI. Baseline EVLWI and PVPI did vary between patients and the reasons for this are likely to be multifactoral – while Vitamin D status may play a role, a number of other factors including smoking status and tumour stage are also likely to contribute. As EVLWI and PVPI tended to have returned to baseline by the day following the operation and therefore the perioperative increases seen are unlikely to be

clinically meaningful, but the data does provide a quantatitive measure of alveolar inflammation.

Alveolar macrophages are the predominant cell in the resting healthy lung and express CYP27B which means they are capable of converting 25-OH Vitamin D to 1,25-$(OH)_2$ Vitamin D and are therefore not reliant on circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D (58). 1,25-$(OH)_2$ Vitamin D plasma concentrations were not as clearly associated with biomarkers of alveolar epithelial dysfunction such as sRAGE and EVLWI, suggesting that 25-OH Vitamin D plasma concentrations may be more important in the alveoli. The action of 25-OH Vitamin D on monocytes and macrophages may also be responsible for the differences in IL8 seen on day 1 post op, as the VDR and IL8 gene binding sites are close in proximity and studies have shown that IL8 gene expression in monocytes and macrophages is dependent on Vitamin D (151, 152).

Our data has limitations. In addition, while blood from the patients with ALI was collected as soon as possible following admission to ITU, we are unable to be sure whether levels of 25(OH) Vitamin D_3 are low prior to the development of ALI in that cohort or whether levels fall because of the development of ALI. The size of the cohort of patients who underwent oesophagectomy is small (although it is the largest cohort to date of patients undergoing oesophagectomy in whom EVLWI and PVPI have been measured to our knowledge) with 14 patients developing lung injury post oesophagectomy. This means that the risk of finding

significant correlations by chance is increased and the data should be regarded as hypothesis generating, rather than conclusive.

CHAPTER 5

EFFECTS OF HIGH-DOSE, ONE-OFF VITAMIN D SUPPLEMENTATION ON PLASMA VITAMIN D STATUS

5.1 Introduction

To my knowledge, there are no studies investigating dosing strategies for pre-operative Vitamin D supplementation in patients awaiting elective surgery. Supplementation in surgical patients presents its own challenges – while many supplementation regimes aim to achieve sufficient circulating plasma concentrations of 25-OH Vitamin D over 8-12 weeks (123), in surgical patients such as those undergoing oesophagectomy, a rapid increase in 25-OH Vitamin D plasma concentrations over a short period of time is required in order to achieve sufficient plasma concentrations without delaying the operation.

The two isoforms of Vitamin D (ergocalciferol/Vitamin D_2 and cholecalciferol/Vitamin D_3) differ only in structure of one of their side chains, but this difference appears to alter their biological potency with supplementation with cholecalciferol more likely to increase circulating total 25-OH Vitamin plasma concentrations than ergocalciferol (153). As early studies showed that supplementation with Vitamin D_2 or D_3 was effective at preventing rickets in children, the two isoforms were traditionally assumed to have biological equivalence. However, it has been shown that the 25-OH Vitamin D_2 isoform has a lower affinity for VDBP and is metabolised differently, resulting in lower conversion of 25-OH Vitamin D_2 to 1,25-$(OH)_2$ Vitamin D_2 than is seen with equivalent plasma concentrations of Vitamin D_3 (154). The difference in biological potency may have clinical effects - a Cochrane review found that supplementation with cholecalciferol reduced mortality in elderly women while

supplementation with ergocalciferol did not (155). Multiple studies have found that 25-OH Vitamin D_3 plasma concentrations decrease in response to supplementation with ergocalciferol, (156, 157) but similar studies of the impact of cholecalciferol have not been published. This is important because studies of supplementation frequently report the effect on total Vitamin D plasma concentrations, but if 25-OH Vitamin D_2 plasma concentrations decrease with an increase in 25-OH Vitamin D_3, the biological effects of supplementation in patients with relatively high plasma concentrations of 25-OH Vitamin D_2 may be underestimated.

A number of factors have been shown to influence the response to cholecalciferol administration, including baseline plasma concentrations of 25-OH Vitamin D, demographic factors such as age, sex and BMI, genetic variation in genes related to Vitamin D metabolism and environmental factors such as season of administration (158). Most studies have examined responses after at least 8 weeks of regular treatment and in many cases response has been measured at 12 months. The short term effects of a one-off high dose supplement on 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients awaiting surgery are not clear. However, a placebo controlled pilot study of high dose cholecalciferol supplementation in critically ill patients found that 25-OH Vitamin D plasma concentrations increased significantly in patients who received cholecalciferol within 24 hours and that this difference was sustained over 7 days post dose (159).

There is limited data concerning the immediate effects of undergoing an operation on 25-OH Vitamin D plasma concentrations. Studies in patients undergoing arthroscopy have found that 25-OH Vitamin D plasma concentrations decrease by day 2 post op and that this is associated with a fall in serum VDBP and an increase in urinary VDBP, suggesting increased renal losses (160, 161). One study of patients undergoing gastric bypass surgery measured baseline 25-OH Vitamin D plasma concentrations 2 weeks post op and found that 32% of patients were deficient, but no pre-operative plasma concentrations are available for comparison (162). Another study in patients undergoing gastric bypass found that 64% of patients were deficient preop. All patients in this study were advised to commence daily supplementation with 800-1200IU Vitamin D preop and continue this post op. There was no significant change in 25-OH Vitamin D plasma concentrations at 3 months post op but no plasma concentrations were taken in the immediate post-op period (163).

5.2 Aims and Hypothesis

5.2.1 Hypothesis

A one-off high dose cholecalciferol supplement given 3-14 days prior to planned oesophagectomy will increase circulating 25-OH Vitamin D plasma concentrations above 50nmol/L in all cases.

5.2.2 Research Questions

1. What is the effect of a one-off dose of oral cholecalciferol, given up to 14 days pre-op, on circulating 25-OH Vitamin D plasma concentrations?
2. What is the effect of oral cholecalciferol on circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D?
3. What factors influence short term response to supplementation with cholecalciferol?
4. What is the effect of pre-operative supplementation on post-operative circulating Vitamin D plasma concentrations?

5.3 Methods

Patients were initially recruited to an open label study to determine the best dose of cholecalciferol to administer preop (see section 3.2.1). Patients were administered the dose of cholecalciferol after consenting to inclusion in the trial at the pre-operative assessment clinic appointment which was 3-14 days prior to the planned date of the operation. Plasma concentrations of 25-OH Vitamin D peak at 7 days post oral dosing and decrease linearly thereafter, returning to baseline around day 84 on average (133). This window was therefore pragmatically chosen in order for the operation to coincide with peak plasma 25-OH Vitamin D concentrations while maximising recruitment of patients. The drug used was Vigantol® 812 oil (Merck Darmstadt Germany), an oily solution containing 20,000IU/ml cholecalciferol. Patients unable to swallow due to the severity of their oesophageal disease are routinely given a nasogastric or

percutaneous endoscopic gastrostomy feeding tube and the drug was administered via that if required.

Following the open-label study, patients were recruited to the VINDALOO trial. (see section 3.2.2). The primary endpoint for the Vindaloo trial was extravascular lung water at the end of the operation and the trial was powered to detect a change of 20% in EVLWI with a power of 80% (p=0.05). This study was conceived as a phase 2, proof of efficacy study and therefore the endpoint chosen was not intended to be clinically meaningful. As a group, patients undergoing oesophagectomy have a mean post-operative EVLWI of 10.1ml/kg and standard deviation of 3.0ml/kg and the power calculation estimated 34 patients were required in each arm to reach the primary endpoint. In order to allow for dropouts such as open/close cases, changes to operative plans and other difficulties with data collection, it was intended to recruit 40 patients in each arm of the study.

Plasma 25-OH Vitamin D_2 25-OH Vitamin D_3 and total 1,25-$(OH)_2$ Vitamin D plasma concentrations were measured in a DEQAS quality controlled laboratory. 25-OH Vitamin D plasma concentrations were measured by LC-MS/MS and 1,25-$(OH)_2$ Vitamin D plasma concentrations were measured by EIA. Plasma Vitamin D Binding Protein plasma concentrations were measured using ELISA. Background and demographic data were collected from the patient's notes or by speaking directly with the patient. Serum calcium, serum bilirubin and eGFR are a routine part of clinical care and were measured by the hospital laboratories.

Statistical significance was defined as $p<0.05$. Statistical tests used are detailed in section 3.10.2. Continuous data in tables is presented as mean (SD) for parametric data and median (IQR) for non-parametric data. Nominative data is expressed as n (%). Statistical significance in the graphs is denoted using * for $p\leq0.05$, ** for $p\leq0.01$, *** for $p\leq0.001$ and **** for $p\leq0.0001$. Non-significance is denoted using ns.

5.4 Results

5.4.1 Dose effects of oral cholecalciferol

18 patients were recruited to an open label dose response trial. 3 doses of oral cholecalciferol were trialled sequentially – the first 6 patients received 100,000 IU, the second 6 patients received 200,000IU and the final 6 patients received 300,000 IU. One patient, who received 200,000IU, withdrew from the trial before post-dose blood for Vitamin D plasma concentrations could be taken (figure 5.1).

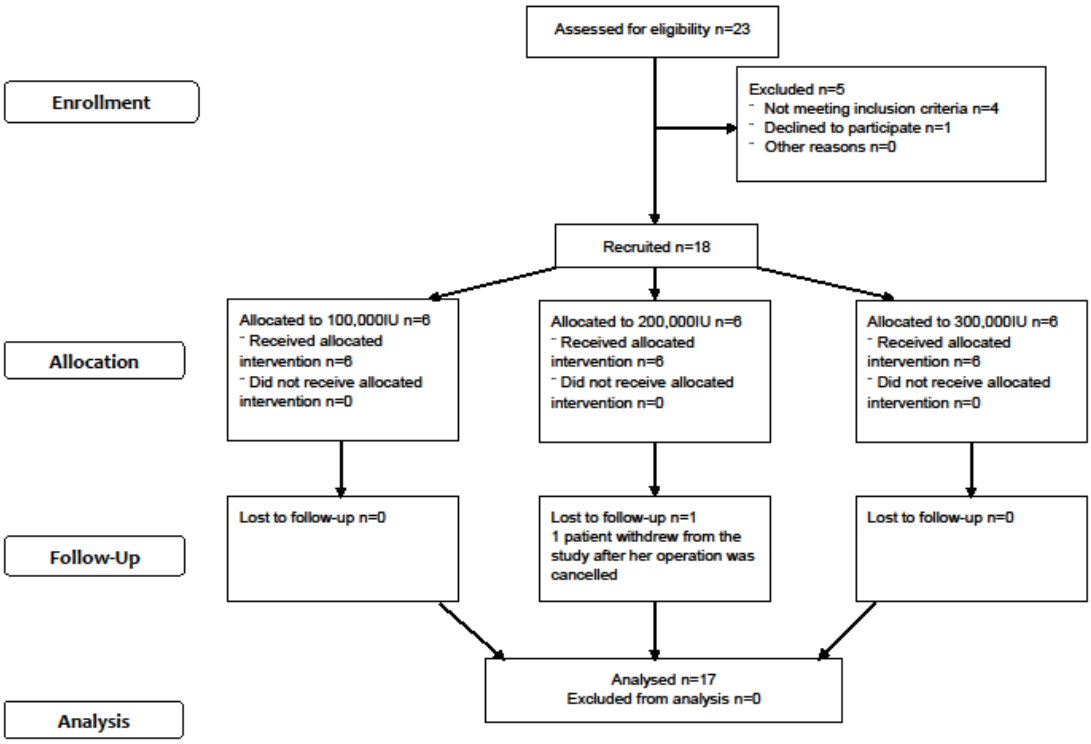


Figure 5.1 Consort diagram of recruitment to open label dosing study

There were no episodes of preoperative hypercalcaemia and the drug was well tolerated, as assessed by patient-reported side effects. None of the three doses increased circulating 25-OH Vitamin D plasma concentrations in all patients. Interpretation of the dose effect was complicated by a significant difference in the pre-dose Vitamin D plasma concentrations between groups, possibly as a result of high incidence of sunny weather conditions at the time the study was undertaken (figure 5.2). All the doses trialled were well tolerated, and the patients who received 300,000IU had the highest median pre-dose 25-OH Vitamin D indicating that this dose is safe in patients who are not deficient.

Therefore, the decision was made to proceed to the randomised controlled trial using 300,000IU cholecalciferol.

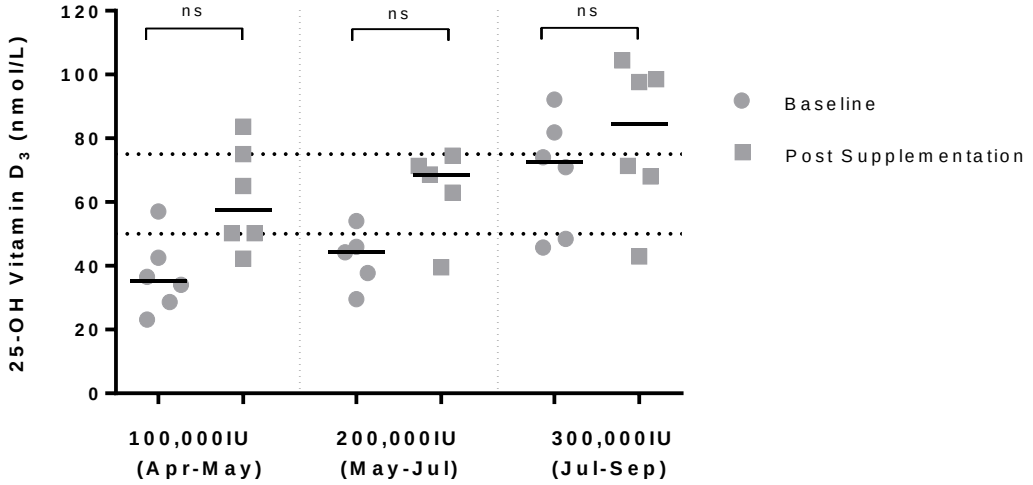


Figure 5.2 Dose effects of supplementation on circulating 25-OH Vitamin D_3 plasma concentrations

n=6 for 100,000IU and 300,000IU and n=5 for 200,000IU. Wilcoxon signed rank test used for each comparison. Black horizontal lines indicate median value for each dataset. Dotted horizontal reference lines indicate 25-OH Vitamin D_3 plasma concentrations 50nmol/L and 75nmol/L. Months of recruitment is indicated in parentheses under each dose.

5.4.2 VINDALOO Trial Patient Characteristics

79 patients were recruited to the trial. 39 were randomised to receive placebo and 40 received active drug (300,000 IU cholecalciferol). Trial recruitment is summarised in the consort diagram (figure 5.3)

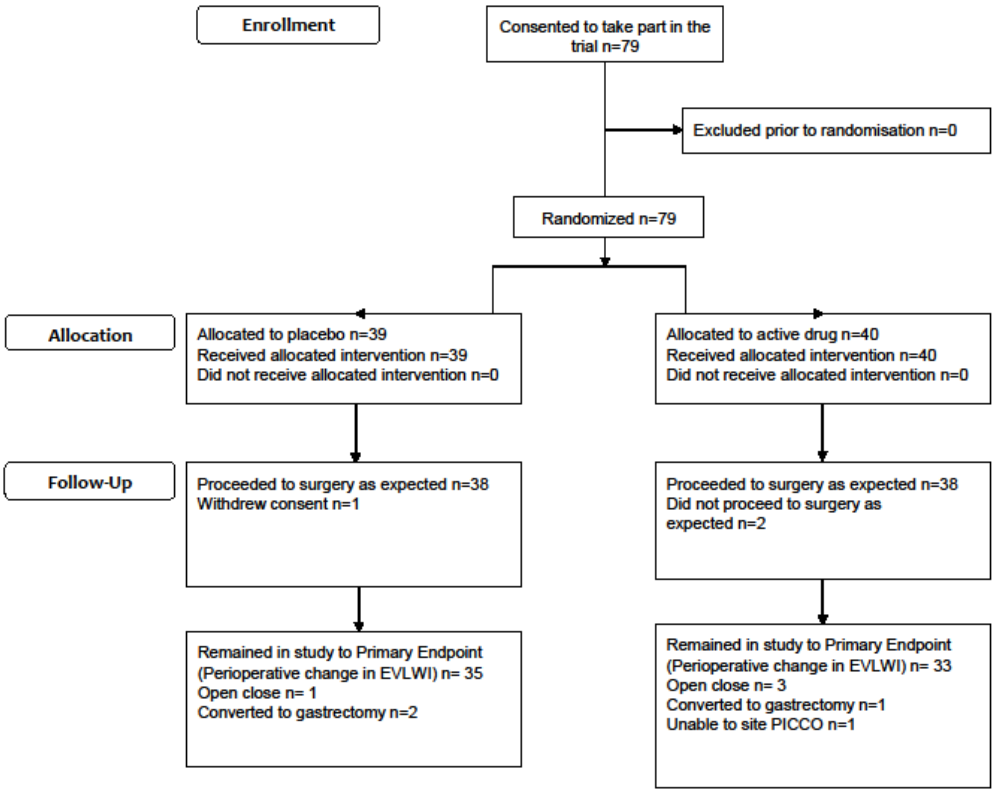


Figure 5.3 Consort Diagram of recruitment to VINDALOO

There were no significant differences in patient characteristics which are summarised in table 5.1.

Table 5.1 Comparison of patient demographics between patients from the historical cohort described in Chapter 4 (BALTI-p cohort) and patients recruited to the Vindaloo trial

		BALTI-p	VINDALOO		P value (Placebo vs active)
			Placebo (n=39)	Active Drug (n=40)	
Age (years) – median (IQR)		64 (53-71)	66 (58-72)	64.5 (58-70)	0.370
BMI (kg/m2) – median (IQR)		24.7 (21.3-28.3)	25.7 (23.6-30.4)	28.2 (24.6-32.5)	0.144
Male – n (%)		47 (85)	31 (79)	36 (90)	0.193
Caucasian – n (%)			36 (92)	40 (100)	0.074
Smoker	Never n (%)	6 (11)	10 (26)	11 (28)	0.630
	Former n (%)	35 (64)	24 (63)	20 (51)	
	Current n (%)	14 (25)	4 (11)	8 (21)	
	Unknown n (%)	0 (0)	1 (2)	1 (3)	
Pack Years – median (IQR)		30 (15-45)	20 (0-40)	15 (0-36)	0.350
Adenocarcinoma – n (%)		40 (73)	32 (82)	35 (88)	0.500
Tumour Stage	0/1 – n (%)	5 (9)	4 (11)	7 (18)	0.335
	2 – n (%)	17 (31)	13 (34)	8 (21)	
	3 – n (%)	33 (60)	22 (55)	25 (62)	
Pre-randomisation plasma 25-OH Vitamin D_3 (nmol/L) – median (IQR)		21 (16-29)	45.3 (35.4-69.0)	43.6 (27.2-67.0)	0.552

5.4.3 Safety and tolerability of high dose preoperative cholecalciferol

4 patients reported developing diarrhoea and vomiting after administration of the trial drug. All episodes were self-limiting and required no medical treatment. 2 patients received placebo and 2 patients received active drug. Circulating 25-OH Vitamin D plasma concentrations increased in the two patients in the cholecalciferol despite their symptoms; one patient's 25-OH Vitamin D_3 plasma concentration increased from 83.8 to 100.8nmol/L and the other patient's 25-OH vitamin D_3 plasma concentration increased from 56.4 to 92.1nmol/L.

No patients reported symptoms of hypercalcaemia following randomisation. Pre-operative serum calcium plasma concentrations were measured in 70 of the 76 patients who proceeded to pre-operative follow up. Pre-operative plasma concentrations were not available in 6 patients (2 received cholecalciferol, 4 received placebo), but post-operative plasma concentrations were not elevated. There was no significant difference in post-randomisation (pre-operative) serum calcium plasma concentrations ($p=0.4419$) or fold change in serum calcium ($p=0.5170$) between groups (figure 5.4 and 5.5). No patient in the cholecalciferol group had hypercalcaemia post randomisation. One patient in the placebo group had post randomisation calcium above the upper limit of normal; their post-randomisation Vitamin D plasma concentration was deficient (37.7nmol/L).

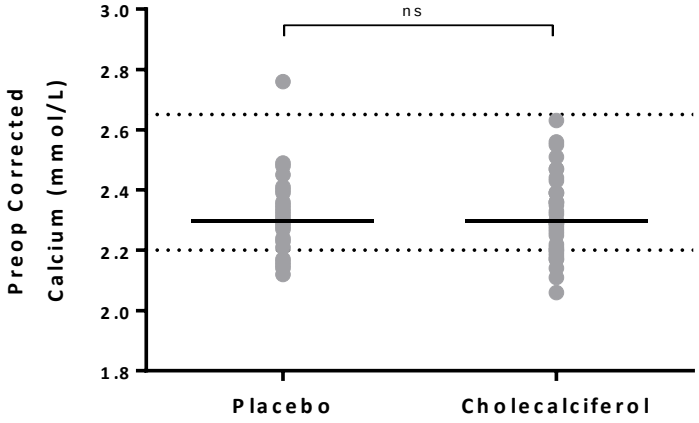


Figure 5.4 Effects of cholecalciferol on post randomisation serum calcium plasma concentrations

n=34 for placebo and n=36 for cholecalciferol. Mann Whitney test used for comparison. Black horizontal lines indicate median value for each dataset. Dotted reference lines indicate the normal range for serum calcium (2.20-2.65mmol/L)

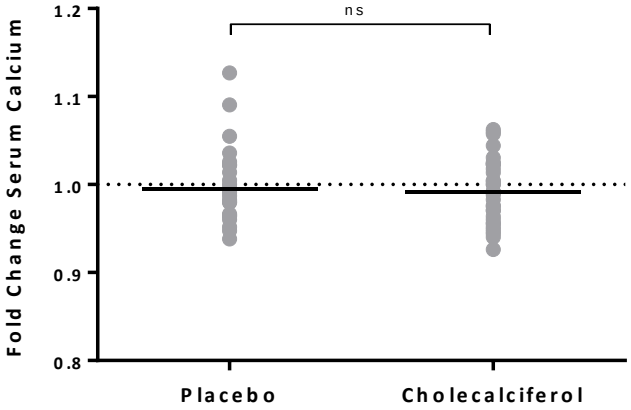


Figure 5.5 Effects of cholecalciferol on fold change in serum calcium plasma concentrations

n=34 for placebo and n=36 for cholecalciferol. Mann Whitney test used for comparison. Black horizontal lines indicate median value for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post randomisation and datapoints above the line increased post randomisation

Due to the nature of the operation, multiple patients developed Serious Adverse Events (SAEs) including two patients who died prior to discharge from hospital,

but there were no Suspected Unexpected Serious Adverse Reactions (SUSARs) reported during the trial.

5.4.4 Effects of cholecalciferol on circulating 25-OH Vitamin D plasma concentrations

55% of patients were deficient in Vitamin D prior to randomisation (25-OH Vitamin D_3 <50nmol/L) and 32% of patients had insufficient plasma concentrations (25-OH Vitamin D_3 50-75nmol/L). Patients recruited to VINDALOO had significantly higher circulating 25-OH Vitamin D_3 plasma concentrations than the BALTI-p cohort described in chapter 4 ($p<0.0001$, figure 5.6). While 36% (20/55) of patients had plasma concentrations lower than 17.85nmol/L in the BALTI-p cohort, 6.5% (5/76) had such severe pre-randomisation deficiency in the Vindaloo cohort.

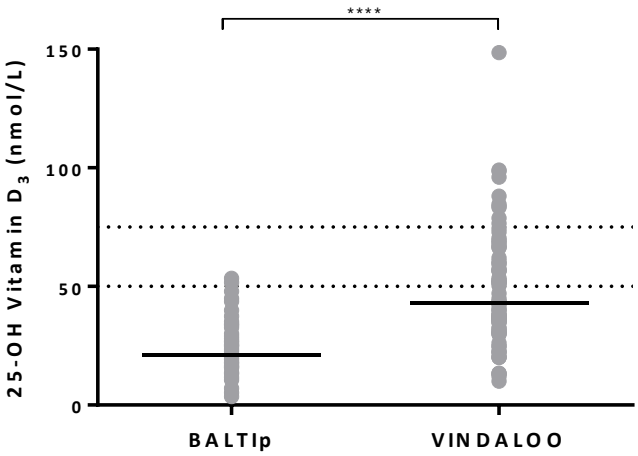


Figure 5.6 Comparison of baseline 25-OH Vitamin D_3 plasma concentrations in patients awaiting oesophagectomy

n=55 for BALTIp dataset and n=76 for VINDALOO dataset. Mann Whitney test used for comparison. Black horizontal lines indicate median value for each dataset. Dotted horizontal reference lines indicate 25-OH Vitamin D_3 plasma concentrations 50nmol/L and 75nmol/L

Patients who received active drug had greater circulating 25-OH Vitamin D_3 plasma concentrations post randomisation than patients who received placebo ($p<0.0001$, figure 5.7). Pre to post randomisation circulating 25-OH Vitamin D_3 plasma concentrations decreased by 11% on average in the placebo group but increased by 64% in the active drug group ($p<0.0001$).

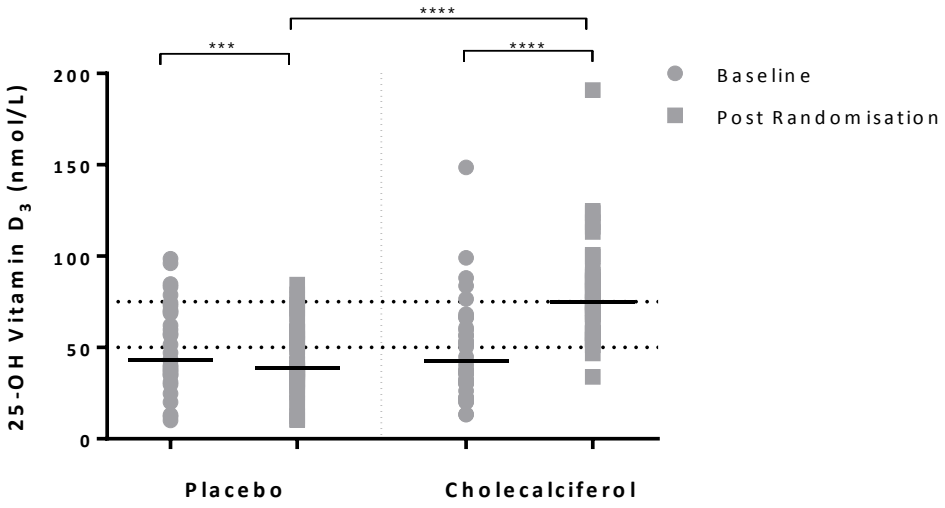


Figure 5.7 Effects of cholecalciferol on 25-OH Vitamin D_3 plasma concentrations

n=38 for each dataset. Wilcoxon signed rank test used to compare within groups and Mann Whitney test used to compare between groups. Black horizontal lines indicate median value for each dataset. Dotted horizontal reference lines indicate 25-OH Vitamin D_3 plasma concentrations 50nmol/L and 75nmol/L

31 patients (42%) had detectable of plasma concentrations of 25-OH Vitamin D_2 at baseline (>2.5nmol/L). Patients with undetectable plasma concentrations were assigned a plasma concentration of 2.49nmol/L for analysis. Post-randomisation plasma concentrations fell significantly in the group that received cholecalciferol (p=0.0005) but not in the placebo group (p=0.0652), with no difference in post randomisation plasma concentrations between groups (p=0.2998, figure 5.8).

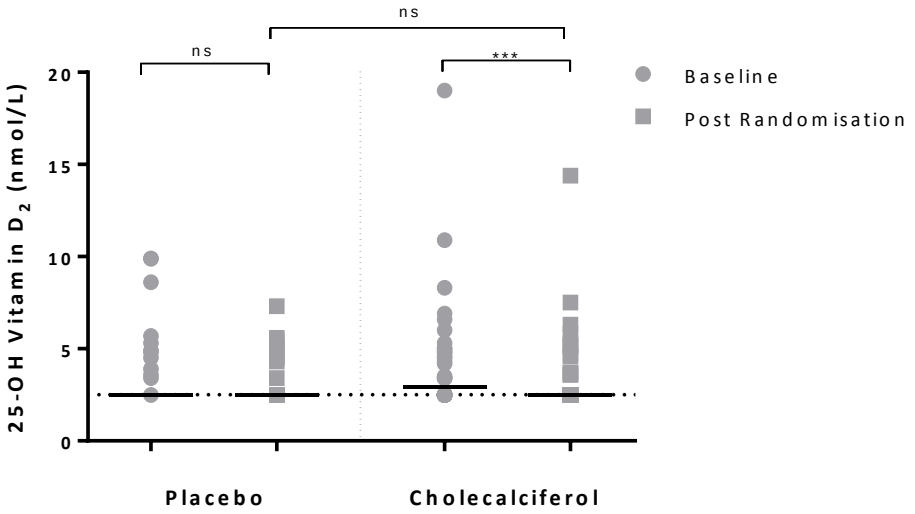


Figure 5.8 Effects of cholecalciferol on 25-OH Vitamin D_2 plasma concentrations

n=38 for each dataset. Wilcoxon signed rank test used to compare within groups and Mann Whitney test used to compare between groups. Black horizontal line indicates median for each dataset. Dotted horizontal reference line indicates lower limit of detection, 2.5nmol/L. Where 25-OH Vitamin D2 plasma concentrations were undetectable a plasma concentration of 2.49nmol/L has been allocated for illustrative and analysis purposes.

For those patients with detectable Vitamin D_2 plasma concentrations the median change post-randomisation was -0.9nmol/L in both groups (p=0.9880).

The low plasma concentrations of 25-OH Vitamin D_2 seen in this cohort meant that total plasma concentrations of 25-OH Vitamin D followed a similar pattern to 25-OH Vitamin D_3 with greater post-randomisation plasma concentrations in the group that received active drug (figure 5.9). 58% of patients who received placebo had total 25-OH Vitamin D plasma concentrations <50nmol/L compared with 5% of patients who received active drug and 11% of patients who received placebo had plasma concentrations >75nmol/L compared with 55% of patients who received active drug (figure 5.10).

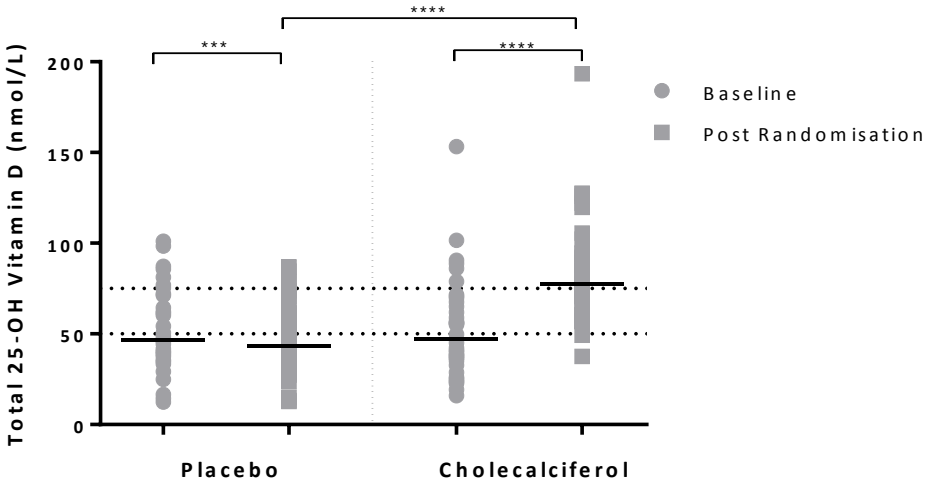


Figure 5.9 Effects of cholecalciferol on total 25-OH Vitamin D plasma concentrations

n=38 for each dataset. Wilcoxon signed rank test used to compare within groups and Mann Whitney test used to compare between groups. Black horizontal lines indicate median value for each dataset. Dotted horizontal reference lines indicate 25-OH Vitamin D plasma concentrations 50nmol/L and 75nmol/L. Where 25-OH Vitamin D_2 plasma concentrations were undetectable (<2.5nmol/L), 2.49nmol/L was added to the total value.

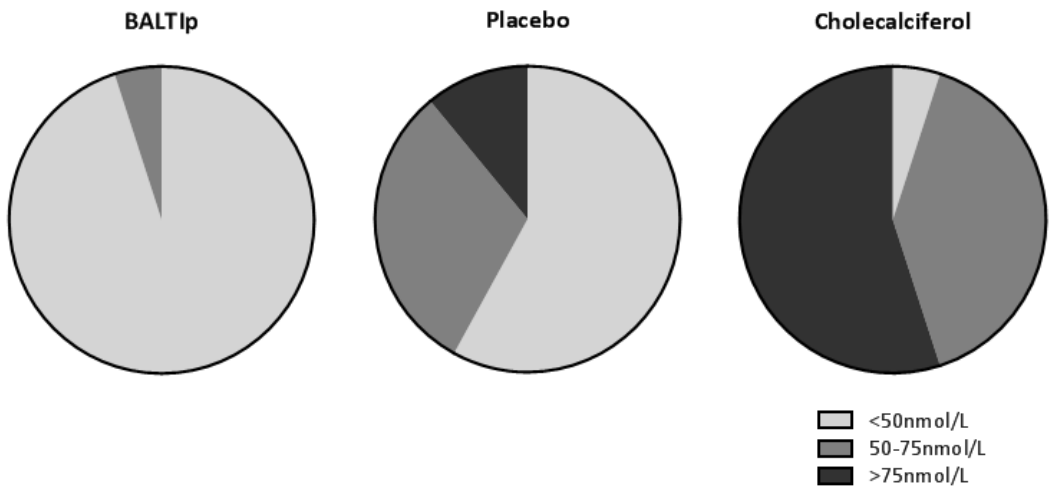


Figure 5.10 Differences between groups in proportions of patients with deficient (<50nmol/L), insufficient (50-75nmol/L) and sufficient (75nmol/L) 25-OH Vitamin D plasma concentrations

5.4.5 Effects of cholecalciferol on circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations

In contrast with 25-OH Vitamin D, there was no significant difference in circulating plasma concentrations of 1,25-$(OH)_2$ Vitamin D between the BALTIp and Vindaloo cohorts (figure 5.11). 27.6% (21/76) patients in the Vindaloo cohort had baseline 1,25-$(OH)_2$ Vitamin D plasma concentrations less than 71.5 pmol/L (as identified by ROC curve analysis in chapter 4) which was similar to the BALTI-p cohort (15/55, 27.2%).

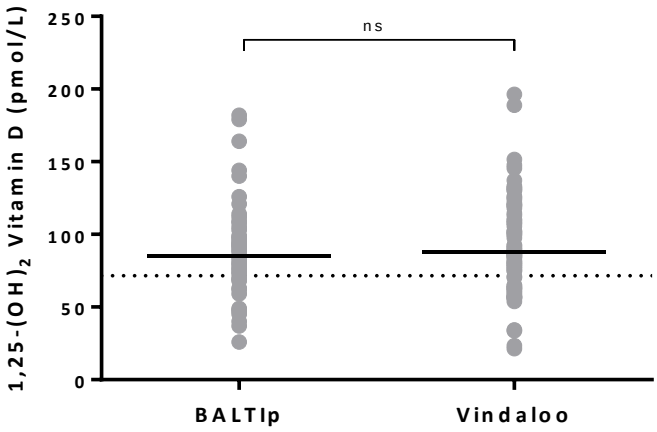


Figure 5.11 Comparison of baseline 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients awaiting oesophagectomy

n=55 for BALTIp dataset and n=76 for VINDALOO dataset. Mann Whitney test used to compare groups. Black horizontal lines indicate median value for each dataset. Dotted reference line indicates 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L.

Cholecalciferol administration did not result in a significant difference in 1,25-$(OH)_2$ Vitamin D plasma concentrations between groups (Figure 5.12). Paired analysis of changes within the two groups showed that while plasma concentrations of 1,25-$(OH)_2$ Vitamin D went up significantly in the cholecalciferol group (p=0.0003), they did not change significantly in the placebo group (p=0.2380). Fold change in 1,25-$(OH)_2$ Vitamin D plasma concentrations was significantly higher in the cholecalciferol group than in the placebo group (p=0.0009 figure 5.13). Post randomisation, 34.2% (13/38) patients who received placebo had 1,25-$(OH)_2$ Vitamin D plasma concentration <71.5pmol/L compared with 18.4% (7/38) of patients who received cholecalciferol (X^2 p=0.1181).

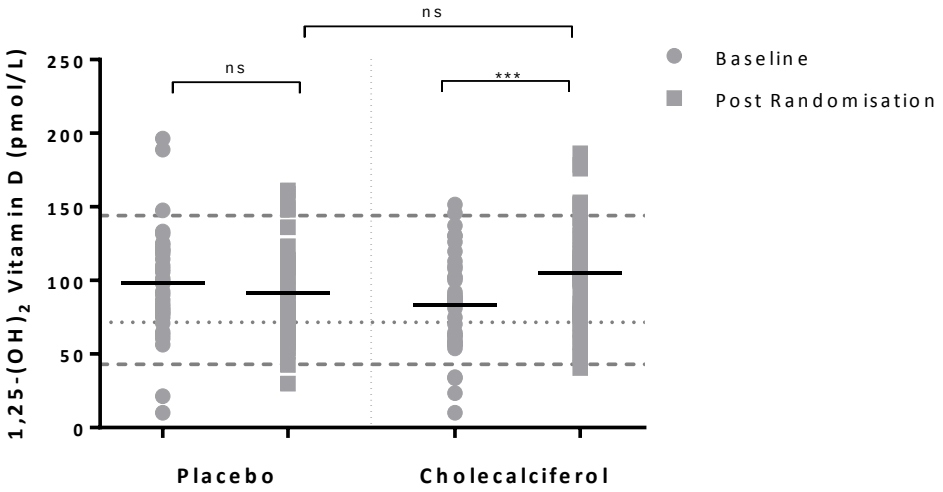


Figure 5.12 Effects of cholecalciferol on 1,25-$(OH)_2$ Vitamin D plasma concentrations

n=38 for each dataset. Paired T test used for comparison within groups and unpaired T test used for comparison between groups. Black horizontal lines indicate mean for each dataset. Dotted reference line indicates 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L, dashed lines indicate normal range for 1,25-$(OH)_2$ Vitamin D (43-144pmol/L).

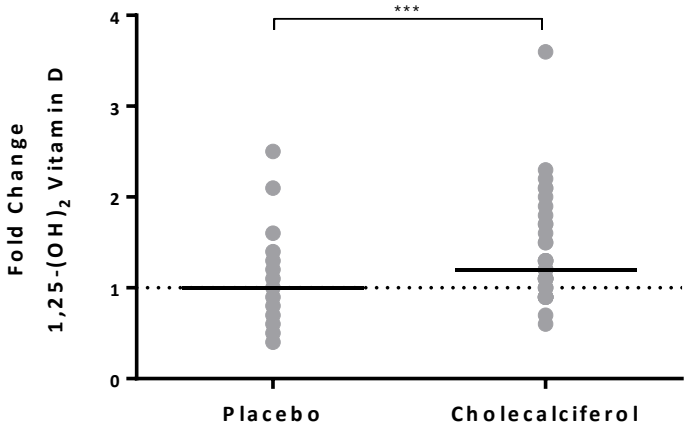


Figure 5.13 Effects of cholecalciferol on change in 1,25-$(OH)_2$ Vitamin D

n=38 for each dataset. Mann Whitney Test used to compare groups. Black horizontal lines indicate median for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post randomisation and datapoints above the line increased post randomisation

5.4.6 Relationship between 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D

There was a significant correlation between post randomisation 25-OH and 1,25-$(OH)_2$ Vitamin D plasma concentrations (Pearson ρ=0.3715, p=0.0008, r^2=0.1380, figure 5.14). 2 (2.6%) patients were deficient in both 25-OH and 1,25-$(OH)_2$ Vitamin D. 23 (30.2%) patients were deficient in 25-OH Vitamin D but had non-deficient 1,25-$(OH)_2$ plasma concentrations (>43pmol/L). 1 (1.3%) patient had deficient 1-25-$(OH)_2$ Vitamin D with non-deficient 25-OH Vitamin D and 50 (65.7%) patients had both non-deficient 25-OH and 1,25-$(OH)_2$ plasma concentrations.

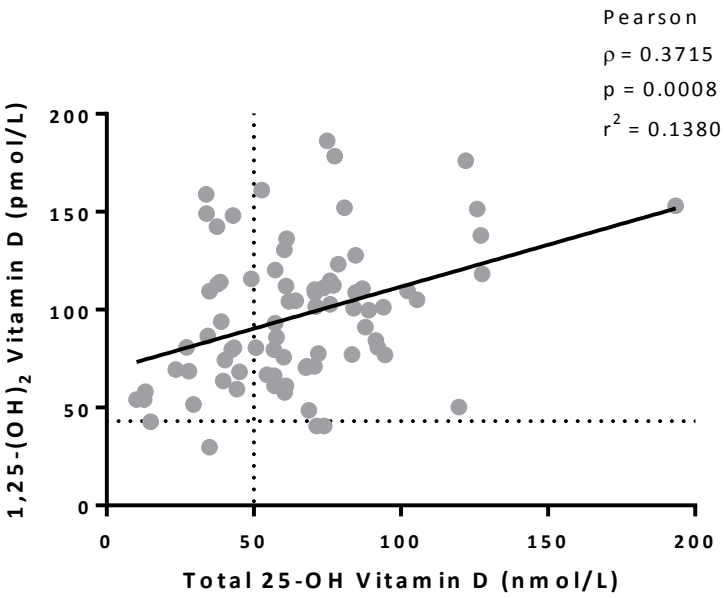


Figure 5.14 Correlation between post randomisation plasma concentrations of 25-OH and 1,25-$(OH)_2$ Vitamin D.

n=76. Vertical dotted reference line indicates 25-OH Vitamin D = 50nmol/L. Horizontal dotted reference line indicates 1,25-$(OH)_2$ Vitamin D 43pmol/L

5.4.7 Effects of cholecalciferol on circulating Vitamin D Binding Protein

Cholecalciferol had no significant short-term effect on circulating plasma concentrations of Vitamin D Binding protein. There was no difference between groups in pre- or post-randomisation plasma concentrations of Vitamin D Binding Protein (VDBP) and no difference in fold change. Median post randomisation plasma concentrations for placebo were 685.1mg/L and for cholecalciferol were 725.0mg/L (p=0.9764, figure 5.15).

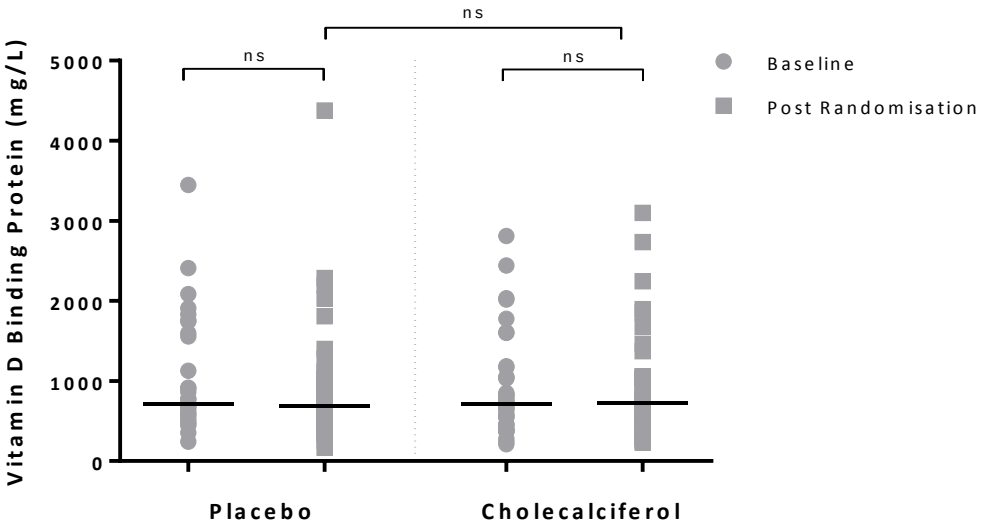


Figure 5.15 Effects of Cholecalciferol on plasma Vitamin D Binding Protein concentration

n=38 for each dataset. Wilcoxon signed rank test used for comparison within groups and Mann Whitney Test used for comparison between groups. Black horizontal lines indicate median for each dataset.

5.4.8 Factors associated with variability in 25-OH Vitamin D response to cholecalciferol administration

The effect of cholecalciferol on circulating 25-OH and 1,25-$(OH)_2$ Vitamin D plasma concentrations was variable. I looked at the effects of a range of factors on response to cholecalciferol administration.

5.4.8.1 Effect of baseline Vitamin D plasma concentration on response to cholecalciferol

Increases in circulating 25-OH Vitamin D plasma concentrations were greatest in patients who had the lowest plasma concentrations pre-randomisation, resulting in a negative correlation (Spearman ρ=-0.7573, p<0.0001, r^2=0.6083 figure 5.16).

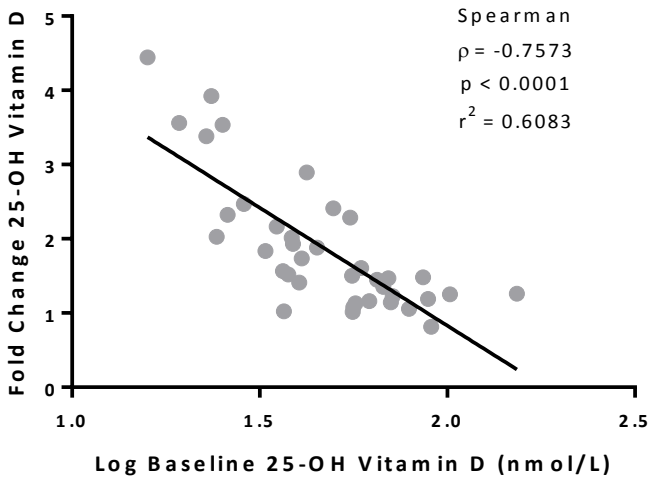


Figure 5.16 Correlation between baseline total 25-OH Vitamin D and fold change in plasma concentrations

n=38. Baseline total 25-OH Vitamin D data is log transformed

However, there was a positive correlation between baseline and post randomisation plasma concentrations of 25-OH Vitamin D with patients with the highest baseline plasma concentrations still tending to have the highest post randomisation plasma concentrations (Spearman ρ=0.5323, p=0.0006, r^2=0.4835 figure 5.17).

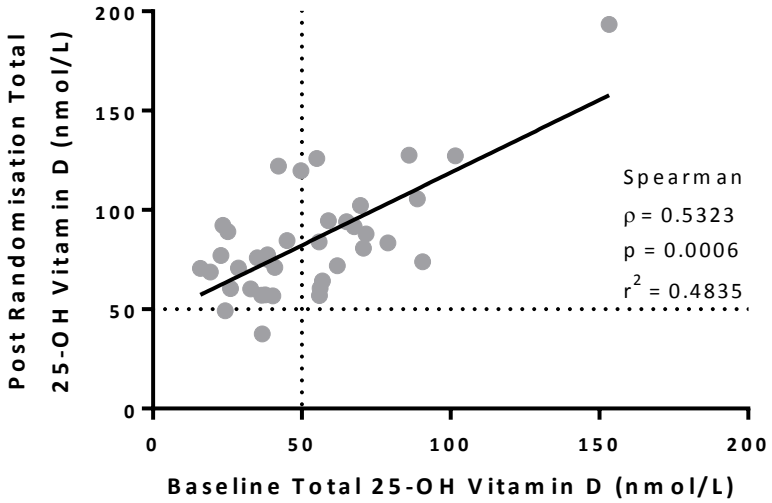


Figure 5.17 Correlation between baseline and post randomisation total 25-OH Vitamin D plasma concentrations

n=38. Reference lines indicate total 25-OH Vitamin D plasma concentrations of 50nmol/L

5.4.8.2 Effect of Demographic Parameters on response to cholecalciferol

Female patients who received supplementation had lower fold change in total 25-OH Vitamin D in response to cholecalciferol than male patients, but this did not translate into a difference in post randomisation Vitamin D plasma concentrations (table 5.2).

Table 5.2 Differences between sexes in Vitamin D response to supplementation

	Male (n=34)	Female (n=4)	P value
Baseline total 25-OH Vitamin D (nmol/L)	43.6 (31.8-68.1)	59.0 (42.3-68.5)	0.4759
Post randomisation total 25-OH Vitamin D (nmol/L)	80.4 (67.6-96.5)	64.6 (57.0-78.6)	0.1284
Fold change in total 25-OH Vitamin D	**1.67 (1.26-2.34)**	**1.15 (1.05-1.43)**	**0.0419**

Older patients tended to have lower post randomisation plasma concentrations of total 25-OH Vitamin D (Spearman ρ=-0.3706, p=0.0220, r^2=0.2717, figure 5.18) and lower response to cholecalciferol administration (Spearman ρ=-0.3580, p=0.0273, r^2=0.0181).

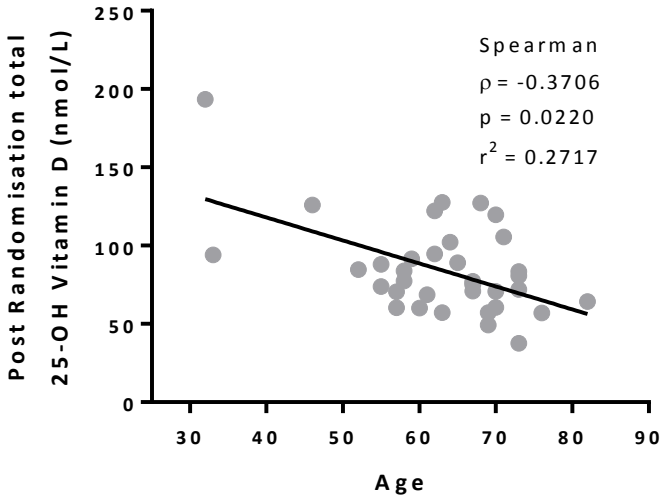


Figure 5.18 Correlation between Age and post randomisation total 25-OH Vitamin D plasma concentrations

n=38. Reference line indicates total 25-OH Vitamin D plasma concentrations of 50nmol/L

There was no correlation between BMI and post randomisation total 25-OH vitamin D plasma concentration (Spearman ρ=-0.2680, p=0.1038, r^2=0.0776) or fold change (Spearman ρ=0.1100, p=0.5110, r^2=0.0029) in response to cholecalciferol.

5.4.8.3 Effect of time between dose and operation on response to cholecalciferol

Patients were administered cholecalciferol between 3 and 14 days of the expected date of their oesophagectomy. On occasion the operation may be delayed at short notice, due to factors such as patient illness, or lack of hospital beds. On 2 occasions there was a gap between the dose and the operation of more than 14 days. Both patients had received active drug, for one the gap was 15 days and for one 29 days. There was no correlation between either fold

change (Spearman ρ=-0.0517 p=0.7577, r^2=0.0025, figure 5.19) or post randomisation total 25-OH Vitamin D plasma concentration (Spearman ρ=-0.1528, p=0.3597, r^2=0.0306) and the number of days between the dose and the operation.

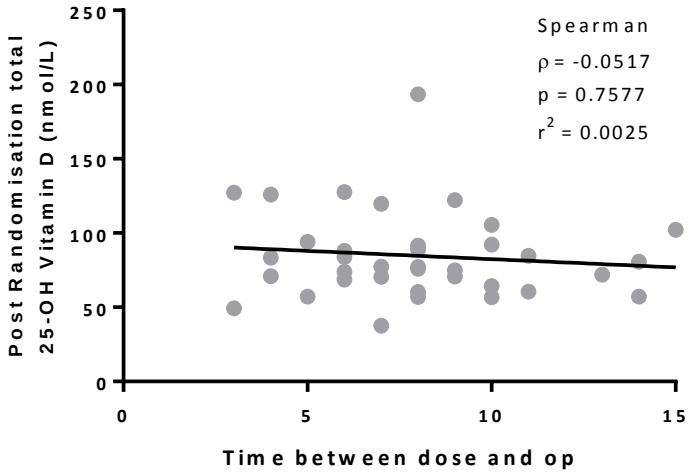


Figure 5.19 Correlation between days between dose and operation and response to cholecalciferol.

n=38. One outlier (29 days) is not shown for illustrative purposes but was included in the analysis.

5.4.8.4 Effect of Season of recruitment on response to cholecalciferol

The season of year when the patient was administered cholecalciferol did not affect the fold change in total 25-OH Vitamin D plasma concentrations (p=0.1505) or post-operative total 25-OH Vitamin D plasma concentrations (p=0.6644). As Vitamin D production in the skin is greatest in the summer, the data was divided into two groups – May-Sept and Oct-April, with no difference in fold change (p=0.6618) or post cholecalciferol total 25-OH Vitamin D (p=0.8190, figure 5.20).

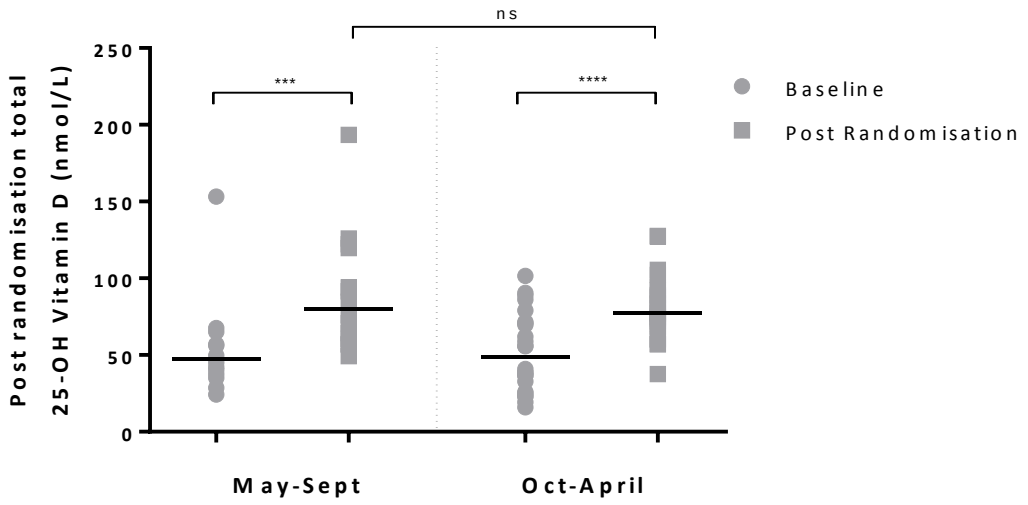


Figure 5.20 Effects of time of year of cholecalciferol administration on total 25-OH Vitamin D plasma concentrations

May-Sept n=14, Oct-April n=24. Wilcoxon signed rank tesst used for comparison within groups and Mann Whitney Test used for comparison between groups. Black horizontal line indicates median for each dataset.

5.4.8.5 Effect of baseline liver function on response to cholecalciferol

As hydroxylation of Vitamin D to form 25-OH Vitamin D takes place in the liver, liver function may impact on post cholecalciferol plasma concentrations of 25-OH Vitamin D. Bilirubin plasma concentrations reflect the liver's ability to take up, process and secrete bilirubin into the bile and is therefore generally useful as a test of liver function. It is the measure of liver function used in the SOFA score (see section 3.8.2) and is generally easily obtainable as part of standard post-operative blood monitoring. There was no significant relationship between baseline bilirubin and fold change in total 25-OH Vitamin D plasma concentrations (Spearman ρ=0.0833, p=0.6289, r^2=0.0243) or post cholecalciferol

total 25-OH Vitamin D plasma concentrations (Spearman ρ=0.0985, p=0.5676, r^2=0.0008).

5.4.8.6 Effect of baseline renal function on response to cholecalciferol

As hydroxylation of 25-OH Vitamin D to form 1,25-$(OH)_2$ Vitamin D occurs predominantly in the kidney and Vitamin D is renally excreted I investigated whether baseline renal function (eGFR) correlates with response to cholecalciferol. There was no correlation between eGFR and fold change in total 25-OH Vitamin D plasma concentrations (Spearman ρ=-0.1571, p=0.3461, r^2=0.0617) or post cholecalciferol total 25-OH Vitamin D (Spearman ρ=-0.1883, p=0.2575, r^2=0.0274).

5.4.8.7 Effect of baseline Vitamin D Binding protein plasma concentrations on response to cholecalciferol

As total 25-OH Vitamin D circulates bound to VDBP I considered the effect of baseline VDBP on response to cholecalciferol. There was no correlation between baseline VDBP and fold change (Spearman ρ=0.1124, p=0.5018, r^2=0.0145) or post cholecalciferol total 25-OH Vitamin D (Spearman ρ=-0.0620, p=0.7114, r^2=0.0262).

5.4.8.8 Linear regression analysis of post cholecalciferol 25-OH Vitamin D_3 plasma concentrations

To further investigate causes in the variance in preoperative total 25-OH Vitamin D plasma concentrations in patients who received cholecalciferol, I performed a

multiple regression. There was no evidence of collinearity between the factors examined (VIF was less than 2.0 for all factors). In view of the small sample size, a forward stepwise regression was used to examine the data. A significant three variable model ($F(3,34) = 21.096$, $p<0.001$) which explained 65% of the variability seen emerged (R square = 0.65). This demonstrated that baseline total 25-OH Vitamin D level ($p<0.001$), age ($p=0.001$) and renal function ($p=0.024$) were significant predictors of post randomisation total 25-OH Vitamin D level in patients who received cholecalciferol (table 5.3). Residuals were checked for normality by visual inspection of the Q/Q plot and found to be acceptable.

Table 5.3 Forward Stepwise linear regression analysis of predictors of post cholecalciferol 25-OH Vitamin D

Variable	Univariate P value	Unstandardised Coefficients		Standardised Coefficient (β)	P value
		B	Std. Error		
Baseline total 25-OH Vitamin D	**0.0006**	**0.605**	**0.112**	**0.574**	**<0.001**
Age	**0.0220**	**-1.137**	**0.306**	**-0.404**	**0.001**
Baseline eGFR	**0.2575**	**-0.321**	**0.136**	**-0.245**	**0.024**

5.4.9 Factors associated with variability in 1,25-$(OH)_2$ Vitamin D response to cholecalciferol administration

The same factors were examined to investigate whether they influenced post randomisation 1,25-$(OH)_2$ Vitamin D following supplementation with cholecalciferol.

5.4.9.1 Effects of Baseline 1,25-$(OH)_2$ Vitamin D on response to cholecalciferol

On univariate analysis, baseline 1,25-$(OH)_2$ Vitamin D plasma concentrations significantly correlated with fold change in 1,25-$(OH)_2$ Vitamin D (figure 5.21) and post cholecalciferol 1,25-$(OH)_2$ Vitamin D (figure 5.22). 1 patient (2.6%) with baseline 1,25-$(OH)_2$ Vitamin D plasma concentrations greater than 71.5pmol/L had post cholecalciferol plasma concentrations less than 71.5pmol/L. 6 patients (15.8%) had baseline plasma concentrations less than 71.5pmol/L at both baseline and post cholecalciferol administration. 8 patients (21.1%) had baseline plasma concentrations less than 71.5pmol/L and post cholecalciferol plasma concentrations greater than 71.5pmol/L. 23 patients (60.5%) had plasma concentrations greater than 71.5pmol/L at both time points.

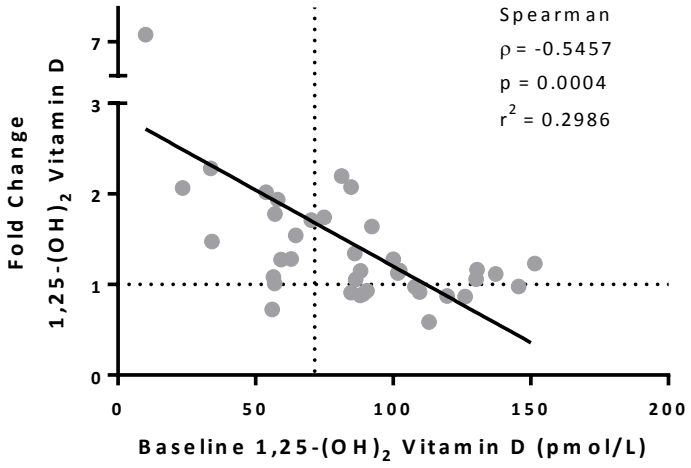


Figure 5.21 Correlation between baseline 1,25-$(OH)_2$ Vitamin D plasma concentration and fold change in 1,25-$(OH)_2$ Vitamin D

n=38. Vertical dotted reference line indicates baseline 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L. Horizontal dotted reference line indicates fold change = 1 – data points below this line decreased post cholecalciferol and data points above the line increased post cholecalciferol

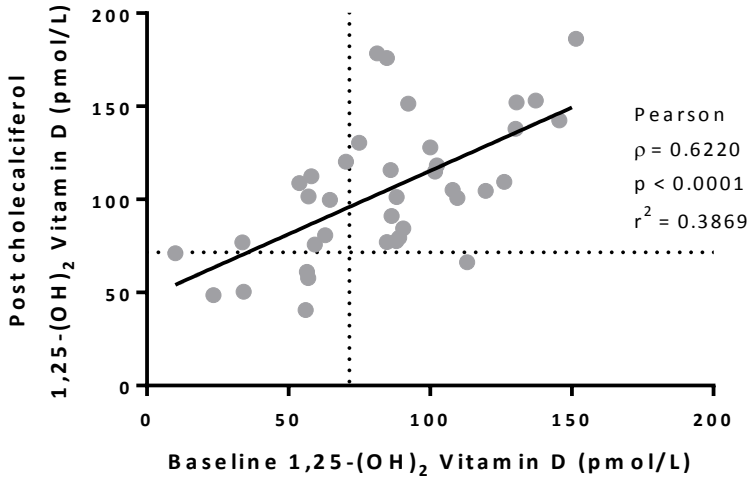


Figure 5.22 Correlation between baseline 1,25-$(OH)_2$ Vitamin D and post cholecalciferol 1,25-$(OH)_2$ Vitamin D

n=38. Dotted reference lines indicate 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L

5.4.9.2 Effects of demographics on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

There was no significant difference between sexes in fold change (p=0.2791) or post cholecalciferol 1,25-$(OH)_2$ Vitamin D (p=0.9816). There was no significant correlation between age and fold change in 1,25-$(OH)_2$ Vitamin D (figure 5.23) with no significant correlation between age and post cholecalciferol 1,25-$(OH)_2$ Vitamin D plasma concentration (Pearson ρ=-0.1508, p=0.3662, r^2=0.0227).

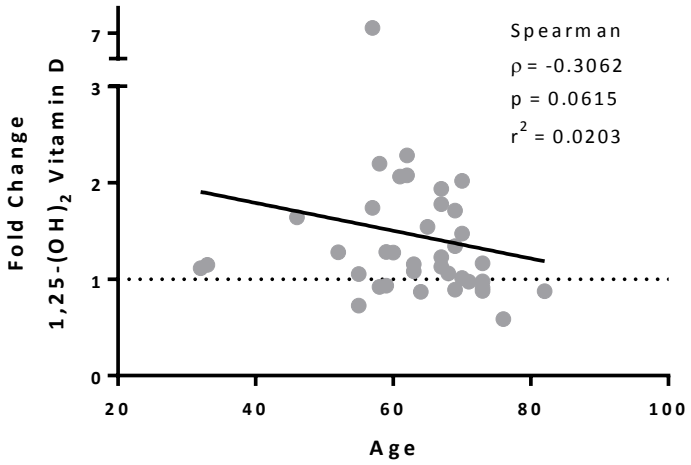


Figure 5.23 Correlation between age and fold change in 1,25-$(OH)_2$ Vitamin D plasma concentrations

n=38. Dotted reference line indicates fold change = 1 – data points below this line decreased post randomisation and data points above the line increased post randomisation

There was no correlation between BMI and fold change (Spearman ρ=-0.0008, p=0.9964, r^2=0.0396) or post cholecalciferol plasma concentrations (Pearson ρ=-0.0725, p=0.6652, r^2=0.0053).

5.4.9.3 Effect of time between dose and operation on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

There was no significant correlation between the number of days between blood sampling and fold change (Spearman ρ=-0.1547, p=0.3538, r^2=0.0089) or post cholecalciferol 1,25-$(OH)_2$ Vitamin D plasma concentrations (Pearson ρ=-0.0299, p=0.8584, r^2=0.0009, figure 5.24).

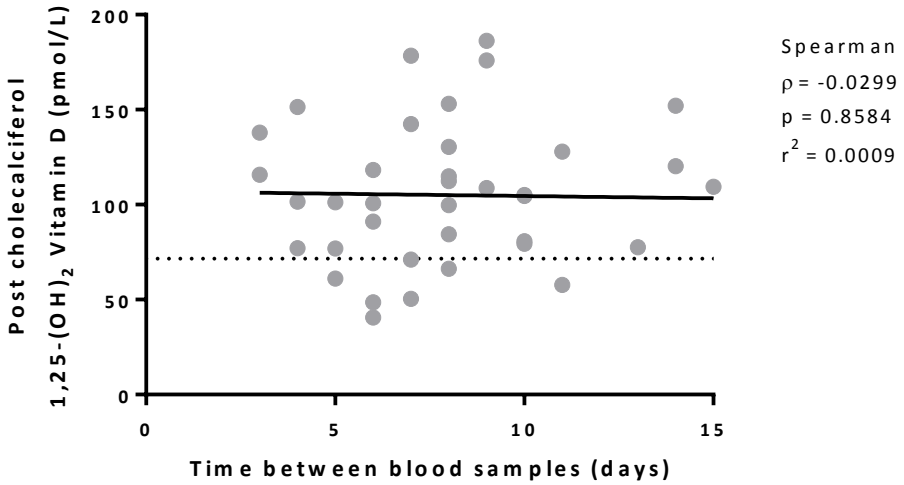


Figure 5.24 Correlation between days between dose and operation and 1,25-$(OH)_2$ Vitamin D response to cholecalciferol.

n=38. One outlier (29 days) is not shown for illustrative purposes but was included in the analysis. Dotted reference line indicates 1,25-OH_2 Vitamin D = 71.5pmol/L

5.4.9.4 Effect of Season on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

The season of year when the patient was administered cholecalciferol did not affect the fold change in 1,25-$(OH)_2$ Vitamin D plasma concentrations (p=0.4654) or post cholecalciferol 1,25-$(OH)_2$ Vitamin D plasma concentration (p=0.2285). There was also no significant difference in fold change (p=0.4925) or post

cholecalciferol 1,25-$(OH)_2$ Vitamin D plasma concentrations (p=0.9820) for patients recruited between May and September compared with October to April.

5.4.9.5 Effect of liver function on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

There was no correlation between baseline bilirubin and fold change in 1,25-$(OH)_2$ Vitamin D (Spearman ρ=0.0450, p=0.7945, r^2=0.0101) or post cholecalciferol 1,25-$(OH)_2$ Vitamin D (Pearson ρ=0.0443, p=0.7974, r^2=0.0020).

5.4.9.6 Effect of renal function on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

There was no correlation between eGFR and fold change in 1,25-$(OH)_2$ Vitamin D plasma concentrations (Spearman ρ=-0.0824, p=0.6229, r^2=0.0572) but baseline and post cholecalciferol 1,25-$(OH)_2$ Vitamin D did correlate with renal function (baseline Pearson ρ=0.3530, p=0.0297, r^2=0.1246, post cholecalciferol Pearson ρ=0.3340, p=0.0404, r^2=0.1116, figure 5.25).

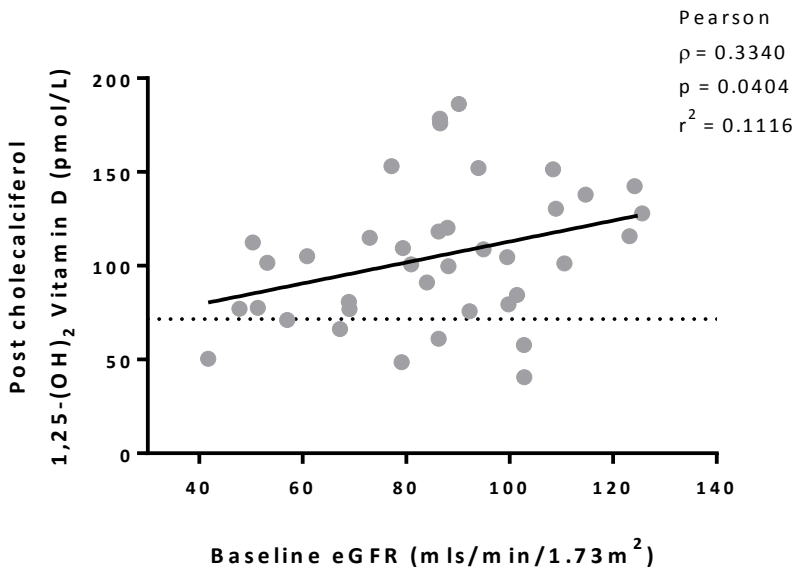


Figure 5.25 Correlation between renal function and post cholecalciferol 1,25-$(OH)_2$ Vitamin D Plasma concentrations

n=38. Dotted reference line indicates 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L

5.4.9.7 Effect of baseline Vitamin D Binding protein plasma concentrations on 1,25-$(OH)_2$ Vitamin D response to cholecalciferol

There was no correlation between baseline Vitamin D Binding Protein and fold change (Spearman ρ=0.1896, p=0.2542, r^2=0.0773) or post cholecalciferol 1,25-$(OH)_2$ Vitamin D (Pearson ρ=-0.0568, p=0.7349, r^2=0.0032).

5.4.9.8 Linear regression analysis of post cholecalciferol 1,25-$(OH)_2$ Vitamin D plasma concentrations in supplemented patients

The above variables were examined using a multiple regression model. There was no evidence of collinearity between the factors examined. In view of the small sample size, a forward stepwise regression was used to examine the data.

This produced a significant model ($F(1,36) = 22.720$ $p<0.001$, R square = 0.37) which demonstrated that, of the examined variables, baseline total 1,25-$(OH)_2$ Vitamin D level was the only significant predictor of post randomisation total 1,25-$(OH)_2$ Vitamin D level in patients who received cholecalciferol ($p<0.001$). Residuals were checked for normality by visual inspection of the Q/Q plot and found to be acceptable.

Table 5.4 Forward Stepwise Linear regression analysis of predictors of post cholecalciferol 1,25-OH_2 Vitamin D in patients who received cholecalciferol

Variable	Univariate p value	Unstandardised Coefficient		Standardised Coefficient (β)	P value
		B	Std. Error		
Baseline 1,25-OH_2 Vitamin D	**<0.0001**	**0.680**	**0.143**	**0.622**	**<0.001**

5.4.10 Effects of cholecalciferol on post-operative plasma 25-OH Vitamin D plasma concentrations

In order to examine perioperative changes in Vitamin D plasma concentrations I measured Vitamin D plasma concentrations in plasma from 52 of the patients on day 3 or 4 post op. This timepoint was chosen for practical reasons as the majority, if not all, patients would be expected to still be receiving inpatient post-operative care at this point. Postop 25-OH Vitamin D_2 was only detectable in 6 of the 52 patients investigated (2 received placebo, 4 received cholecalciferol). Average fold change in 25-OH Vitamin D_2 plasma concentrations in these 6 patients was 0.66. 17 of the 52 patients had had detectable plasma

concentrations of 25-OH Vitamin D_2 preop, therefore it had fallen to undetectable plasma concentrations in 11 patients (64.7%).

The very low plasma concentrations of 25-OH vitamin D_2 meant that results for Vitamin D_3 and total vitamin D were very similar. There was a significant perioperative fall in total 25-OH Vitamin D plasma concentrations in both groups. Patients who received placebo had significantly lower circulating plasma concentrations of total 25-OH Vitamin D post op than at baseline but patients who received cholecalciferol had no significant difference between post op plasma concentrations than at baseline, although the trend was towards higher post op plasma concentrations (p=0.0918). Patients who received cholecalciferol had higher plasma concentrations of total 25-OH Vitamin D on day 3/4 post op compared to patients who received placebo (figure 5.26).

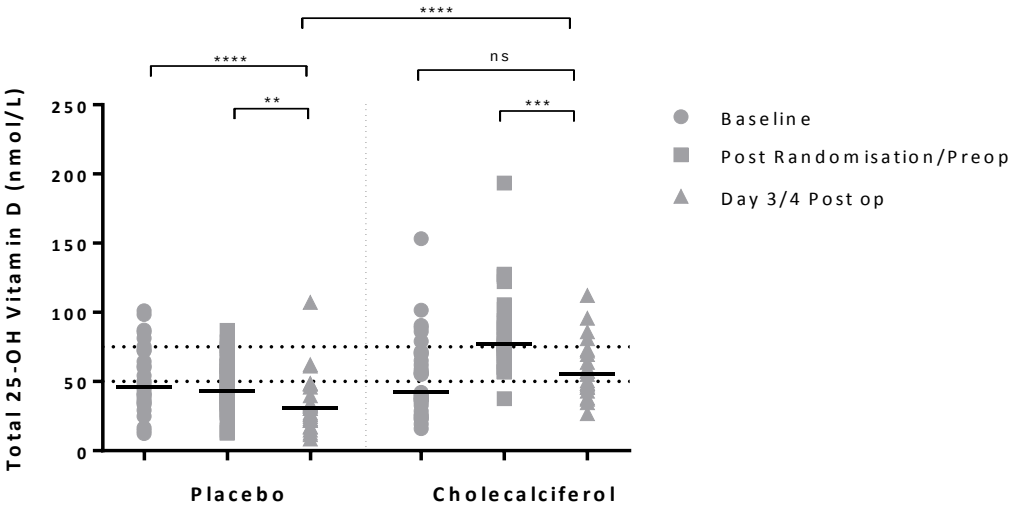


Figure 5.26 Perioperative Changes in total 25-OH Vitamin D plasma concentrations in patients randomised to placebo or cholecalciferol

n=38 for both preop datasets, n=29 for post op placebo dataset and n=23 for post op cholecalciferol dataset. Black horizontal lines indicate median value for each dataset. Dotted horizontal reference lines indicate 25-OH Vitamin D plasma concentrations 50nmol/L and 75nmol/L. Friedman test for both placebo and cholecalciferol groups p<0.0001, with mulitple comparisons within the groups as indicated. Mann Whitney test was used to compare data between groups.

3 (10%) of patients in the placebo group had 25-OH Vitamin D plasma concentrations >50nmol/L on day 3/4 post op compared with 13 (57%) in the cholecalciferol group (p=0.0006).

5.4.11 Effects of cholecalciferol on post-operative plasma 1,25-$(OH)_2$ Vitamin D plasma concentrations

While there was a significant perioperative fall in plasma concentrations of 1,25-$(OH)_2$ Vitamin D in the group that received placebo (p=0.0055), there was no significant change in patients who received active drug (p=0.7210). Plasma concentrations of 1,25-$(OH)_2$ Vitamin D fell significantly between baseline and

day 3/4 postop in the placebo group but there was no significant change compared to baseline in the group that received cholecalciferol (p=0.0921), with a trend towards plasma concentrations being higher. There was a significant difference in plasma concentrations of 1,25-$(OH)_2$ Vitamin D on day 3/4 (figure 5.27).

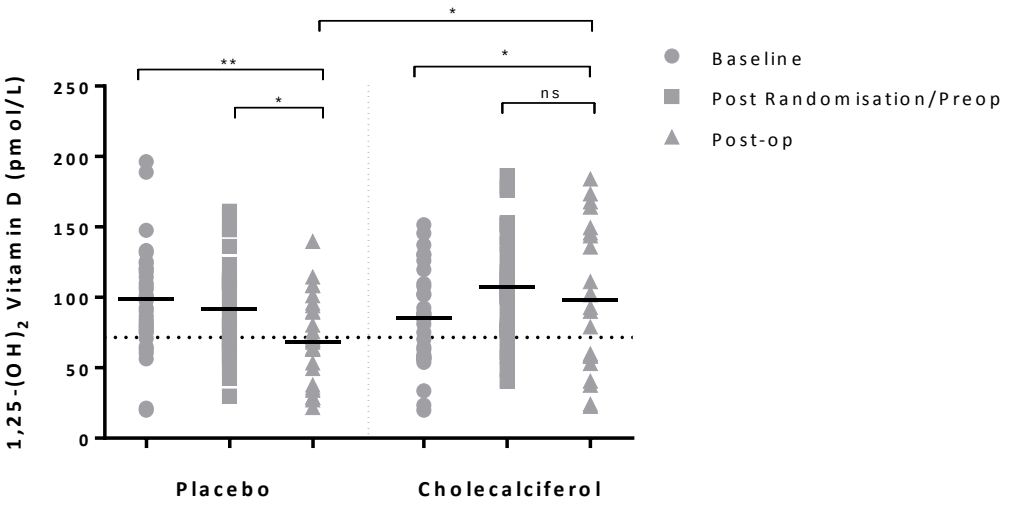


Figure 5.27 Perioperative changes in 1,25-$(OH)_2$ Vitamin D plasma concentrations in patients randomised to placebo or cholecalciferol

n=38 for both preop datasets, n=29 for post op placebo dataset and n=23 for post op cholecalciferol dataset. Black horizontal lines indicate median value for each dataset. Dotted reference line indicates 1,25-$(OH)_2$ Vitamin D = 71.5pmol/L. RM one way ANOVA for placebo p= 0.0010, RM one way ANOVA for cholecalciferol p=0.0018 with multiple comparisons within groups as indicated. Unpaired t test was used to compare data between groups.

5.5 Discussion

Supplementation with a one off dose of 300,000IU oral cholecalciferol 3-14 days before planned oesophagectomy was a safe and effective method of increasing pre-operative plasma concentrations of circulating 25-OH Vitamin D in our

patients. There were no clinically significant increases in serum calcium in the patients who received cholecalciferol and no SUSARs during the trial. The low circulating plasma concentrations of 25-OH Vitamin D_2 in this cohort of patients makes the effects of cholecalciferol on Vitamin D_2 plasma concentrations hard to assess but we did not find any evidence of a significant effect. Total 25-OH Vitamin D plasma concentrations were significantly higher in patients who received cholecalciferol with 95% of patients having plasma concentrations greater than 75nmol/L compared with 42% of patients who received placebo.

Cholecalciferol administration also resulted in significant increases in 1,25-$(OH)_2$ Vitamin D plasma concentrations, although post randomisation absolute plasma concentrations were not significantly different between groups. Post randomisation plasma concentrations of 1,25-$(OH)_2$ Vitamin D were similar in the VINDALOO cohort were similar to preoperative plasma concentrations seen in the BALTI-prevention cohort described in chapter 4. This is in contrast to 25-OH Vitamin D plasma concentrations where VINDALOO patients had significantly higher plasma concentrations than BALTIp patients.

The majority of 25-OH Vitamin D circulates bound to Vitamin D Binding Protein, but unbound (or “free”) and albumin bound fractions also circulate in the plasma. The plasma concentration of circulating free 25-OH Vitamin D can be calculated using the following formula:

$$\text{Free 25OHD} = \frac{\text{total 25OHD}}{1 + (6 \times 10^3 \times \text{ALB}) + (7 \times 10^8 \times \text{DBP})} \quad (164)$$

25OHD = 25-OH Vitamin D

DBP = Vitamin D Binding Protein

ALB = Serum Albumin

As free 25-OH Vitamin D is inversely proportional to circulating concentrations of Vitamin D binding protein and cholecalciferol administration did not have any significant short term effect on circulating plasma concentrations of Vitamin D Binding Protein, patients who received Vitamin D are likely to have had substantial increases in free 25-OH Vitamin D. This may be an important finding as while renal cells express the endocyclic receptor megalin and are able to process 25-OH Vitamin D whilst bound to Vitamin D binding protein (165), most extra-renal tissues do not express megalin (166). Indeed, monocytes cultured in serum from Vitamin D binding protein knockout mice generate greater quantities of cathelicidin than controls and cathelicidin production under these conditions can be suppressed by addition of increasing quantities of Vitamin D Binding Protein (167). If high dose cholecalciferol administration results in substantial increases in the free component of circulating 25-OH Vitamin D, this may significantly enhance its immune effects, without this necessarily being reflected by circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations.

Increases in Vitamin D plasma concentrations were greatest in patients with lower initial Vitamin D plasma concentrations although patients with higher baseline plasma concentrations of vitamin D also had the highest post

randomisation plasma concentrations. In addition to baseline Vitamin D plasma concentrations, the linear regression analysis suggested that age and renal function significantly impacted on total 25-OH Vitamin D plasma concentrations post cholecalciferol. However, we did not find any factors that had a significant impact on 1,25-$(OH)_2$ Vitamin D other than baseline plasma concentrations. It is interesting that renal function negatively correlated with 25-OH Vitamin D plasma concentrations but not 1,25-$(OH)_2$ Vitamin D plasma concentrations as classically the kidney produces the majority of circulating 1,25-$(OH)_2$ Vitamin D (48). One possible explanation for this is if increased production of 1,25-$(OH)_2$ Vitamin D in the kidney leads to increased onward metabolism and excretion, but in patients with lower glomerular filtration rates less 25-OH Vitamin D is hydroxylated to form 1,25-$(OH)_2$ Vitamin D, resulting in a negative correlation. None of the patients recruited to the trial had a history of renal failure, and this finding would suggest that use of high doses of cholecalciferol in patients with renal failure could have a greater impact on 25-OH Vitamin D plasma concentrations than in patients with normal renal function, which may have implications for future studies.

It is likely that genetic variations also impact response to cholecalciferol and one weakness of this data is that patient numbers were not high enough to adequately investigate that variable. In particular, for 1,25-$(OH)_2$ Vitamin D the variables examined did not explain a lot of the variance seen, suggesting that other factors are likely to be significantly contributing. A number of studies have reported links between single nucleotide polymorphisms (SNPs) in Vitamin D

pathway genes and vitamin D metabolite concentrations. The majority of these relate to DBP, the gene encoding Vitamin D binding protein, although polymorhisms in CYP2R1 which is involved in hydroxylation of Vitamin D to 25-OH Vitamin D have also been reported to affect plasma concentrations. A recent review article found reports linking a total of 35 SNPs in 7 different genes to Vitamin D concentrations (168). However, a recent study in 220 older adults in the UK which investigated the impact of 15 SNPs in six genes did not find that they made any impact after adjusting for multiple comparisons (169).

Post-op, 25-OH Vitamin D plasma concentrations significantly fell in both groups compared to preoperative plasma concentrations. While plasma concentrations returned to baseline in patients who received cholecalciferol, in the placebo group plasma concentrations were significantly below baseline with only 10% of patients having 25-OH Vitamin D plasma concentrations greater than 50nmol/L. Perioperative fold change in 25-OH Vitamin D plasma concentrations was similar in both groups suggesting that the fall relates to the operation rather than the cholecalciferol administration. Intake of Vitamin D (via the skin or gut) will naturally be low during the perioperative period as patients typically have no nutritional intake on the day of the operation and feeding only slowly increases over the course of the first post-operative week as the gut heals. Oesophagectomy elicits a catabolic metabolic response characterised by lipolysis and protein breakdown (170) which theorectically could lead to increased release of Vitamin D stored in fat cells, but I have not found any evidence that this occurs. Patients often receive large volumes of intravenous fluids over the

perioperative period leading to plasma dilution. Serum total protein and albumin concentrations also fall during the perioperative period, supporting dilution as a potential factor. A further potential cause of perioperative falls in 25-OH Vitamin D is increased metabolism during the perioperative period. Patients in the placebo group also saw similar falls in circulating 1,25-$(OH)_2$ Vitamin D, but plasma concentrations did not change perioperatively in patients who had received cholecalciferol. This could indicate that patients in the placebo group have insufficient circulating 25-OH Vitamin D to maintain plasma concentrations of 1,25-$(OH)_2$ vitamin D in the perioperative period, whereas patients who received cholecalciferol supplementation are better able to maintain circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations post op.

CHAPTER 6

EFFECTS OF VITAMIN D SUPPLEMENTATION ON ALVEOLAR OEDEMA AND SYSTEMIC INFLAMMATION

6.1 Introduction

In chapter 4 I demonstrated that preoperative vitamin D deficiency is common in patients undergoing oesophagectomy and associated with increased risk of post-operative ARDS. I also demonstrated that 25-OH Vitamin D plasma concentrations relate to biomarkers of systemic and alveolar inflammation in the post-operative period. In chapter 5 I demonstrated that patients recruited to the Vindaloo trial who received cholecalciferol had significantly higher preoperative circulating 25-OH vitamin D plasma concentrations than patients who received placebo.

This chapter will consider the effects of high dose cholecalciferol on post-operative markers of systemic and alveolar inflammation. Patients undergoing oesophagectomy experience a marked inflammatory response with cytokines such as IL-1β, IL6, IL-10 and thrombin increasing during the operation while other markers of inflammation such as C reactive protein (CRP) and complement factors such as C3a increase shortly after the operation (171, 172). Marked perioperative neutrophilia and lymphopenia have also been noted in patients undergoing oesophagectomy (172).

Pre and post-operative scoring systems to help predict patients who will suffer increase morbidity and mortality post-op have been developed. A number of preoperative scoring systems consider the ratio between inflammatory cells – the neutrophil lymphocyte ratio, lymphocyte to monocyte ratio and platelet to lymphocyte ratio have all been suggested by various groups (173-177). Other

scores which aim to quantify the level of preoperative inflammation using serum albumin and CRP such as the modified Glasgow prediction score (mGPS) have also been suggested (178-180). However, none of these scoring systems are currently routinely used in clinical practice.

APACHE-II is a commonly used severity scoring system and mortality prediction derived from a large sample of ICU patients in the United States. It is uses 12 physiological measurements, age and previous health status to generate a severity score between 0 and 71 which can be converted to predicted mortality when reason for ICU admission is taken into account (181). The full scoring system is detailed in chapter 3. SOFA scoring aims to quantify the degree of organ dysfunction and morbidity in ICU patients and is based on 6 organ systems. It can be used serially to help track patient's progress during their ICU admission (139).

6.2 Hypothesis and Research Questions

6.2.1 Hypothesis

Supplementation with cholecalciferol reduces perioperative inflammation, resulting in lower perioperative increases in alveolar oedema and circulating cytokines

6.2.2 Research Questions

1. What is the effect of supplementation with cholecalciferol on PICCO biomarkers of alveolar oedema and inflammation?

2. What is the effect of supplementation with cholecalciferol on perioperative changes in circulating cytokines?
3. What is the clinical effect of supplementation with cholecalciferol in patients undergoing oesophagectomy?

6.3 Methods

Patients were recruited to the VINDALOO trial (see section 5.4.2 for consort diagram). Clinical data including development of complications were collected prospectively using a custom designed case report form. Full blood count and CRP were measured by the hospital laboratories as a routine part of clinical care. PICCO measurements were collected perioperatively according to the method detailed in chapter 3. Inflammatory cytokines were measured by Luminex except LL37 which was measured by ELISA. APACHE-II and SOFA score were calculated using data gathered from the clinical notes according to the scoring systems detailed in chapter 3.

Statistical significance was defined as $p<0.05$. Statistical tests used are detailed in section 3.10.2. Continuous data in tables is presented as mean (SD) for parametric data and median (IQR) for non-parametric data. Nominative data is expressed as n (%). Statistical significance in the graphs is denoted using * for $p\leq0.05$, ** for $p\leq0.01$, *** for $p\leq0.001$ and **** for $p\leq0.0001$. Non-significance is denoted using ns.

6.4 Results

6.4.1 Perioperative inflammatory response in patients undergoing oesophagectomy and effects of cholecalciferol

To demonstrate the level of inflammatory response in our cohort, I compared plasma concentrations of circulating white cells pre and post op. The white cell count fell marginally between the preoperative assessment clinic and immediately prior to the operation and then rose post op and remained elevated more than 7 days. There was no difference in WCC between placebo and cholecalciferol groups at any time point (figure 6.1).

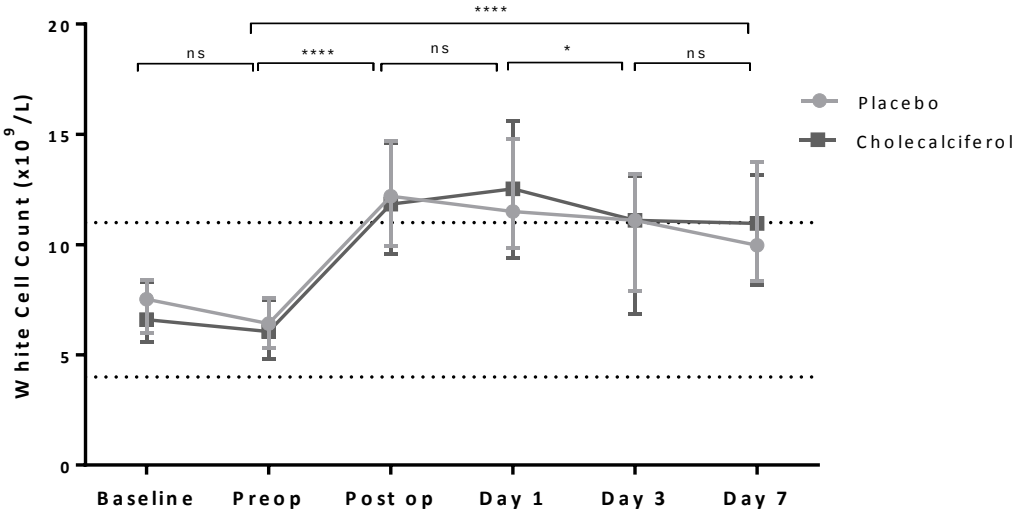


Figure 6.1 Perioperative changes in white cell count in patients undergoing oesophagectomy

Placebo: n=34 (baseline), n=31 (preop), n=32 (post op), n=35 (day 1), n=31 (day 3) and n=29 (day 7). Cholecalciferol: n=33 (baseline), n=29 (preop), n=31 (post op), n=31 (day 1), n=31 (day 3) and n=24 (day 7). Symbol represents median for each group, error bars represent inter quartile range. Dotted reference lines indicate the normal range for white cell count (4.0-11.0x10^9/L). There was no significant difference between groups at any timepoint. Friedman test for combined data p<0.0001 with multiple comparisons using Dunn's test used to compare individual timepoints as shown on graph. P values shown on graph indicate differences between combined data.

In the immediate preoperative period, the number of circulating neutrophils and eosinophils did not significantly change while the number of circulating lymphocytes and monocytes significantly decreased. Perioperatively, the number of circulating neutrophils and monocytes significantly increased while the number of lymphocytes and eosinophils significantly decreased, with eosinophils being undetectable in all bar 7 cases at the post op time point. Numbers of circulating neutrophils and monocytes remained elevated 1 week post op and numbers of circulating lymphocytes remained depressed 1 week post op, but

numbers of circulating eosinophils were significantly higher on day 7 post op than they were preop. There were no differences between the placebo and cholecalciferol groups in the differential white cell count (figure 6.2).

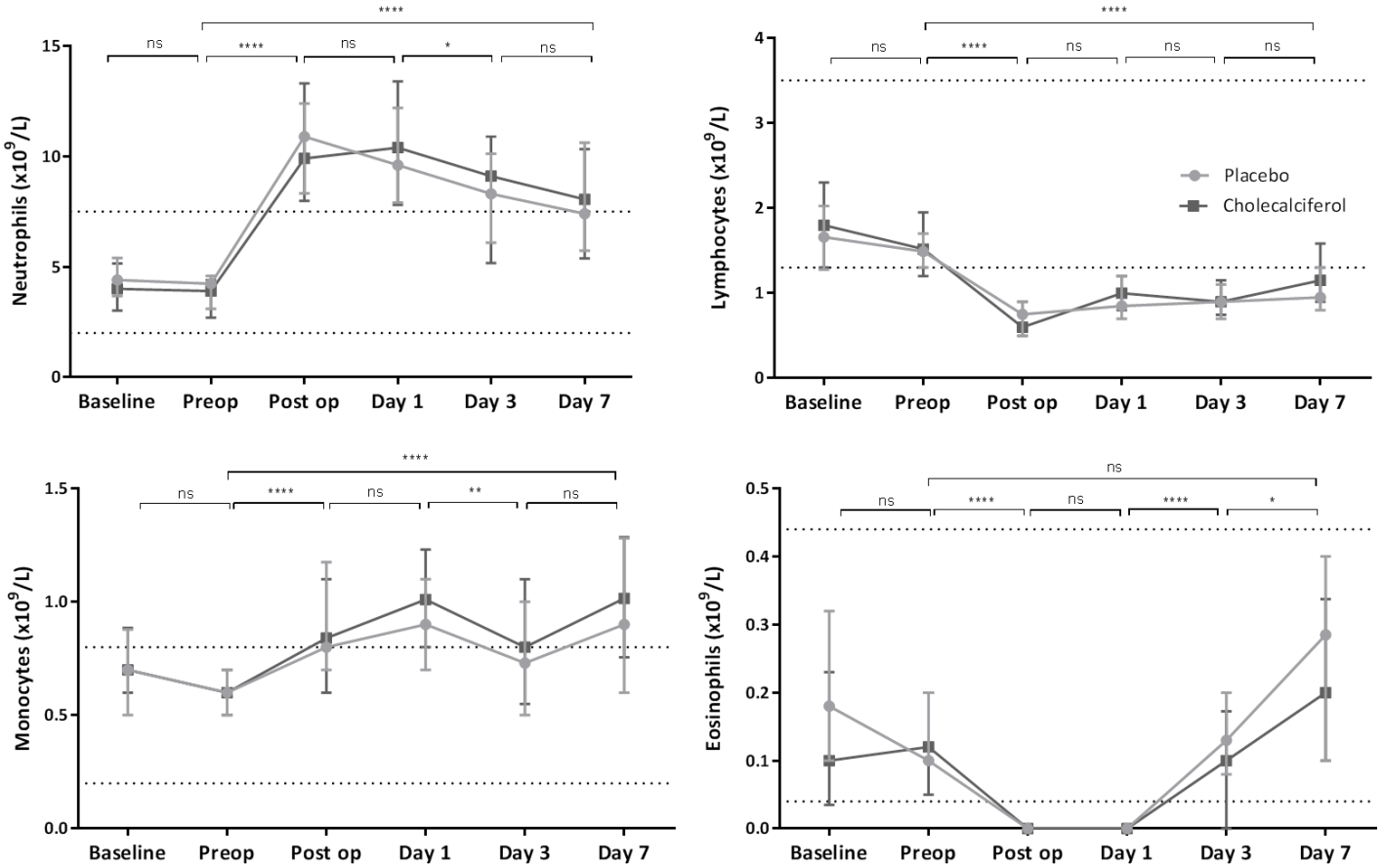


Figure 6.2 Effects of cholecalciferol on perioperative neutrophil, lymphocyte, monocyte and eosinophil counts

Placebo: n=34 (baseline), n=31 (preop), n=32 (post op), n=35 (day 1), n=31 (day 3) and n=29 (day 7). Cholecalciferol: n=33 (baseline), n=29 (preop), n=31 (post op), n=31 (day 1), n=31 (day 3) and n=24 (day 7). Symbols represent median for each group with interquartile range represented by error bars. Dotted reference lines represent normal range for each parameter. There was no significant difference between groups at any timepoint for any of the 4 cell types. Friedman test for combined data p<0.0001 for each cell type with multiple comparisons using Dunn's test used to compare individual timepoints as shown on graph between combined data.

In comparison, circulating levels of CRP, a generalised marker of inflammation showed a delayed response to oesophagectomy, with levels frequently undetectable immediately post op (27%), but then increasing rapidly in the immediate post-operative period and peaking on day 3 post op. Again, there was no difference in CRP between placebo and cholecalciferol groups at any timepoint (figure 6.3).

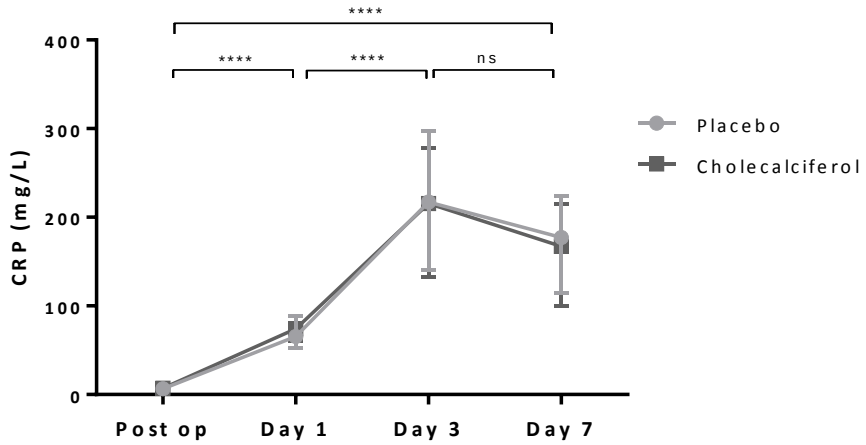


Figure 6.3 Post-operative changes in post-operative CRP in patients undergoing oesophagectomy

Placebo: n=28 (post op), n=26 (day 1), n=33 (day 3), n=19 (day 7). Cholecalciferol: n=20 (post op), n=19 (day 1), n=24 (day 3), n=14 (day 7). Symbol represents median for each group, error bars represent inter quartile range. Where plasma concentrations were undetectable (<3mg/L) a value of 2.9mg/L has been used for analysis. Kruskal Wallis p<0.0001 for combined data.

6.4.2 Effects of Cholecalciferol on pre-operative blood cell ratios

There were no significant differences between the cholecalciferol and placebo groups in preoperative neutrophil:lymphocyte ratio (placebo median = 2.7 IQR 1.9-3.4, cholecalciferol median = 2.3 IQR = 1.7-3.6, p=0.4380),

lymphocyte:monocyte ratio (placebo median = 2.4 IQR = 1.7-3.4, cholecalciferol median = 2.8 IQR = 2.0-3.7, p=0.5012) or platelet:lymphocyte ratio (placebo median = 153 IQR = 108-184, cholecalciferol median = 130 IQR = 95-187, p=0.4920).

6.4.3 Effects of cholecalciferol on perioperative changes in cytokine plasma concentrations

To investigate the effect of cholecalciferol on levels of systemic inflammation prior to oesophagectomy, I measured levels of cytokines at baseline and preop. The analytes were chosen because they were identified during analysis of the BALTI-prevention trial as significantly changing over the course of oesophagectomy, or because they have previously been shown to be affected by Vitamin D. There was a significant difference in levels of IL-1β at baseline, but no significant difference in levels of other cytokines (table 6.1).

Table 6.1 Comparison of Cytokine Plasma concentrations at Baseline

Cytokine	Baseline plasma concentration (pg/ml)		p-value
	Placebo (n=38)	Cholecalciferol (n=38)	
IL6	11.00 (9.00-13.13)	11.50 (9.50-15.06)	0.4510
IL8	55.88 (45.81-83.63)	57.50 (46.38-69.13)	0.7942
IL10	15.50 (14.00-16.50)	15.50 (14.50-17.00)	0.6165
TNF-α	13.25 (10.94-14.50)	12.50 (10.50-14.06)	0.5958
s-RAGE	48.50 (36.88-58.00)	44.50 (37.88-66.94)	0.9239
s-TNF-R1	468.5 (269.8-617.6)	447.4 (309.0-970.0)	0.4518
s-TNF-R2	2758 (2287-4521)	3316 (2245-4965)	0.4965
LL37*	5.06 (1.81-12.32)	4.96 (1.46-10.05)	0.4572
IL2	13.3 (11.8-15.1)	13.5 (12.0-15.1)	0.990
IL1ra	479.0 (401.8-621.5)	483.1 (375.3-623.7)	0.944
IL1β	8.0 (6.5-9.5)	7.1 (5.9-8.5)	0.046
IL17	13.9 (12.4-15.3)	13.5 (13.0-15.3)	0.961

**For LL37 placebo n=34 and cholecalciferol n=32 due to no EDTA plasma being available for the missing cases*

Plasma concentrations of the majority of cytokines fell slightly between baseline and preoperative measurements but there was no difference between groups in fold change of any of the cytokines measured. A significant difference in plasma concentrations of IL-1β remained, but there was still no difference in plasma concentrations of other cytokines (table 6.2).

Table 6.2 Preoperative differences in absolute cytokine plasma concentrations and fold change (preop/baseline)

Cytokine	Preoperative Concentration (pg/ml)			Fold change (Preop/Baseline)		
	Placebo (n=38)	Cholecalciferol (n=38)	p-value	Placebo (n=38)	Cholecalciferol (n=38)	p-value
IL6	10.25 (8.50-11.88)	9.88 (8.50-12.50)	0.7739	0.94 (0.80-1.06)	0.87 (0.69-0.98)	0.2451
IL8	48.63 (35.88-58.63)	46.00 (38.06-53.50)	0.3217	0.81 (0.65-0.98)	0.76 (0.69-0.90)	0.5572
IL10	14.88 (14.00-16.50)	14.88 (14.00-17.25)	0.4286	0.97 0.90-1.04)	0.97 (0.92-1.03)	0.8748
TNF-α	12.50 (11.00-14.13)	12.50 (11.00-13.50)	0.4988	0.97 (0.92-1.08)	0.96 (0.89-1.04)	0.5853
s-RAGE	43.25 (36.38-61.44)	42.00 (33.50-52.63)	0.5892	0.97 (0.83-1.06)	0.90 (0.77-1.02)	0.2580
s-TNF-R1	396.1 (260.1-596.6)	414.4 (315.0-612.8)	0.4273	0.84 (0.77-1.06)	0.82 (0.67-1.10)	0.7896
s-TNF-R2	2365 (2003-3677)	2865 (2211-4340)	0.2559	0.85 (0.80-1.00)	0.93 (0.79-1.09)	0.2737
LL37	3.91 (1.80-13.50)	5.58 (2.11-11.08)	0.9156	1.12 (0.66-1.31)	1.02 (0.68-1.43)	0.9797
IL2	12.63 (11.38-14.75)	13.00 (11.94-14.06)	0.6103	0.92 (0.88-1.00)	0.97 (0.90-1.04)	0.1979
IL-1ra	462.3 (321.4-666.1)	432.8 (332.9-617.9)	0.7626	0.98 (0.76-1.26)	0.90 (0.78-1.10)	0.4153
IL-1β	**7.75 (7.00-10.00)**	**7.13 (6.00-8.00)**	**0.0073**	1.00 (0.93-1.10)	1.00 (0.92-1.05)	0.1800
IL-17	13.50 (12.69-14.63)	13.50 (12.50-14.63)	0.9938	0.96 (0.93-1.04)	0.97 (0.95-1.00)	0.7977

**For LL37 placebo n=35 and cholecalciferol n=32 due to no EDTA plasma being available for the missing cases*

Perioperatively, plasma concentrations of most cytokines increased, although little change in IL-2, IL-17 and LL37 was seen. Fold change in plasma concentrations of IL-8, TNF-α and IL-1β was greater in the cholecalciferol group (table 6.3).

Table 6.3 Perioperative differences in absolute cytokine plasma concentrations and fold change (postop/preop)

Cytokine	Post-operative Concentration (pg/ml)			Fold change (Post-op/Preop)		
	Placebo (n=34)	Cholecalciferol (n=31)	p-value	Placebo (n=34)	Cholecalciferol (n=31)	p-value
IL6	296.4 (188.5-488.5)	546.3 (202.8-920.0)	0.0527	27.62 (21.85-43.45)	45.52 (21.73-87.16)	0.0537
IL8	82.8 (59.9-134.7)	102.0 (75.3-156.0)	0.1659	**1.88 (1.38-2.58)**	**2.30 (1.76-3.76)**	**0.0331**
IL10	60.13 (31.3-107.1)	43.0 (31.0-76.0)	0.3690	3.81 (1.94-6.83)	2.59 (1.90-5.22)	0.2940
TNF-α	10.5 (9.5-11.5)	11.0 (10.0 12.5)	0.0959	**0.85 (0.80-0.95)**	**0.92 (0.85-1.05)**	**0.0140**
s-RAGE	51.8 (38.1-86.8)	51.0 (37.8-67.4)	0.8409	1.15 (0.92-1.45)	1.26 (1.08-1.57)	0.1682
s-TNF-R1	806.6 (520.4-1302)	1146 (653.1-1440)	0.1565	2.14 (1.42-3.16)	2.40 (1.41-3.25)	0.7422
s-TNF-R2	3112 (2429-4697)	4140 (3122-5510)	0.0964	1.24 (1.00-1.52)	1.29 (1.03-1.43)	0.5767
LL37*	5.71 (2.45-7.98)	4.61 (2.30-11.58)	0.9336	0.99 (0.62-1.67)	1.02 (0.75-1.48)	0.9029
IL2	13.0 (11.4-13.8)	13.5 (12.0-15.0)	0.1547	1.01 (0.96-1.05)	1.06 (0.97-1.08)	0.1591
IL-1ra	2812 (1573-4766)	4256 (2404-5431)	0.1662	6.49 (2.97-13.39)	10.35 (4.03-12.17)	0.3622
IL-1β	9.3 (8.0-10.5)	9.0 (7.5-10.5)	0.7812	**1.12 (0.99-1.34)**	**1.28 (1.17-1.62)**	**0.0066**
IL-17	13.0 (12.0-14.0)	13.5 (13.0-14.0)	0.2703	0.96 (0.93-1.01)	0.96 (0.93-1.03)	0.6689

**For LL37 placebo n=31 and cholecalciferol n=26 due to no EDTA plasma being available for the missing cases*

By day 1 post op plasma concentrations of inflammatory cytokines were generally falling again, with no difference seen in plasma concentrations of most of the inflammatory cytokines. However, patients who received cholecalciferol had significantly higher plasma concentrations of TNFR1 and TNFR2 on day 1 post op. There was a trend towards greater fold change in LL37 in the patients who received placebo, but no difference in absolute plasma concentrations (table 6.4).

Table 6.4 Post-operative differences in absolute cytokine plasma concentrations and fold change (day 1/post-op)

Cytokine	Day 1 Concentration (pg/ml)			Fold change (Day 1/Post-op)		
	Placebo (n=33)	Cholecalciferol (n=33)	p-value (Mann Whitney)	Placebo (n=33)	Cholecalciferol (n=31)	p-value (Mann Whitney)
IL6	240.5 (133.8-444.4)	249.0 (153.4-451.4)	0.8860	0.90 (0.46-1.32)	0.53 (0.29-1.00)	0.1010
IL8	81.5 (68.0-121.0)	85.0 (68.5-151.6)	0.9568	1.04 (0.78-1.31)	1.01 (0.52-1.30)	0.2954
IL10	27.0 (21.0-36.8)	26.5 (23.5-37.0)	0.7188	0.59 (0.33)	0.71 (0.42)	0.2267
TNF-α	11.5 (10.5-13.4)	12.0 (10.5-13.0)	0.4145	1.06 (0.99-1.20)	1.09 (1.00-1.15)	0.6190
s-RAGE	35.3 (28.8-44.3)	40.0 (30.8-72.1)	0.0774	0.71 (0.22)	0.78 (0.22)	0.1779
s-TNF-R1	**716.1 (447.6-1157)**	**886.0 (606.8-1844)**	**0.0279**	0.93 (0.62-1.19)	1.07 (0.62-1.46)	0.2956
s-TNF-R2	**3666 (2402-4828)**	**5130 (3607-6865)**	**0.0112**	1.07 (0.89-1.27)	1.18 (0.96-1.52)	0.2715
LL37*	14.63 (5.17-23.03)	9.30 (3.56-15.83)	0.4487	2.12 (1.51-4.21)	1.50 (0.96-2.55)	0.0576
IL2	13.0 (12.0-14.4)	13.3 (12.0-14.5)	0.7962	1.03 (0.96-1.11)	1.00 (0.92-1.04)	0.0687
IL-1ra	1266 (854-1651)	1255 (1023-22.16)	0.4889	0.49 (0.31-0.67)	0.35 (0.23-0.58)	0.2541
IL-1β	8.5 (7.8-10.0)	8.0 (7.0-10.0)	0.2319	1.00 (0.87-1.05)	0.85 (0.73-1.07)	0.1109
IL-17	13.0 (12.3-14.5)	13.5 (12.7-15.0)	0.3932	1.00 (0.98-1.06)	1.03 (0.96-1.08)	0.9867

**For LL37 placebo n=28 and cholecalciferol n=24 due to no EDTA plasma being available for the missing cases*

Between D1 and D3 post op, there was little change in plasma concentrations of many cytokines. Plasma concentrations of LL37 increased significantly in both groups and fold change was greater in the group who received cholecalciferol although absolute plasma concentrations on day 3 were not significantly different. For IL2, there was a significant difference in fold change between the two groups, although the absolute concentrations did not differ significantly (table 6.5).

Table 6.5 Post-operative differences in absolute cytokine plasma concentrations and fold change (day 3/day 1)

Cytokine	Day 3 Concentration (pg/ml)			Fold change (Day 3/Day 1)		
	Placebo (n=28)	Cholecalciferol (n=25)	p-value (Mann Whitney)	Placebo (n=28)	Cholecalciferol (n=25)	p-value (Mann Whitney)
IL6	124.5 (68.1-329.3)	162.0 (127.5-360.4)	0.2473	0.54 (0.30-1.06)	0.69 (0.28-1.20)	0.8487
IL8	92.6 (64.7-135.8)	88.0 (71.5-172.8)	0.3881	0.91 (0.68-1.39)	1.06 (0.90-1.47)	0.1972
IL10	17.8 (16.0-22.3)	20.0(16.3-24.5)	0.3496	0.72 (0.55-0.87)	0.65 (0.55-0.87)	0.6353
TNFa	12.0 (10.6-13.5)	13.0 (11.3-17.4)	0.1638	1.05 (0.96-1.18)	1.13 (0.98-1.36)	0.4178
s-RAGE*	36.5 (33.0-52.0)	35.8 (28.1-45.5)	0.3270	1.10 (0.85-1.19)	0.87 (0.70-1.04)	0.0705
s-TNF-R1*	1044 (518.3-1689)	958.3 (617.1-1531)	0.8546	1.12 (0.87-1.34)	0.99 (0.72-1.54)	0.3224
s-TNF-R2*	5043 (3391-7505)	5000 (4134-7627)	0.3418	1.20 (0.95-1.64)	1.13 (0.91-1.49)	0.6156
LL37**	12.54 (5.37-41.99)	24.08 (9.91-43.88)	0.4472	**1.76 (0.98-2.41)**	**2.11 (1.73-4.59)**	**0.0269**
IL2	13.3 (12.3-14.9)	13.5 (12.4-17.3)	0.2971	**0.98 (0.90-1.05)**	**1.04 (0.99-1.12)**	**0.0391**
IL-1ra	1038 (746-1402)	1486 (890-2171)	0.0633	0.81 (0.67-1.13)	1.12 (0.61-1.56)	0.3237
IL-1B	9.3 (8.0-11.5)	8.8 (7.6-12.5)	0.6395	1.06 (0.96-1.20)	1.09 (0.91-1.32)	0.8390
IL-17	13.8 (12.5-14.5)	14.5 (13.3-16.0)	0.2954	1.04 (0.99-1.08)	1.05 (0.98-1.16)	0.3103

**n=27 for placebo and n=24 for cholecalciferol as there was insufficient plasma to carry out these tests for 2 patients*

***n=23 for placebo and n=18 for cholecalciferol as EDTA plasma was not available for all patients*

6.4.4 Effects of cholecalciferol on PICCO biomarkers of alveolar oedema and inflammation

While post-operative EVLWI was significantly higher than preoperative values in patients who received placebo, there was no significant perioperative difference in EVLWI in patients who received cholecalciferol. However this did not translate into a significant difference in absolute post-operative EVLWI (figure 6.4).

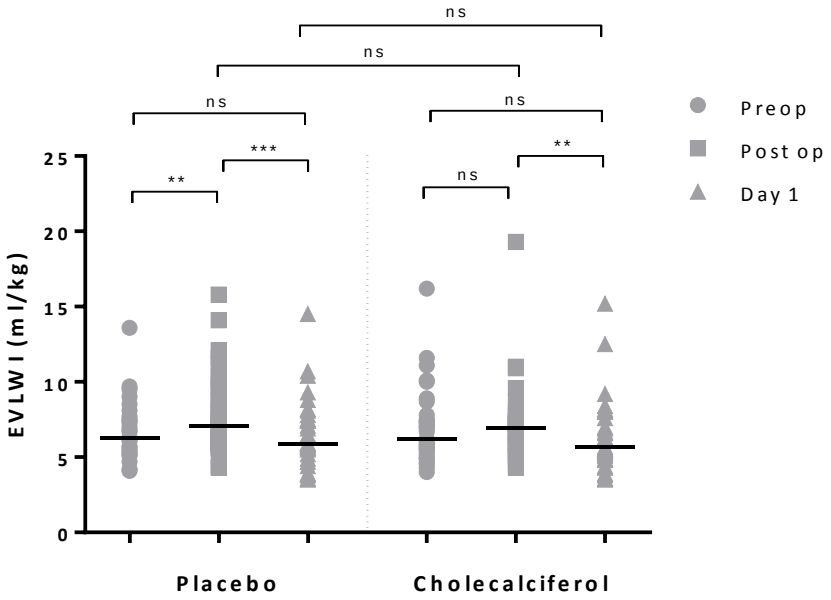


Figure 6.4 Effects of cholecalciferol on perioperative EVLWI

n=35 for placebo preop and postop and 32 for placebo day 1, n=33 for cholecalciferol preop and postop and 31 for day 1. Black horizontal lines indicate median for each dataset. Overall Friedman test p<0.0001 for placebo and p=0.0057 for cholecalciferol. Dunn's multiple comparisons test used to look for differences within the groups, Mann Whitney test used to compare between groups.

Patients who received cholecalciferol smaller post-operative fold change in EVLWI, but this was not significant at the 5% level (p=0.0528, figure 6.5).

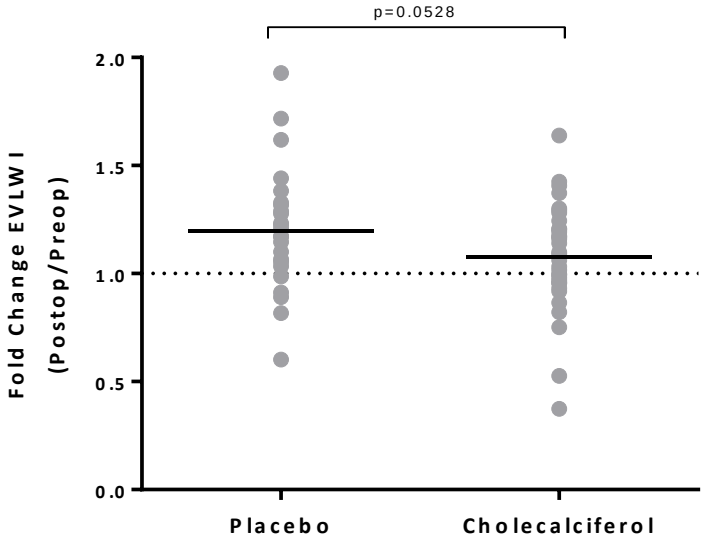


Figure 6.5 Effect of cholecalciferol on post-operative fold change EVLWI

n=35 for placebo and n=33 for cholecalciferol. Unpaired T test used to compare groups. Black horizontal lines indicate mean for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

Comparison of patients who were Vitamin D deficient preop compared with those who had insufficient or sufficient plasma concentrations showed a significant difference in fold change (p=0.0076, figure 6.6).

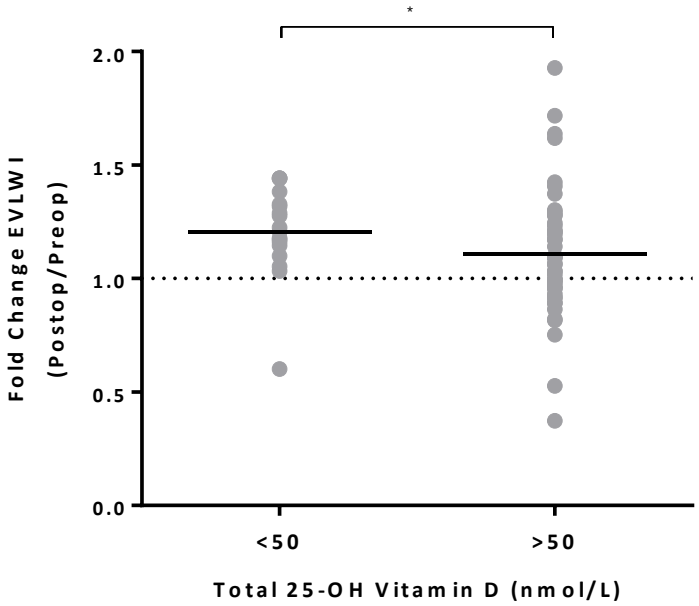


Figure 6.6 Differences in post-operative fold change in EVLWI according to 25-OH Vitamin D status

n=21 for 25-OH Vitamin D <50nmol/L and n=47 for 25-OH Vitamin D >50nmol/L. Mann Whitney test used to compare groups. Black horizontal lines indicate median for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

By day 1 post-op, changes in EVLWI had largely resolved with median day 1/preop fold change = 0.96. There were no significant differences between groups at this point (p=0.6954, figure 6.7)

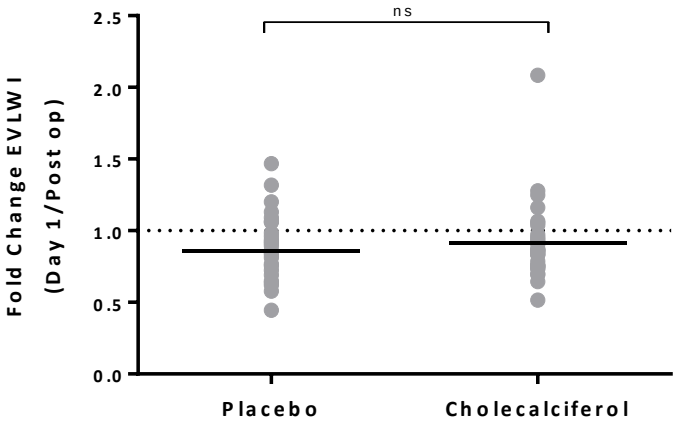


Figure 6.7 Effect of cholecalciferol on day 1 post-op fold change EVLWI

n=32 for placebo and n=31 for cholecalciferol. Unpaired T test used to compare groups. Black horizontal lines indicate mean for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

There was no correlation between preoperative 25-OH Vitamin D or 1,25-$(OH)_2$ Vitamin D and post op or day 1 EVLWI or PVPI. Analysis of PVPI showed that while in the placebo group post-operative levels were significantly higher than preoperative levels, in the cholecalciferol group there was no significant difference. On day 1 post op, levels in the placebo group were not significantly different to pre-operative levels, but in the cholecalciferol group day 1 levels were significantly lower than preoperative levels (figure 6.8).

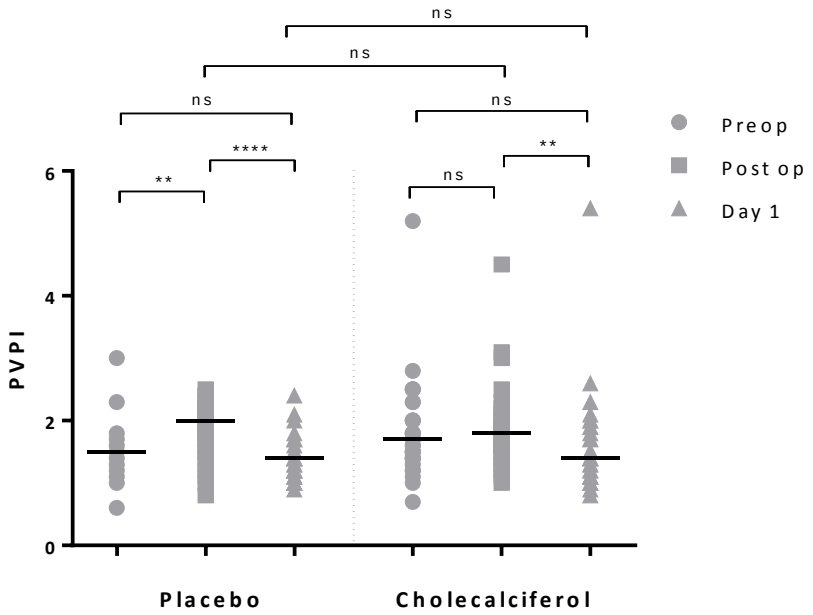


Figure 6.8 Effects of cholecalciferol on perioperative PVPI

n=35 for placebo preop and postop and 32 for placebo day 1, n=33 for cholecalciferol preop and postop and 31 for day 1. Day 1 measurements could not be taken for 2 patients because the PICCO line had been removed by the clinical team overnight and for 1 patient because the central line was not working. Black horizontal lines indicate median for each dataset. Friedman test p<0.0001 for placebo, p=0.0015 for cholecalciferol.Dunn's multiple comparisons test used to look for differences within the groups, Mann Whitney test used to compare between groups.

There was a significant difference in post-operative fold change between the groups (p=0.0274, figure 6.9).

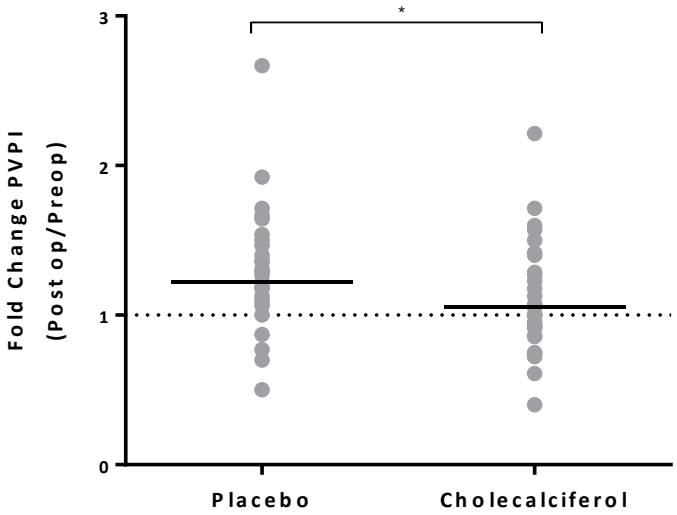


Figure 6.9 Effect of cholecalciferol on post-operative fold change PVPI

n=35 for placebo and n=33 for cholecalciferol. Mann Whitney test used to compare groups. Black horizontal lines indicate median for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

This difference persisted when Vitamin D deficient patients were compared with patients with insufficient or sufficient levels (p=0.0142, figure 6.10).

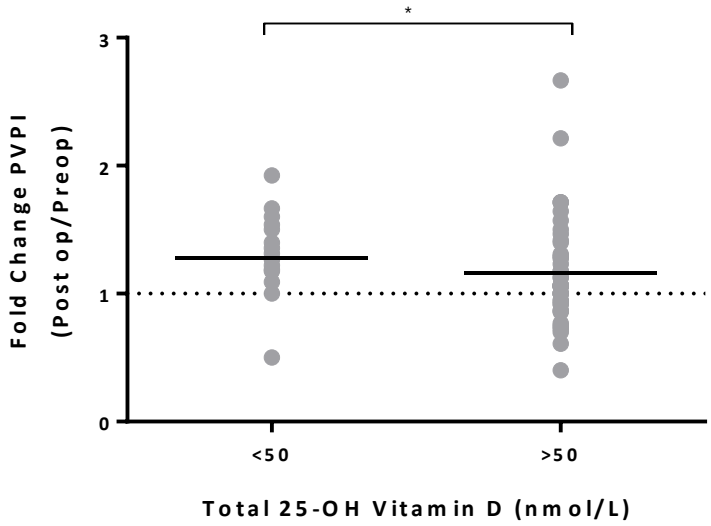


Figure 6.10 Differences in post-operative fold change in PVPI according to 25-OH Vitamin D status

n=22 for 25-OH Vitamin D <50nmol/L and n=46 for 25-OH Vitamin D >50nmol/L. Mann Whitney test used to compare groups. Black horizontal lines indicate median for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

Comparison of changes on day 1 showed that the previously seen increases in PVPI had largely resolved and there was no longer a significant difference between groups (p= 0.2290, figure 6.11).

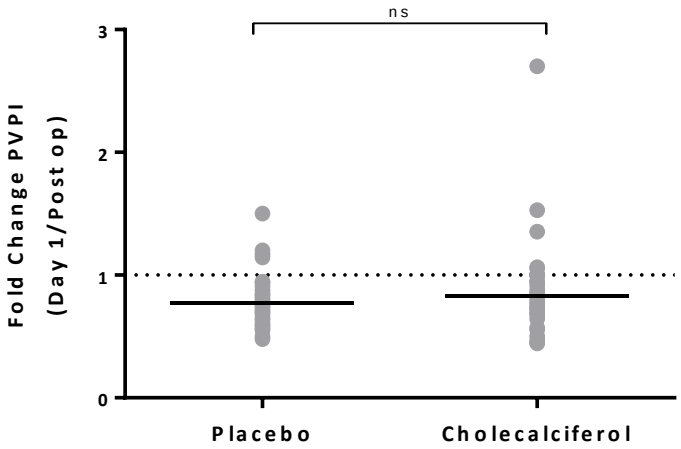


Figure 6.11 Effect of cholecalciferol on day 1 post-op fold change PVPI

n=32 for placebo and n=31 for cholecalciferol. Unpaired T test used to compare groups. Black horizontal lines indicate mean for each dataset. Dotted reference line indicates fold change=1; datapoints below the line decreased post op and datapoints above the line increased post op

Analysis of other PICCO parameters showed no significant difference in fold change (table 6.6).

Table 6.6 Effects of cholecalciferol on perioperative fold change in Cardiac Output (CO), Global End Diastolic Volume (GEDV), and Systemic Vascular Resistance (SVR)

PICCO Parameter	Post op/Preop			Day 1/Post op		
	Placebo (n=35)	Chole-calciferol (n=33)	P value	Placebo (n=32)	Chole-calciferol (n=31)	P value
CO	1.25 (1.07-1.41)	1.29 (1.02-1.57)	0.658	1.05 (0.86-1.19)	0.93 (0.81-1.08)	0.414
GEDV	1.00 (0.89-1.10)	0.98 (0.88-1.10)	0.785	1.05 (0.97-1.13)	1.08 (0.95-1.22)	0.463
SVR	0.74 (0.59-0.96)	0.79 (0.64-1.01)	0.690	1.11 (0.74-1.35)	0.98 (0.86-1.09)	0.240

6.4.5 Effects of cholecalciferol on post-operative oxygenation

There was no difference P/F ratio between the groups post-op (figure 6.12). In addition, there was no significant correlation between preop total 25-OH Vitamin D levels and post op P/F ratio (Pearson r =0.0913, p=0.4588, r^2=0.0083), but there was a weak correlation between preop 1,25-$(OH)_2$ Vitamin D plasma concentrations and post op P/F ratio (Pearson r =0.2580, p=0.0337, r^2=0.0665).

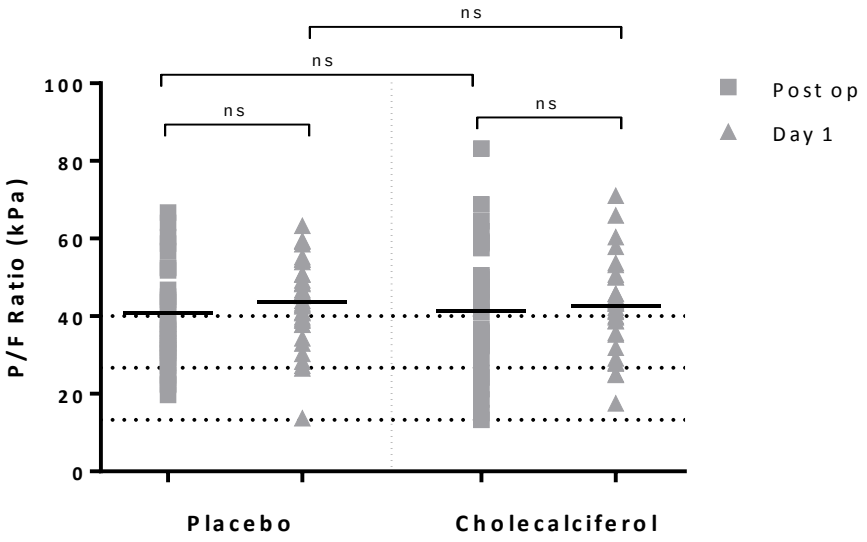


Figure 6.12 Comparison of Post op and Day 1 P/F ratio between groups

n=35 for placebo post op and n=34 for day 1, n=33 for cholecalciferol post op and n=32 for day 1. Paired T tests were used for comparison within groups and unpaired T tests were used for comparison between groups. Black horizontal lines indicate median for each dataset. Dotted horizontal reference lines indicate P/F ratios of 40kPa, 26.6kPa and 13.3kPa

As PaO2 was not measured routinely in patients after discharge from the Intensive Care Unit, I used the ratio of oxygen saturations to inspired oxygen concentration (S/F ratio) to compare oxygenation between groups over the first 7 days post op. An S/F ratio of 315 is equivalent to a P/F ratio of 40kPa while an

S/F ratio of 235 is equivalent to a P/F ratio of 26.6kPa (182). There was no significant difference in S/F ratios between groups in the first 7 days post op (figure 6.13)

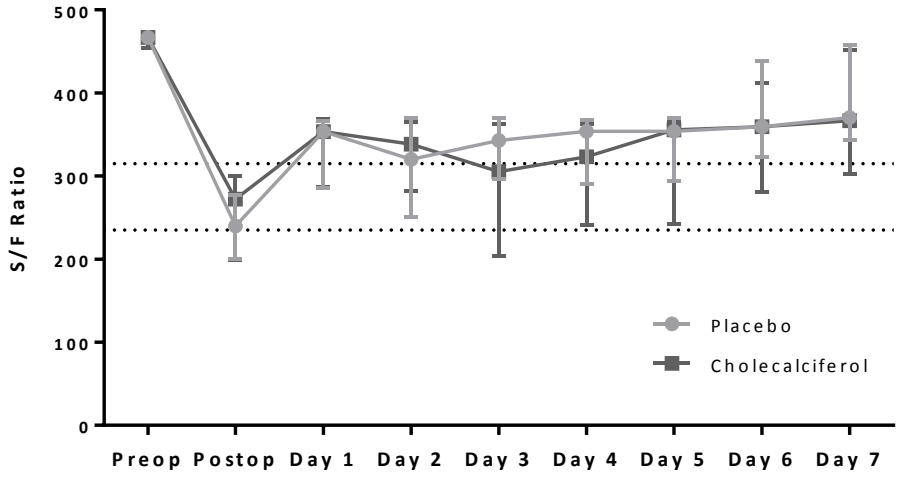


Figure 6.13 Comparison of S/F ratios between groups for 7 days post op

n=35 for placebo (all timepoints), n=33 for cholecalciferol Preop - day 1 and n=32 for cholecalciferol day 2 - day 7. Symbols represent median for each group with interquartile range represented by error bars. Dotted horizontal reference lines represent S/F ratio 315 and 235. Friedman test for combined data p<0.0001 for each cell type with multiple comparisons using Dunn's test used to compare individual timepoints as shown on graph.

There was a significant correlation between preop total 25-OH Vitamin D and S/F ratio on day 1 (Pearson r=0.2513, p=0.0387, r^2=0.0632) but not at any other timepoint up to day 7 post op. There was also a significant correlation between preop 1,25-$(OH)_2$ Vitamin D plasma concentrations and S/F ratio post op

(Spearman r=0.3838, p=0.0012, r^2=0.1146), on day 1 (Spearman r=0.2871, p=0.0176, r^2=0.0749), on day 2 (Pearson r=0.3192, p=0.0085, r^2=0.1166) and on day 4 (Spearman r=0.2574, p=0.0379, r^2=0.0463). On day 3 the relationship was not significant (Spearman r=0.2084, p=0.0906, r^2=0.0436). There was no correlation between either day 3 total 25-OH Vitamin D or day 3 1,25-$(OH)_2$ Vitamin D and S/F ratio on days 3-7.

6.4.6 Effects of cholecalciferol on clinical diagnoses of ARDS

7 of the patients developed ARDS as defined by the Berlin criteria. A further 2 patients met AECC criteria, but were not ventilated with PEEP/CPAP at least 5cmH_2O. 4 of the patients received placebo and 3 received cholecalciferol (p=1.000). The prevalence of ARDS in the Vindaloo cohort was lower than in the BALTI cohort (10% vs 25% p=0.0263). There was no difference in plasma concentrations of total 25-OH Vitamin D (ARDS median = 61.9nmol/L IQR=60.2-73.7nmol/L, No ARDS median=60.99nmol/L IQR=41.1-79.7nmol/L, p=0.9969) or 1,25-$(OH)_2$ Vitamin D (ARDS median = 104.2pmol/L IQR = 75.7-111.1pmol/L, No ARDS median=100.7pmol/L, IQR=69.9-119.3pmol/L, p=0.8761) between patients who developed ARDS and those who did not.

6.4.7 Effects of cholecalciferol on post op adverse events

There were no differences in rates of adverse events between the groups, as summarised in table 6.7.

Table 6.7 Adverse Events in patients recruited to the Vindaloo Trial

	Placebo (n=35)	Cholecalciferol (n=33)	P value
Hospital/Ventilator acquired Pneumonia	12 (34%)	13 (39%)	0.6624
Reintubation	7 (20%)	8 (24%)	0.6733
Mechanically Ventilated on day 1 post op	4 (11%)	7 (21%)	0.2735
Any respiratory complication	17 (49%)	17 (52%)	0.8083
Any infection	15 (49%)	18 (55%)	0.3351
Any cardiac complication	12 (34%)	6 (18%)	0.1325
Required inotropes in first 7 days post op	18 (51%)	16 (48%)	0.8083
Renal replacement therapy	0 (0%)	1 (3%)	0.4853
Returned to theatre	1 (3%)	1(3%)	1.0000
Any surgical complication	8 (23%)	12 (36%)	0.2218

Patients in the cholecalciferol group tended to develop pneumonia later in the postoperative course (median day of pneumonia development in the placebo group was 3.5 whereas in the cholecalciferol group it was 5.0). This difference did not meet significance (p=0.0891). 1/12 patients in the placebo group and 5/13 patients in the cholecalciferol group developed pneumonia after day 7 post op (p=0.1602). The majority of patients were not ventilated on any of the first 7 days post op and there was no significant difference between groups in

ventilator free days in the first 7 days post op (median for both groups 7 days ventilator free, p=0.2799). 10 (29%) patients in the placebo group and 12 (36%) patients in the cholecalciferol group required ventilation at some point in the first 7 days after the operation (p=0.4924).

6.4.8 Effects of cholecalciferol on post-operative morbidity and mortality prediction scores

APACHE-II score was calculated on admission to the intensive care unit. There was no significant difference in APACHE-II scores between groups (placebo mean=8.8 SD=3.15, cholecalciferol mean = 8.7 SD=4.51, p=0.8636). The median mortality predicted by the APACHE-II scores was 4.7% for placebo and 4.1% for cholecalciferol (p=0.4201). There was no significant correlation between preop total 25-OH Vitamin D and APACHE-II score (Pearson ρ=-0.2225, p=0.0683, r^2=0.0495), and no correlation between 1,25-$(OH)_2$ Vitamin D and APACHE-II score (Pearson ρ=-0.101, p=0.3773, r^2=0.0118).

There was no significant difference in SOFA score on day 1 post op (placebo median=3 IQR = 2-5, cholecalciferol median=4.0 IQR = 2-6, p=0.4071) and no correlation between preop vitamin D plasma concentration and day 1 SOFA score (25-OH Vitamin D: Spearman ρ=-0.0954, p=0.4392, r^2=0.0116, 1,25$(OH)_2$ Vitamin D: Spearman ρ=0.0414, p=0.7376, r^2=0.0028).

6.4.9 Effect of cholecalciferol on length of ITU and Hospital Stay

On average, patients who received cholecalciferol spent 5 days on ITU post op while patients who received placebo spent 4 days on ITU (p=0.0469, figure 6.14).

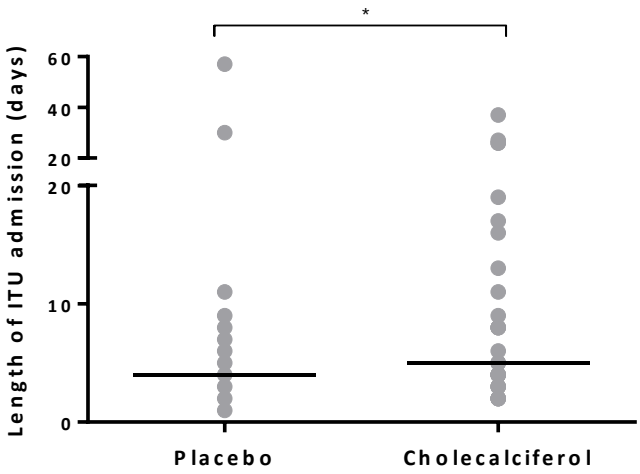


Figure 6.14 Effects of cholecalciferol on length of ITU admission post oesophagectomy

n=35 for placebo and n=33 for cholecalciferol. Mann Whitney test used to compare between groups. Black horizontal lines indicate median for each dataset

However, there was no significant difference between groups in length of hospital admission with median length of stay 13 days for both groups (p=0.6710, figure 6.15)

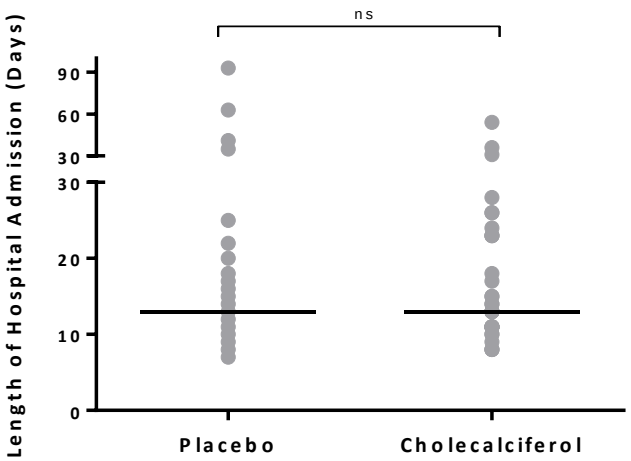


Figure 6.15 Effects of cholecalciferol on length of hospital admission post oesophagectomy

n=35 for placebo and n=33 for cholecalciferol. Mann Whitney test used to compare between groups. Black horizontal lines indicate median for each dataset

6.4.10 Effects of cholecalciferol on post op mortality

2 patients died prior to hospital discharge. Both had received placebo. One patient died 24 days post randomisation (preop total 25-OH Vitamin D= 13.2nmol/L and 1,25-$(OH)_2$ Vitamin D=58.0pmol/L) and the other 73 days post randomisation (preop total 25-OH Vitamin D = 73.7nmol/L and 1,25-$(OH)_2$ Vitamin D=111.1pmol/L).

The only patient who died within 30 days of randomisation was the patient detailed above. 2 further patients (4 in total) died within 90 days of randomisation. Both received cholecalciferol. One patient died 75 days post randomisation (preop total 25-OH Vitamin D= 74.9nmol/L and 1,25-$(OH)_2$

Vitamin D=186.3pmol/L) and the other died 82 days post randomisation (preop total 25-OH Vitamin D= 83.4nmol/L and 1,25-$(OH)_2$ Vitamin D=77.1pmol/L).

So far, overall, there is no difference in post randomisation survival between the two groups (p=0.9952, figure 6.16).

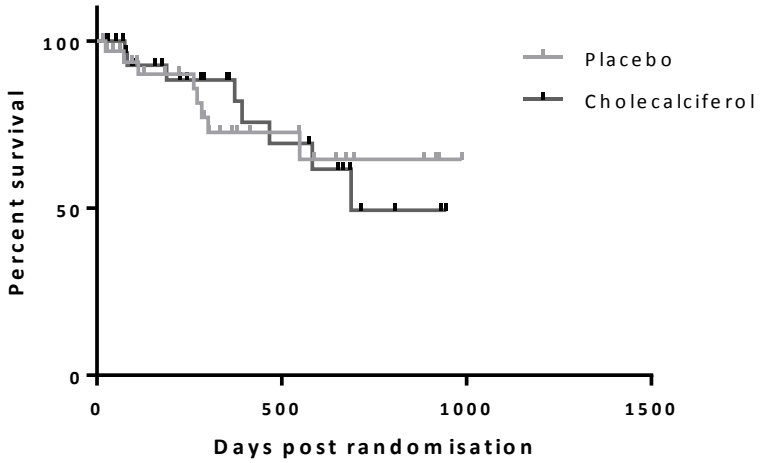


Figure 6.16 Kaplan Meier Survival Curve demonstrating survival post randomisation.

n=35 for placebo and n=33 for cholecalciferol. Vertical symbols on survival curves represent censored values (patients who were alive at last known time point).

6.5 Discussion

The results in this chapter demonstrate that patients undergoing oesophagectomy have a marked inflammatory response with changes in circulating white cells, inflammatory cytokines and alveolar oedema. This is in line with findings in the BALTI-prevention trial (42), from which the cohort examined in chapter 4 were drawn as well as other published literature (171, 172). The results suggest there are two main peaks of inflammation in the

perioperative period – changes in white cells and inflammatory cytokines including IL-6 and IL-8 occur during the operation whilst plasma concentrations of C-reactive protein and LL-37, an antimicrobial peptide, appear to peak around day 3 post op. This demonstrates that the inflammatory response to surgical stress evolves over several post-operative days.

There was little difference in absolute plasma concentrations of inflammatory cytokines during the perioperative period, with the exception of s-TNF-RI and s-TNF-RII on day 1 post op when patients who received cholecalciferol had significantly higher plasma concentrations. In addition, patients who received cholecalciferol had significantly greater perioperative fold change in plasma concentrations of inflammatory cytokines, although this did not translate into a difference in overall plasma concentrations and had resolved by day 1 post op. Other studies have found little evidence Vitamin D supplementation leads to changes in circulating cytokines (183, 184) although a study of Vitamin D supplementation in patients with multiple sclerosis did show upregulation of gene expression of IL-6 in peripheral blood mononuclear cells (PBMCs) (185).

By day 3 plasma concentrations of LL37, an antimicrobial peptide, had increased significantly more in the cholecalciferol group than in the placebo group, although, again there was no difference in absolute plasma concentrations. This coincides with peak plasma concentrations of CRP for the cohort as a whole, which were not different between groups and also the peak period for development of post op pneumonia (over 70% of patients who developed post

op pneumonia did so between days 2 and 5 post op). While there was no difference in numbers of infections between groups, there was a trend towards patients in the cholecalciferol group developing pneumonia at a later time point than patients in the placebo group. This result suggests that patients who received cholecalciferol may have an enhanced response to the presence of microbes, resulting in post-operative pneumonia developing later in the post-operative course.

Cholecalciferol administration significantly reduced perioperative increases in inflammatory alveolar oedema, as shown by reductions in extra vascular lung water and pulmonary vascular permeability index. The differences seen were more apparent when the cohort was divided according to preoperative 25-OH Vitamin D plasma concentration. Interestingly, in a significant proportion of patients who received cholecalciferol levels of EVLWI decreased during the operation. The mechanism behind this is not clear but could be related to a direct effect of vitamin D on the alveolar capillary barrier. Activated alveolar macrophages and respiratory epithelial cells are capable of converting 25-OH Vitamin D to 1,25-$(OH)_2$ Vitamin D (60, 186) . My group has shown in-vitro that physiologically relevant doses of 25-OH Vitamin D stimulates wound repair and cellular proliferation and reduces sFasL induced cell death of human type 2 alveolar epithelial cells (187). In addition to its antimicrobial actions, LL37 has been shown to stimulate wound vascularization and re-epithelialization of healing skin (188) 1,25-$(OH)_2$ Vitamin D has been shown to reduce expression of

ICAM-1 on pulmonary endothelial cells and reduce platelet activating factor induced neutrophil adherence to endothelial cells (189). While there was no significance difference between groups in post op oxygenation, there was a correlation between 1,25-$(OH)_2$ Vitamin D plasma concentrations and S/F ratio on a number of post-operative days.

The trial was not powered to detect differences in clinical diagnoses of ARDS or respiratory complications and there was no difference between groups in incidence of these clinical outcomes. While patients who received cholecalciferol spent 1 day longer on ITU on average, there was no significant difference in ventilator free days, numbers of patients requiring reintubation or total length of hospital stay. The increased length of ITU stay may reflect the later development of pneumonia in the cholecalciferol group, as both average around 5 days and it is common to delay discharge from ITU is there is a suspicion that patients are starting to develop a chest infection so treatment can be commenced before transfer to a new clinical team.

CHAPTER 7

EFFECTS OF VITAMIN D ON NATURAL KILLER CELLS

7.1 Introduction

We've shown that patients with and at risk of ARDS are highly likely to be Vitamin D deficient and that a one-off administration of 300,000IU cholecalciferol is a safe and effective way of increasing Vitamin D plasma concentrations in a short period of time preop. We have also demonstrated an association between circulating 1,25-$(OH)_2$ Vitamin D plasma concentrations and post-operative mortality in patients undergoing oesophagectomy. The majority of patients who undergo oesophagectomy die following recurrence of the disease (section 4.4.10).

Natural Killer (NK) Cells are lymphocytes that form part of the innate immune system. They are part of the first line of defence against both infection and cancer and are widely distributed in blood, spleen, liver, lung and bone marrow (190). They act in two ways; they are able to directly kill cells by forming synapses with target cells and releasing perforin and granzyme and they are able to co-ordinate immune responses via cytokine release (191).

NK Cells are defined by a lack of CD3 expression and by the presence of CD56 (192). There are two major subsets of natural killer cells which are distinguished by their levels of CD56 expression. CD56dim cells make up approximately 90% of NK cells in the peripheral blood and mediate cytotoxicity responses. CD56bright cells are primarily cytokine producers and are better able to leave the vasculature. They constitute the majority of natural killer cells found in lymph nodes (191).

NK cells originate in the bone marrow from CD34+ haematopoietic cells. They mature in secondary lymphoid tissue which contains a highly enriched population of CD34+CD45RA+ pre-NK cells (193). Antigen presenting cells such as dendritic cells in secondary lymphoid tissue express membrane bound IL-15 which is required for NK cell maturation. All NK cells are initially CD56bright. Some CD56bright cells remain within the lymph nodes and interact with dendritic cells while others undergo a further maturation step forming CD56dim cells which return to the circulation via the efferent lymphatic system (194) (figure 7.1).

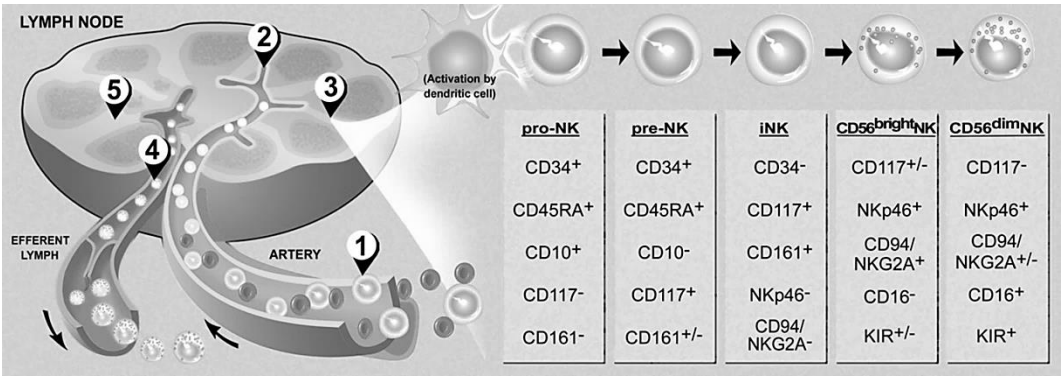


Figure 7.1 NK cell development (194)

(1) Bone marrow derived $CD34^{+}CD45RA^{+}$ cells circulate in the blood and (2) extravasate across lymph node high endothelial venules to enter the parafollicular space. There, (3) pro-NK cells are activated by dendritic cells to progress through distinct stages of maturation to create both $CD56^{bright}$ and $CD56^{dim}$ NK cells, as detailed on the right. Mature $CD56^{dim}$ NK cells return to the circulation via the efferent lymph (4), whereas some $CD56^{bright}$ NK cells remain within the secondary lymphoid tissue to interact with dendritic cells (5)

Production of cytokines and chemokines by NK cells in early innate immune responses is a key step in recruitment and function of other haematopoietic cells (195). While CD56bright cells produce greater amounts of cytokine per cell,

CD56dim cells produce a greater proportion of the total level of cytokine secreted into peripheral blood due to their greater numbers (194).

NK cells make up around 10% of resident lymphocytes in the lung (190). Survival of NK cells in the lung may be promoted by bronchial epithelial cells which spontaneously produce IL15 (196). In the resting state, cytotoxicity of pulmonary NK cells is suppressed – the cells form synapses with target cells but killing is suppressed (197, 198). Lung NK cells regain their activity after 24 hours in culture or after stimulation with IL-2 (198). Within hours of infection or other inflammatory stimulus large numbers of NK cells are recruited to the lungs from the blood (199-201). Peripheral blood NK cells can be suppressed by culture with BAL fluid or alveolar macrophages (but not macrophages from other tissues) (202, 203).

Numbers of circulating cytotoxic NK cells are reported to fall by day 1 post oesophagectomy and remain low on day 3 post-op (172) and low pre- and post-operative NK cell activity is associated with poor outcomes following cancer surgery (204, 205). NK cells are reportedly more severely affected by surgical stress than other lymphocytes. Low NK cell activity has been reported to be related to high concentrations of plasma catecholamines in the perioperative period and is exacerbated by use of norepinephrine as a result of interactions with beta-2 adrenoceptors (206, 207). Some groups have suggested that Natural Killer cell transfer (208), or administration of an influenza vaccination to boost

Natural Killer Cell activity (209) could be used in the perioperative period to improve long term outcomes

Studies have suggested that Vitamin D affects NK cell function, but findings have not been consistent. While some groups have found that adding 1,25-$(OH)_2$ Vitamin D to culture media enhances the ability of NK cells to kill K562 cells (a cancer cell line) (73), other groups have suggested that 1,25-$(OH)_2$ Vitamin D reduces NK cell cytotoxicity by reducing IL2 release from PBMCs (210). A study in healthy Italian nonagenarians found a positive correlation between serum 1,25-$(OH)_2$ Vitamin D plasma concentrations and circulating NK cell numbers and cytotoxicity (211), while a group investigating the effect of 1,25-$(OH)_2$ Vitamin D on in vitro NK cell development from haematopoetic stem cells found that NK cell development was impaired and those cells that did develop had significant reductions in function. This group found no effect of Vitamin D on mature NK cell function (212). Finally, a study of NK cell function in women with recurrent pregnancy losses found that 1,25-(OH)2 Vitamin D reduced NK cell cytotoxicity in cells from women with this condition, but had no effect on cells from healthy controls, suggesting that different disease states may alter the association seen (213).

In view of the suggested link between Vitamin D and Natural Killer Cell function, and the importance of this cell in defence against cancer and infection (both of which are highly relevant in patients undergoing oesophagectomy), we examined changes in NK cell numbers in our cohort.

7.2 Research Hypothesis and Questions

7.2.1 Hypothesis

Vitamin D supplementation results in alteration in the perioperative number and function of circulating NK cells making development of recurrence post-op less likely.

7.2.2 Research Questions

1. How do NK cell numbers change perioperatively?

2. Is there a relationship between development of adverse event post-op and NK cell numbers?

3. What is the effect of supplementation with cholecalciferol on circulating NK cell numbers?

4. What is the effect in-vitro of culture in the presence of Vitamin D on NK cell cytotoxicity?

5. How does expression of the Vitamin D receptor in NK cells compare with other cell types?

7.3 Methods

PBMCs were isolated from heparinised whole blood, stained for surface markers of NK cells, then analysed by flow cytometry. Comparison of NK cell numbers was made between patients who survived two years and those who died within two

years. This timepoint was chosen as it was the maximum possible prior to submission of this thesis. In vitro, NK cell function was assessed using cells from healthy volunteers for a cytotoxicity assay as outlined in chapter 3. Cell expression of the Vitamin D receptor was assessed in three cell types – natural killer cells, monocytes/alveolar macrophages and alveolar type 2 cells using qPCR. The method of purification of all three cell types in described in the methods chapter (chapter 3).

Continuous data was assessed for normality and data was assessed repeated measures ANOVA for parametric data and Friedman test for non-parametric data. For comparison between groups across multiple timepoints 2 way ANOVA was used with multiple T tests to examine difference s between individual timepoints. Where data was missing, the last known figure was brought forward for the purposes of statistical analysis.

7.4 Results

7.4.1 Perioperative changes in total circulating NK cells

Numbers of circulating NK cells were expressed as both absolute number and as a percentage of the total lymphoid pool. The absolute number of circulating NK cells increased preop, in contrast with the absolute number of lymphocytes which did not significantly change preop, but trended towards a fall in numbers (figure 6.2). Perioperatively, absolute numbers of NK cells fell (in line with the overall fall in total lymphocyte numbers), and by day 3 post-op numbers of

circulating NK cells were significantly lower than at either preoperative timepoint (figure 7.2).

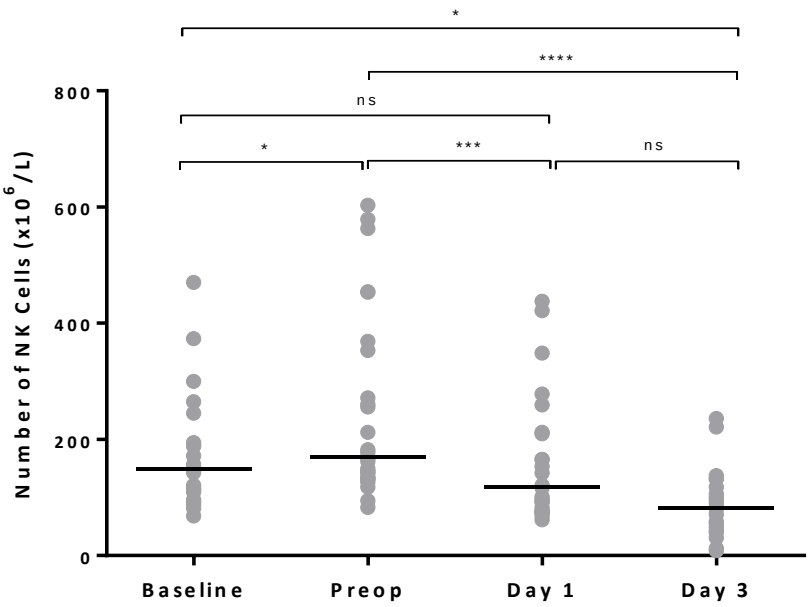


Figure 7.2 Perioperative changes in circulating NK cell numbers

Baseline n=28, Preop n=29, Day 1 n=24, Day 3 n=21. Friedman test p<0.0001 with Dunn's multiple comparison test used to compare individual timepoints as shown on graph. Black horizontal line indicates median for each dataset

When expressed as a percentage of the total lymphoid pool, the proportion of NK cells increased preop, did not change perioperatively and then decreased back to baseline levels by day 3 post op (figure 7.3).

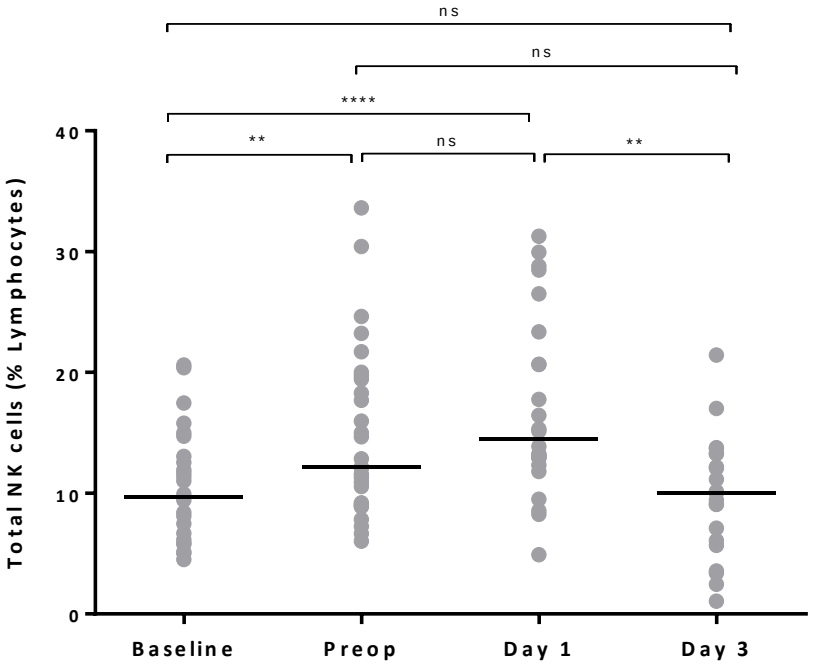


Figure 7.3 Perioperative changes in circulating NK cells expressed as % of total lymphoid pool

Baseline n=28, preop n=29, day 1 n=24, day 3 n=21. Friedman test p<0.0001 with Dunn's multiple comparison test used to compare individual timepoints as shown on graph. Black horizontal line indicates median for each dataset

7.4.2 Perioperative changes in the ratio between cytotoxic and cytokine secreting NK cells

The preop increase in total circulating NK cells occurred as a result of increases in circulating numbers of $CD56^{dim}CD16^{+}$ (cytotoxic) cells; Numbers of $CD56^{bright}CD16^{-}$ (cytokine secreting) cells did not significantly change preop. Absolute numbers of both types of cell significantly decreased perioperatively (figure 7.4).

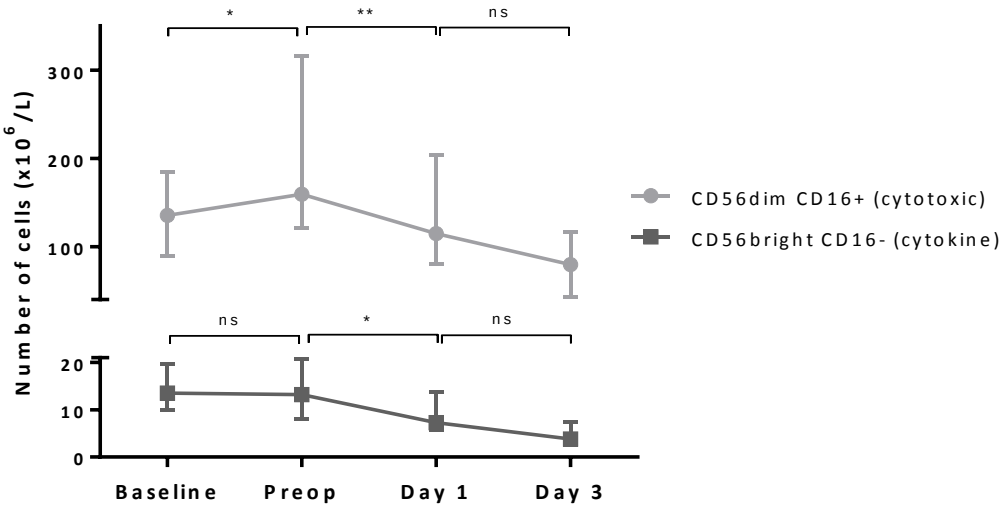


Figure 7.4 Perioperative changes in cytotoxic and cytokine secreting NK cells

Baseline n=28, Preop n=29, Day 1 n=24, Day 3 n=21 for both cell types. Friedman test p<0.0001 for both cell types with Dunn's multiple comparison test used to compare individual timepoints as shown on graph. Median and IQR for each timepoint shown

When expressed as a percentage of the total lymphoid pool, $CD56^{dim}CD16^{+}$ cells show a similar pattern to total cell numbers increasing preop, remaining unchanged perioperatively and falling back to baseline proportions by day 3 post op. $CD56^{bright}CD16^{-}$ cells however do not change preop but significantly increase in proportion perioperatively and significantly decrease between day 1 and day 3 post op (figure 7.5)

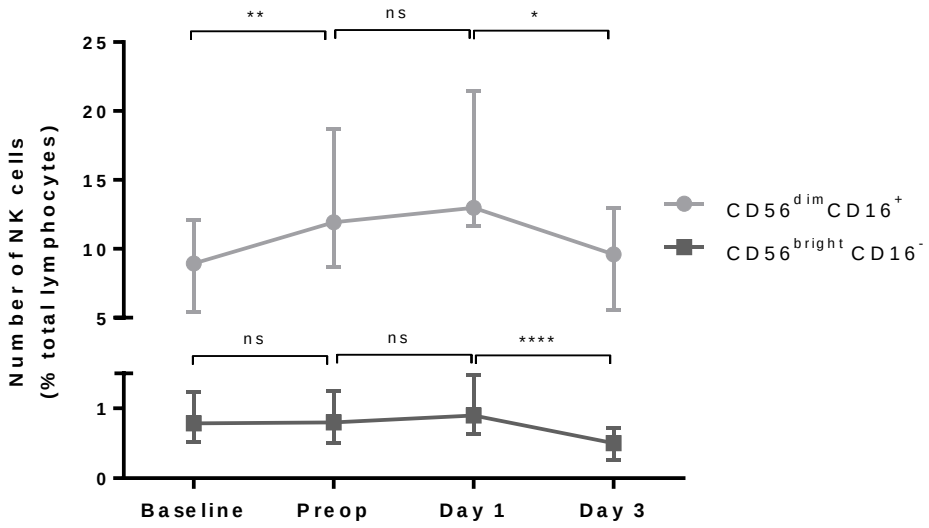


Figure 7.5 Perioperative changes in cytotoxic and cytokine secreting NK cells expressed as % of total lymphoid pool

Baseline n=28, preop n=29, day 1 n=24, day 3 n=21. Friedman test p<0.0001 for both cell types with Dunn's multiple comparison test used to compare individual timepoints as shown on graph. Median and IQR for each timepoint shown

7.4.3 Comparison of circulating NK cells and development of post-operative adverse events

There was no difference in total numbers of NK cells at any timepoint in patients who developed post-operative pneumonia, any infection or any respiratory complication. Patients who developed a surgical complication had significantly higher circulating absolute and proportionate NK cells on day 3 post op but there was no difference prior to this timepoint (table7.1).

Table 7.1 Comparison of total circulating NK cells and post-operative adverse events

Complication		Baseline (n=26)	Preop (n=27)	Day 1 (n=24)	Day 3 (n=21)
Hospital Acquired Pneumonia	Yes	10.2 (4.3)	13.9 (5.0)	15.7 (6.8)	10.8 (6.0)
	No	11.0 (5.0)	16.2 (8.2)	17.9 (8.0)	9.1 (4.5)
	P value	0.677	0.402	0.502	0.472
	n (Yes)	11	11	9	8
Any Infection	Yes	10.4 (4.0)	15.0 (6.7)	17.1 (7.3)	9.9 (5.3)
	No	10.9 (5.6)	15.7 (8.0)	17.0 (8.1)	9.6 (4.9)
	P value	0.806	0.793	0.973	0.895
	n (Yes)	15	16	13	13
Respiratory Complication	Yes	10.2 (4.2)	14.6 (5.2)	15.7 (6.1)	9.0 (5.7)
	No	11.2 (5.4)	16.2 (9.1)	18.4 (8.7)	10.8 (4.1)
	P value	0.598	0.575	0.377	0.416
	n (Yes)	15	15	12	12
Surgical Complication	Yes	11.2 (3.8)	16.4 (7.7)	17.6 (6.5)	14.4 (3.7)
	No	10.4 (5.1)	14.7 (6.9)	16.8 (8.2)	7.4 (3.9)
	P value	0.669	0.556	0.7378	**0.0008**
	n (Yes)	8	9	8	7

Mean (SD) of absolute NK cell numbers (x10^6) shown for each parameter.

7.4.4 Differences between total NK cell numbers in patients who die within two years of their operation

The timepoint of 2 years was chosen as this was the maximum time we could follow up for prior to the submission of this thesis. Data was available for 16 patients who were thought to have had curative surgery. 7 had died within 2 years of their operation and 9 had not. There was a significant difference in

preoperative total numbers of circulating NK cells between the groups and a trend towards patients who died within 2 years having lower numbers of NK cells overall (figure 7.6).

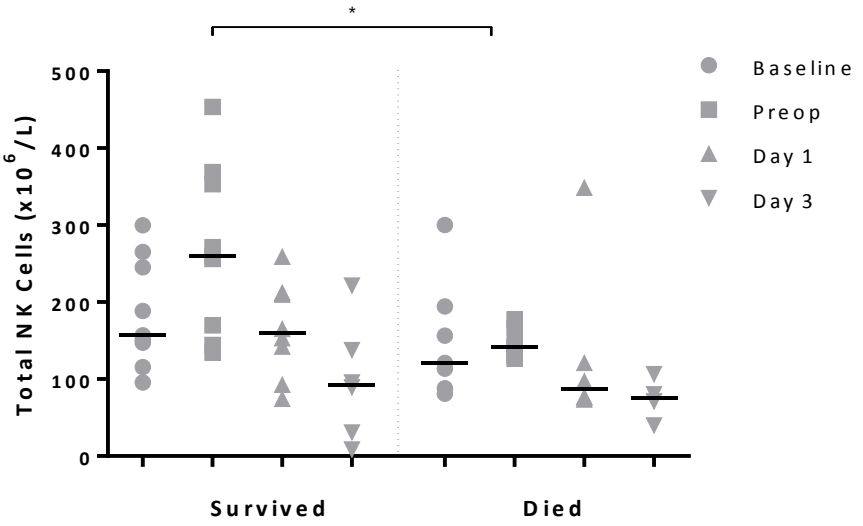


Figure 7.6 Comparison of circulating absolute numbers of NK cells in patients who survived at least 2 years post op and those who did not

Survived: n=9 at baseline and preop,n=8 on day 1, n=6 on day 3, Died: n=7 at baseline and preop, n=6 on day 1 and n=4 on day 3. Black horizontal lines indicate median for each dataset. Friedman test p=0.0014 for survived, p=0.0508 for died. Mann Whitney test used to compare between groups.

Preoperative data was also available for 3 patients who were found to have incurable cancer during their operation; average preop NK cells in these patients was also low – 113.7x10^6/L compared with 150.97x10^6/L in patients who died and 288.27x10^6/L in patients who survived.

There was no difference in smoking status, number of patients undergoing preoperative chemotherapy, TNM staging or rate of post-operative respiratory or surgical complications between the patients who died within 2 years and the

patients who survived. 43% of patients who died received cholecalciferol compared with 67% of patients who survived. This difference was not statistically significant and there was no significant difference at any timepoint between 1,25-$(OH)_2$ Vitamin D plasma concentrations between patients who died within 2 years and those who survived.

The changes seen were replicated in both cell types with cytotoxic and cytokine secreting cells being higher on average in patients who survived than patients who died and the greater difference being seen at the preoperative timepoint.

7.4.5 Effects of cholecalciferol on NK cells

There was no significant difference between patients who received placebo and patients who received cholecalciferol in the number of circulating NK cells at any timepoint (figure 7.7). When changes between the timepoints for the individual groups were considered, there was greater variability in the patients who received cholecalciferol. Patients who received placebo only had significantly different levels between the preop and day 3 timepoint, while there were significant differences between preop and both post-operative timepoints for the cholecalciferol groups.

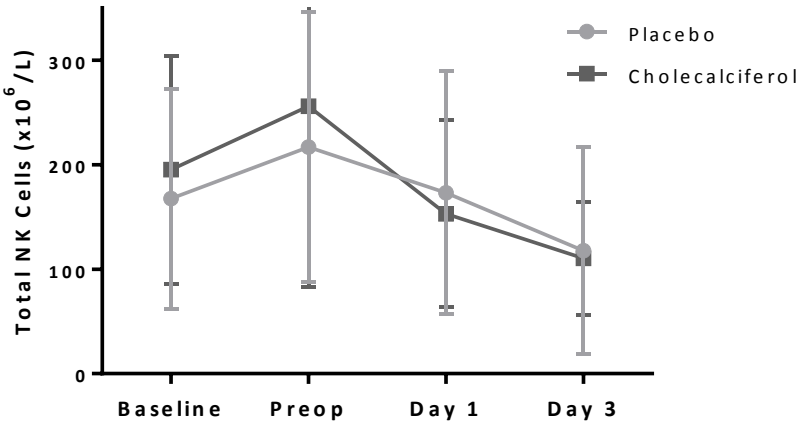


Figure 7.7 Effects of cholecalciferol on circulating NK cell numbers

Placebo: Baseline n=12, Preop n=13, Day 1 n=11, Day 3 n=10. Cholecalciferol: Baseline n=16, Preop n=16, Day 1 n=13, Day 3 n-11. Mean and SD for each timepoint shown. Friedman test p=0.0020 for placebo, p=0.0004 for cholecalciferol. No significant difference between groups at any timepoint (Unpaired T test).

When NK cells were divided into cytotoxic ($CD56^{dim}CD16^{+}$) and cytokine secreting ($CD56^{bright}CD16^{-}$) subtypes, there was no difference in numbers of circulating cells at any of the 4 timepoints but greater variability in cytotoxic cell numbers in the patients who received cholecalciferol. Changes in cytokine secreting cell numbers were similar across the two groups (figure 7.8 and 7.9).

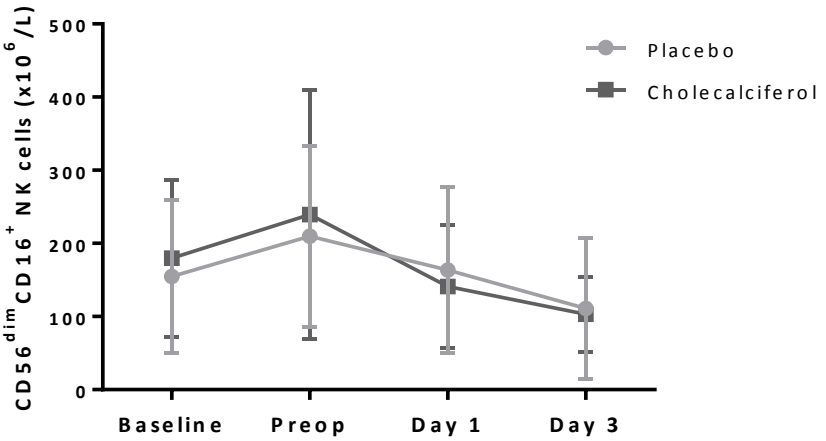


Figure 7.8 Effects of cholecalciferol on circulating cytotoxic NK cell numbers

Placebo: Baseline n=12, Preop n=13, Day 1 n=11, Day 3 n=10. Cholecalciferol: Baseline n=16, Preop n=16, Day 1 n=13, Day 3 n-11. Mean and SD for each timepoint shown. Friedman test p=0.0027 for placebo, p=0.0015 for cholecalciferol. No significant difference between groups at any timepoint (unpaired T test).

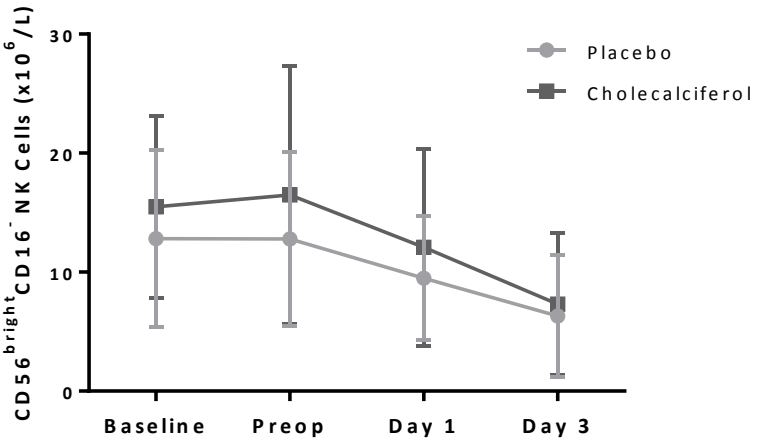


Figure 7.9 Effects of cholecalciferol on circulating cytokine secreting NK cell numbers

Placebo: Baseline n=12, Preop n=13, Day 1 n=11, Day 3 n=10. Cholecalciferol: Baseline n=16, Preop n=16, Day 1 n=13, Day 3 n-11. Mean and SD for each timepoint shown. 1way ANOVA p=0.0089 for placebo, p=0.0010 for cholecalciferol. No significant difference between groups at any timepoint (unpaired T test).

7.4.7 Correlations between vitamin D plasma concentrations and numbers of circulating NK cells

At baseline, there was a positive correlation between 1,25-$(OH)_2$ Vitamin D plasma concentrations and absolute numbers of total NK cells. On the day of the operation (preop), there was no significant correlation between Vitamin D plasma concentrations and numbers of NK cells. Post-op (day 3) there was a positive correlation between 25-OH Vitamin D plasma concentrations and total NK cells, but no relationship with 1,25-$(OH)_2$ Vitamin D plasma concentrations was found (table 7.2). There were no significant correlations between change in vitamin D plasma concentrations and change in NK cell numbers (data not shown).

Table 7.2 Correlations between Vitamin D plasma concentrations and Total NK cells

		Baseline (n=28)	Preop (n=28)	Day 3 (n=21)
Total 25-OH Vitamin D	Spearman ρ p r^2	0.0799 0.6860 0.0515	-0.0301 0.8791 <0.0001	**0.5411** **0.0113** **0.1589**
1,25-$(OH)_2$ Vitamin D	Spearman ρ p r^2	**0.3898** **0.0403** **0.0696**	0.0909 0.6456 0.0580	0.3273 0.1476 0.1082

7.4.8 In-vitro effects of Vitamin D on NK cells function

To investigate the effects of Vitamin D on NK cell function, I carried out cytotoxicity experiments in the presence of 100nM 25-OH Vitamin D. I used both unstimulated cells and cells stimulated with IL2. As baseline %K562 cell death varied the results from the experiments were compared with results from a control well which did not contain vehicle or Vitamin D and reported as fold difference. No significant difference in cytotoxicity was seen in cells exposed to 25-OH Vitamin D compared to vehicle control (Wilcoxon paired test unstimulated p=0.461, stimulated p=0.375, figure 7.10).

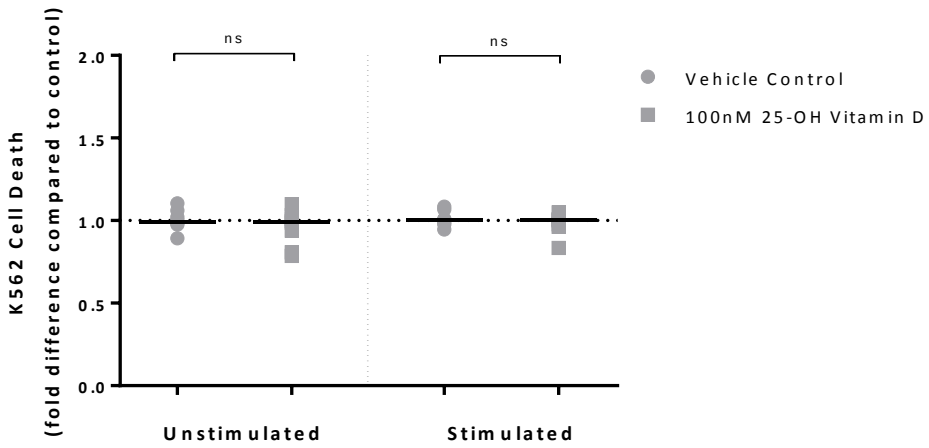


Figure 7.10 Effects of 25-OH Vitamin D on NK cell Cytotoxicity

n=8 for unstimulated cells and n=7 for stimulated cells. Wilcoxon signed rank test used to compare within groups. Black horizontal line indicates median for each dataset. Dotted reference line indicates fold difference=1; datapoints below the line were lower than control and datapoints above the line were higher than control

I also carried out the same experiments using 100nM 1,25-$(OH)_2$ Vitamin D. 1,25-$(OH)_2$ Vitamin D did not significantly affect cytotoxicity compared to vehicle

control (Wilcoxon paired test unstimulated p=0.945, stimulated p=0.109 figure 7.11)

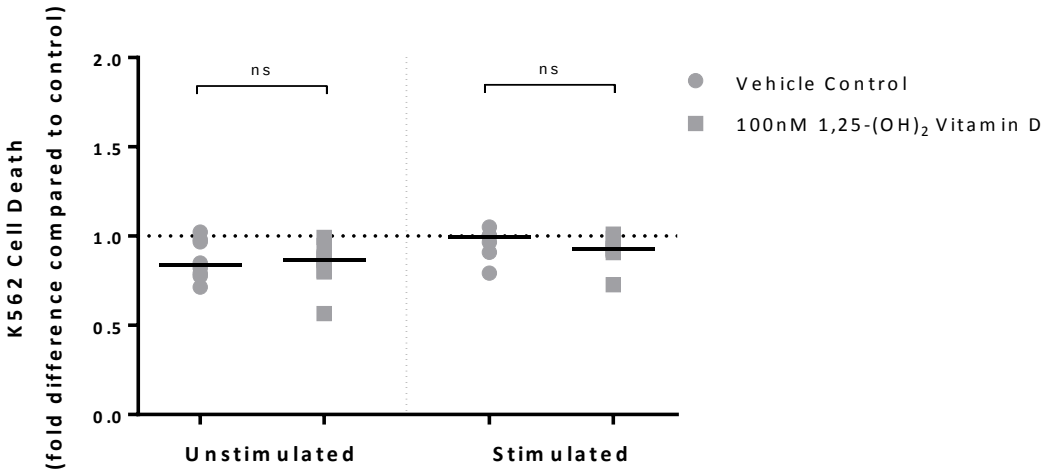


Figure 7.11 Effects of 1,25-$(OH)_2$ Vitamin D on NK cell Cytotoxicity

n=8 for unstimulated cells and n=7 for stimulated cells. Wilcoxon signed rank test used to compare within groups. Black horizontal line indicates median for each dataset. Dotted reference line indicates fold difference=1; datapoints below the line were lower than control and datapoints above the line were higher than control

7.4.9 Expression of VDR in NK cells

To further investigate the above results we measured expression of the Vitamin D receptor (VDR) in purified NK cells. Expression of VDR was significantly lower in unstimulated NK cells than in type 2 alveolar epithelial cells or monocytes and macrophages (figure 7.12).

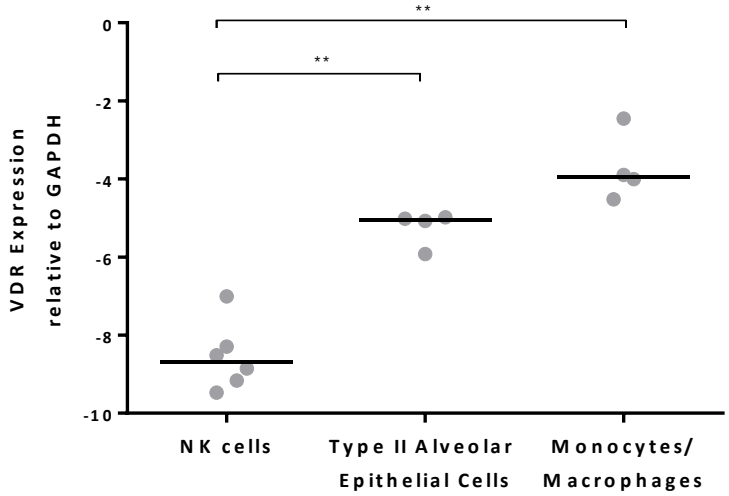


Figure 7.12 Expression of VDR in NK cells, Type II alveolar epithelial cells and monocytes/macrophages

n=6 for NK cells, n=4 for Type II Alveolar Epithelial Cells, n=4 for monocytes/macrophages. Black horizontal line indicates median for each dataset. Kruskal Wallis p<0.0001

Stimulation of the purified NK cells with IL2 did not result in a significant change in VDR expression (figure 7.13).

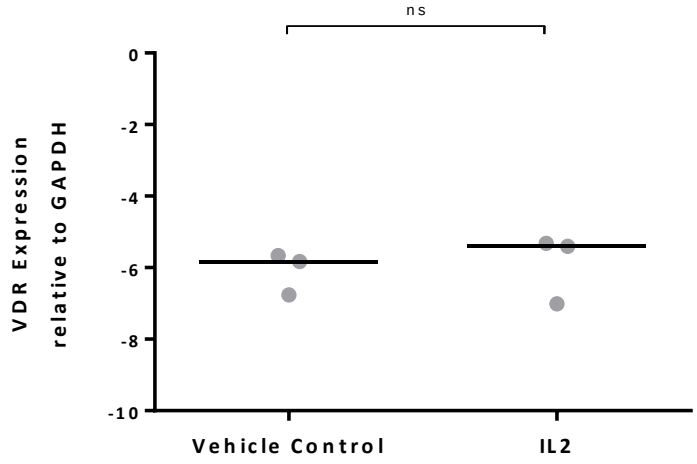


Figure 7.13 VDR expression in NK cells stimulated with IL2 compared with vehicle control

Datasets are paired. n=3. Mann Whitney Test used to compare groups. Black horizontal line indicates median for each dataset.

7.5 Discussion

We've demonstrated that NK cells increase prior to oesophagectomy, but that this was not as a result of Vitamin D supplementation. Previous studies have demonstrated that acute psychological stress has marked effects on NK cell numbers and that NK cell numbers correlate with plasma concentrations of noradrenaline (214). In vitro and ex-vivo work has also suggests that adrenaline (epinephrine) has a marked effect on NK cell cytotoxicity, although there is controversy as to whether the effect is suppressive or enhancing (215-217). One group have suggested that in-vitro and ex-vivo experiments may overestimate effect of stress hormones in vivo (218). Interestingly, patients who survived at least 2 years following their operation had greater preoperative circulating NK cells than patients who died within two years, suggesting that the effect of stress on NK cells offers a survival advantage.

Overall, there was a trend towards lower circulating NK cells in patients who died at all timepoints. All of 8 of the patients analysed died following diagnosis of recurrence or metastasis of their tumour suggesting that reduced NK cells during the perioperative period may have played a role in their reduced survival. This finding means that NK cell count immediately prior to cancer surgery may highlight patients who would benefit from increased post-op surveillance.

Contrary to some previous reports, I found little evidence that Vitamin D affects NK cell numbers or function. While there was a correlation between $1,25(OH)_2$ Vitamin D and NK cell numbers at baseline, this did not continue after

randomisation. The baseline findings replicate the findings in Italian nonagenarians (211) but suggest that changes in 1,25-$(OH)_2$ Vitamin D do not directly result in a linear change in NK cell numbers over the short term. Our finding that preoperative NK cell numbers increase in both the cholecalciferol and placebo groups seems at odds with Weeres et al's finding that NK cell development is reduced in the presence of 1,25$(OH)_2$ Vitamin D (212) and might reflect in-vivo interplay between different stress hormones, including adrenaline which cannot be fully replicated in ex-vivo and in-vitro settings.

Our finding that Vitamin D does not affect in vitro NK cell functions in cells from healthy controls and that relative VDR expression is lower in NK cells compared with epithelial cells and monocyte/macrophages support the in vivo findings. While the reduced expression of VDR in NK cells does not rule out a paracrine effect of Vitamin D on NK cells (for example by increasing IL2 release by other cells), the in-vitro NK cell killing experiment was carried out in the presence of other PBMCs and also did not find any effect.

Our data has weaknesses. The n numbers for some of the analysis are small and there is a risk that the significant relationships seen are due to chance and that more extensive analysis may not confirm the findings. It would have been informative to examine NK cell function in the ex-vivo samples, but we were limited by the amount of material available for analysis. For our q-PCR experiments we used GAPDH as a single housekeeping gene, as per our standard laboratory method. However, expression of GAPDH has been shown to differ

across different cell types (219), and while we found no difference in levels of expression across the three cell types examined (figure 3.8), use of more than one reference gene may have strengthened our findings. Our PCR findings showing that type 2 alveolar epithelial cells and monocytes/macrophages have relatively higher expression of VDR suggests that future work focussing on these cell types may yield more positive results.

In conclusion, Natural Killer cell numbers change markedly over the perioperative period and patients who survive 2 years post op have greater circulating NK cells than patients who die within 2 years. This correlates with the association I found between long term survival and 1,25-$(OH)_2$ Vitamin D plasma concentrations, but I have found little evidence that these two observations are linked.

CHAPTER 8

SUMMARY AND CONCLUSIONS

8.1 Overview of investigations into Vitamin D supplementation in patients at risk of ARDS

8.1.1 Vitamin D deficiency is common in patients with and at risk of ARDS

ARDS is a severe form of respiratory failure which results in significant morbidity and mortality. We have demonstrated that Vitamin D deficiency is highly prevalent in patients with ARDS and severe Vitamin D deficiency in patients who undergo oesophagectomy is associated with increased risk of developing ARDS post op.

8.1.2 Preoperative supplementation with 300,000IU cholecalciferol is a safe and effective method of rapidly increasing preoperative Vitamin D plasma concentrations

We recruited patients to a placebo controlled trial of Vitamin D replacement prior to oesophagectomy and demonstrated that high dose cholecalciferol administration up to 2 weeks pre-op is a safe and effective method of increasing preoperative Vitamin D plasma concentrations, despite patients in the VINDALOO cohort having higher pre-supplementation Vitamin D plasma concentrations than expected. Patients who received cholecalciferol had significantly greater increases in 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D between baseline and the day of their operation.

Perioperatively, Vitamin D plasma concentrations decreased markedly in both the placebo and cholecalciferol groups post-op, so that patients who achieved

Vitamin D sufficiency preop were still at risk of developing Vitamin D deficiency in the post op period.

8.1.3 Patients with lower 25-OH Vitamin D plasma concentrations have evidence of greater perioperative inflammatory alveolar oedema and systemic inflammation

Patients with the lowest Vitamin D plasma concentrations had greater perioperative increases in EVLWI, and patients who received cholecalciferol had lower increases in EVLWI and PVPI than patients who received placebo.

Comparison of the two cohorts is complicated by the general difference in 25-OH Vitamin D plasma concentrations between the two groups; patients who received placebo in the Vindaloo trial had similar preoperative plasma concentrations of 25-OH Vitamin D_3 to patients with the highest 25-OH Vitamin D_3 plasma concentrations in the BALTI-p cohort. Perioperative fold change in IL-6 was similar between the BALTI-p cohort with 25-OH Vitamin D_3 plasma concentrations >17.85nmol/L and the Vindaloo patients who received placebo, while patients with 25-OH Vitamin D_3 plasma concentrations <17.85 in the BALTIp cohort had much greater fold change than any of the other patients. This suggests that patients with very severe vitamin D deficiency have a hyper-inflammatory response to surgical stress. In both cohorts IL-6 differences between groups resolved by day 1 post op but by this timepoint in the Vindaloo cohort patients, the cholecalciferol group had elevated circulating plasma

concentrations of soluble TNF receptors suggesting enhanced resolution of inflammation.

While there was no difference in incidence of diagnoses of pneumonia between patients who received placebo and those who received cholecalciferol, there was a trend towards pneumonia developing later in patients who received cholecalciferol. Patients who received cholecalciferol also had greater increases in LL37 between day 1 and day 3. This, combined with the significant falls in circulating Vitamin D plasma concentrations seen perioperatively suggests that cholecalciferol administration may support mechanisms that prevent the development of infection, but as post-operative plasma concentrations fall this ability is lost. Other studies of cholecalciferol supplementation in the acute setting have started to consider whether initial high doses should be followed up with smaller regular doses to help maintain Vitamin D plasma concentrations. As nutrition during the immediate post-operative course in patients undergoing oesophagectomy can be difficult (due to the nature of the operation), consideration should possibly be given as to whether the recommended daily intake of Vitamin D provided in enteral and parenteral feeds is sufficient to maintain plasma concentrations and future studies should consider dosing regimens carefully using pilot studies.

8.1.4 Patients with lower 1,25-$(OH)_2$ Vitamin D plasma concentrations have reduced long term post-operative survival

I found that patients recruited to the BALTI cohort with low plasma concentrations of 1,25-$(OH)_2$ Vitamin D have lower post op survival than patients with higher plasma concentrations. As the majority of patients who died did so following recurrence of their oesophageal tumour I investigated whether this finding was due to an effect of Vitamin D on NK cells. While I found that patients who survived at least two years have greater numbers of preoperative NK cells than patients who died, and there was a trend towards patients who survived being more likely to have received cholecalciferol, I found no evidence of an effect of Vitamin D on NK cell numbers or function. One limitation of this part of my study is that I was unable to test NK cell function in cells from patients undergoing oesophagectomy, due to restrictions in the amount of blood we could collect from the patients (around 20ml blood would be required in order to isolate sufficient cells to carry out the functional experiment, especially in the post-operative samples where total numbers of lymphocytes are low). It is possible that cells from patients with oesophageal cancer react differently in the presence of Vitamin D to cells from healthy controls as Ota et al have demonstrated in women with recurrent pregnancy loss (213).

8.1.5 Incidence of ARDS post oesophagectomy has decreased over past 5 years

Whilst we found no difference in clinical diagnoses of ARDS between groups in patients recruited to VINDALOO, the incidence of ARDS in patients undergoing

oesophagectomy was markedly lower in patients recruited to VINDALOO compared with patients recruited to BALTIp. This may reflect the differences in Vitamin D plasma concentration between the two groups, but is also likely to be due to improvements in perioperative care with greater awareness among anaesthetists around the importance of protective lung ventilation and the dangers of liberal IV fluid administration. This change in the incidence of ARDS post oesophagectomy is important for the design of trials using this model and may mean that larger cohorts need to be recruited in future, particularly where the primary endpoint is development of ARDS.

8.2 Future Work

There are many possibilities for development of my findings. As patients with ARDS have significantly lower plasma concentrations of both 25-OH Vitamin D and 1,25-$(OH)_2$ Vitamin D than patients undergoing oesophagectomy and I have demonstrated that high dose Vitamin D is capable of rapidly increasing circulating Vitamin D plasma concentrations, trials of cholecalciferol supplementation in patients with ARDS should be considered. Trials of oral supplementation in general ITU populations have already been undertaken using high dose cholecalciferol (220) but found that any effect on circulating Vitamin D plasma concentrations was limited, suggesting that in this population intramuscular or intravenous dosing should be considered, along with regular "top-up" dosing throughout the ITU admission. However, another group investigating cholecalciferol supplementation in patients with severe sepsis and

septic shock (who are at high risk of ARDS) found that a one-off dose bolus of 200,000IU or 400,000IU rapidly increased circulating Vitamin D plasma concentrations (221). Another group has investigated intravenous 1,25-$(OH)_2$ Vitamin D administration in critically ill patients and found an effect on cathelicidin and IL10 mRNA expression by leucocytes but did not measure circulating 1,25-$(OH)_2$ plasma concentrations in order to directly observe the effect of the dose administered on Vitamin D plasma concentrations (222). These studies highlight the importance of undertaking small scale dosing studies prior to undertaking large scale clinical trials in relevant patient groups.

My findings support the literature showing that NK cell number and function are important determining factors in survival post cancer surgery. Whilst I found no evidence that Vitamin D affects this parameter, my finding that preoperative NK cell numbers were higher in patients who survived at least 2 years post op highlights the potential of NK cells as a therapeutic target in patients undergoing cancer surgery. In addition, future work investigating other therapies in patients undergoing cancer surgery should consider measuring impact on NK cells.

8.3 Conclusions

In conclusion, a one off high dose preoperative cholecalciferol supplement is a safe and effective way of increasing preoperative Vitamin D plasma concentrations and is associated with lower perioperative increases in inflammatory alveolar oedema. Preoperative Vitamin D supplementation should be considered in patients with and at risk of Vitamin D deficiency. Trials of the

effect of cholecalciferol in other patients with conditions placing them at risk of ARDS (such as sepsis or pneumonia) should be considered, but will require careful dose response studies prior to large scale trials.

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