OESOPHAGECTOMY AS A MODEL OF THE ACUTE RESPIRATORY

DISTRESS SYNDROME

by

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ABSTRACT

Acute Respiratory Distress Syndrome (ARDS) is a life-threatening illness which can follow major surgery, including oesophagectomy. This thesis aimed to confirm the importance of ARDS in this cohort and assess the effects of GSK2862277

Methods

Analysis of previous oesophagectomy trials modelling ARDS sought differences between the studies and identified risk factors. The immunomodulatory effects of oesophagectomy and critical illness and novel therapeutic GSK2862277 on macrophage and neutrophil function were investigated using *in vitro* assays.

Results

Previous trials showed the harm to patients associated with ARDS, but falling ARDS rates more recently. Active smoking and pre-operative dihydropyridine use were risk factors for ARDS. Oesophagectomy and critical illness modulate neutrophil extracellular trap formation but not phagocytosis. GSK2862277 appears to cause an off-target effect increasing neutrophil extracellular trap formation. GSK2862277 increases alveolar macrophage phagocytosis.

Discussion

Perioperative ARDS has decreased following oesophagectomy although it is harmful to patients who develop it. Oesophagectomy is no longer useful as a model of ARDS. Major surgery and critical illness effect neutrophil function, which may drive complications in these cohorts. Macrophage function was modulated by GSK2862277, suggesting it may have promise in future for preventing or treating ARDS and other post-operative pulmonary complications.

DEDICATION

This work is dedicated to my daughter Nina Kamlesh Howells.

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Chapter 1

INTRODUCTION

1.1 Surgery, perioperative risk and outcome

Surgery is performed with the aim of curing, improving or palliating disease. The modern era of surgery has only been possible with the advent of anaesthesia, permitting optimisation of the surgical field and making invasive procedures both physiologically and psychologically tolerable for the patient. Perioperative mortality is now low, especially in developed nations with advanced healthcare [1], but some patients and procedures are associated with increased risk of mortality and post-operative morbidity [2, 3]. Risk of death attributable specifically to anaesthesia fell over ten-fold from before the 1970s to the 1990s [4]. For very high risk patients, surgery remains much more dangerous, with a 48-fold increase in risk for those who are American Society of Anesthesiologists' (ASA) Score IV-V, compared to I-III [1]. The proportion of high risk patients has increased over the decades [1], due to the increased frailty and medical complexity of patients requiring surgery [5]. Perioperative complications adversely affect the patient and increase healthcare cost [2, 6], therefore prevention, rescue, complication limitation and mitigation are all important strategies to develop for optimal care and outcome [7].

1.2 Oesophagectomy

One such high-risk surgical intervention is oesophagectomy [8, 9]. Oesophagectomy is usually performed for carcinoma of the oesophagus or pre-neoplastic lesions, but also occasionally for severe benign diseases. The sources of perioperative risk are multifactorial. Patients affected are typically middle-aged or older. Squamous cell carcinoma is associated with alcohol and cigarette consumption and poor oral hygiene and therefore, often there is comorbid

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ischaemic heart disease, vascular disease and/or COPD, whilst adenocarcinoma is associated with obesity and gastro-oesophageal reflux [10].

Upper gastrointestinal surgery was associated with the highest risk of complications in the International Surgical Outcome Study (ISOS) [2]. Multi-cavity surgery is required for most oesophagectomy surgery, frequently necessitating one-lung ventilation, associated with a risk of respiratory complications [11]. The anastomosis is formed at the extreme end of the supply of the foregut and is therefore vulnerable to ischaemia (Figure 1) [12]. Infections in the mediastinum can be devastating [13]. Post-operatively, it is challenging to manage pain, nutrition, thromboembolic risk and rehabilitation back to normal activity.

Figure 1: The formation of the gastric conduit and its blood supply following oesophagectomy. Note the anastomosis is formed at the extreme end of the foregut, therefore at the point furthest from the origin of its arterial supply from the coeliac axis [13].
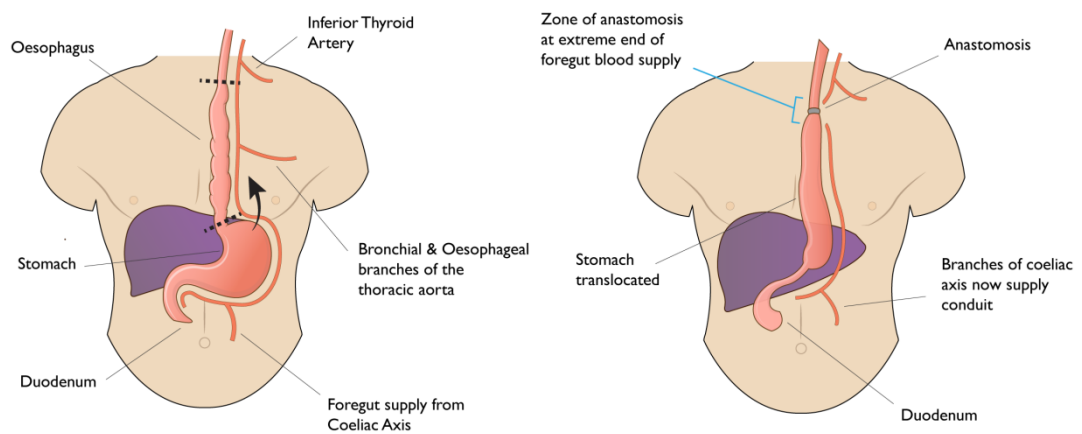
Efforts have been made to reduce risk and optimise outcome. Moving oesophagectomy to high volume centres is associated with lower mortality [14], although best surgical technique remains to be resolved [13]. Minimally invasive techniques (involving laparoscopic and thoracosopic or even robotic techniques) are increasingly used and associated with lower pain, pulmonary complications, length of stay and better patient quality of life score in experienced centres [15].

1.3 Post-operative pulmonary complications

Post-operative pulmonary complications (PPCs) are the most common complication following oesophagectomy [16]. What qualifies as a PPC varies between studies [17], although attempts have been made to produce international consensus definitions [18]. A range of patient, disease and surgical factors contribute to the high risk of PPCs in the oesophagectomy cohort.

Patients are harmed by PPCs. There is increased mortality in both the short- [19, 20] and long-term [21, 22]. This has been demonstrated in patients undergoing oesophagectomy [23]. Morbidity is also increased, for example increased length of stay and intensive care utilisation [22, 24].

1.3.1 Patient related factors

Major risk factors for both oesophageal cancer and respiratory disease (in general and post-operatively) include smoking and alcohol consumption [10, 17]. Chronic obstructive pulmonary disease has been demonstrated to be a risk factor in a thoracic surgical cohort [22]. Both smoking and alcohol use have been associated with Acute Respiratory Distress Syndrome (discussed further below) [25].

1.3.2 Surgical and anaesthetic factors

Surgery for oesophagectomy involves both abdominal and thoracic phases [26]. The surgical intervention is by definition pro-inflammatory [27], and even minimally invasive techniques represent a significant "hit" to the patient [13, 15, 28].

1.3.2.1 Perioperative ventilation

General anaesthesia, especially with neuromuscular blocking drugs, is associated with a number of processes that adversely affect the respiratory system, including loss of respiratory drive, altered lung mechanics, atelectasis, impairment of the mucociliary escalator, adverse effects of hyperoxia and denitrogenation and post-operative respiratory dysfunction [17, 29]. Traditional anaesthetic techniques for perioperative ventilation included using a large tidal volume as a method to reduce atelectasis [30, 31]. Some anaesthetists have felt relatively short periods of ventilation, even without a lung protective strategy, were too brief to cause harm [31]. A meta-analysis of available controlled trial data, including 2127 patients in total [32], showed there were fewer PPCs in the lung protective group and those who developed a PPC had longer ICU and hospital stays and higher mortality. ASA score, surgical type, body mass index and gender did not modify effects. Lower tidal volume in those with PEEP was associated with fewer PPCs, but had no effect on length of stay or mortality. A recent meta-analysis demonstrated reduced ARDS in elective surgical patients provided with lung protective ventilation (low tidal volume and high PEEP), although no difference in pneumonia or atelectasis [33].

There are benefits to protecting the lung with low tidal volume ventilation intra-operatively even in circumstances where the lung is healthy, although the role of PEEP remains to be better elucidated by further trials [34, 35]. An intriguing retrospective study has suggested that there may be benefit of volume-controlled

over pressure-controlled ventilation [36] but this has been criticised [37] and requires assessment in prospective trials. In patients starting surgery with healthy lungs, much of the damage to the lung is due to the harmful effects of ventilation.

1.3.2.2 Ventilator Induced Lung Injury (VILI)

During conventional mechanical ventilation, intermittent positive pressure ventilation exposes regions in the lung with differing mechanics to excess overload (volutrauma), excess pressure (barotrauma) and periods of repeated recruitment and airway closure (atelectrauma) which can primarily cause injury to the alveolar epithelium and endothelium or exacerbate the inflammatory process in an already-vulnerable lung (the multi-hit hypothesis) [38]. The worsening of ARDS by inflammatory processes is termed biotrauma [39]. More recent work has unified these mechanisms. Collapsed areas act as alveolar stress concentrators, driving damage in adjacent areas of lung [40].

Raised capillary pressure has been shown in vitro to be associated with pro-inflammatory endothelial signalling [41]. Mechanical overstretch leads to mediator release, then disruption of intercellular contacts, causing leak and, if severe, rupture of plasma cell membranes causing necrosis [39]. There is complex interplay between mechanical stress, immunological and coagulation processes driving

1.3.2.3 One Lung Ventilation

One Lung Ventilation (OLV), required for the thoracic phase in most surgical techniques for oesophagectomy [13] is injurious to the lung. Ventilating one lung subjects the ventilated lung to volutrauma, barotrauma and biotrauma and potentially atelectrauma as well as high inspired oxygen tension. The deflated lung

will become atelectatic and form alveolar stress concentrators, then re-recruited with high airway pressures, with ischaemia-reperfusion and biotrauma [40, 42]. Handling may cause physical trauma, although this tends to be less injurious than resection [42].

1.4 Strategies to prevent PPCs.

Recommendations for lung protection specifically for OLV include recruitment manoeuvres before OLV, minimising the duration of lung isolation, application of CPAP to the deflated lung if possible, protective tidal volumes in the ventilated lung (4-5mlkg^{-1}), the application of PEEP and permissive hypercarbia. With the restoration of two-lung ventilation, hyperoxia should be avoided [42].

Post-operative ventilation has received little attention, perhaps because of the adoption of lung protective ventilation for most patients in ICU. Ventilation practices following cardiac surgery are more heterogeneous, with concerns about raised arterial carbon dioxide levels adversely affecting right heart function [43]. However, one study in patients undergoing cardiac surgery showed increased organ failure, longer duration of mechanical ventilation and haemodynamic instability in those with "standard" ventilation (10-12mlkg^{-1}), compared to low tidal volume as less than 10 mlkg^{-1} [44].

1.5 The Acute Respiratory Distress Syndrome

Amongst the most severe respiratory complications is the Acute Respiratory Distress Syndrome (ARDS). Post-operative ARDS is associated with a mortality of 23.9%, a risk exceeded only by cardiac arrest [2]. The current clinical definition (the Berlin Definition) [45] consists of:

- Acute hypoxia (arterial oxygen tension to inspired oxygen fraction (P:F) ratio of less than 40kPa).

- 5cmH$_2$O or more of positive end-expiratory pressure (PEEP)/continuous positive airways pressure.

- Bilateral chest x-ray infiltrates (not fully explained by lung collapse, effusions or nodules), and not fully explained by cardiac failure or fluid overload.

- Within a week of onset or worsening of respiratory symptoms.

Previously, the North American European Consensus Definition (NAEC) was used [46]. Acute Lung Injury (ALI) defined as:

- A known acute cause.

- A P:F ratio less than 40kPa.

- Bilateral chest infiltrates.

- A pulmonary capillary wedge pressure of less than 15mmHg.

ARDS was defined by NAEC as a more severe subset of ALI with a P:F ratio of less than 26.7kPa [46]. For the purposes of this thesis, ARDS is used as an overarching term to refer to both ARDS as defined by the Berlin criteria and what was previously defined as ALI, unless otherwise explicitly stated.

Some studies suggest mortality has fallen over the last 20 years [47], whilst a recent cohort study showed mortality remains around 40% [48]. Most studies of therapeutic interventions in ARDS have been performed in critical care patients, with fewer in the perioperative setting.

Ventilation using low tidal volumes (6 rather than 12mlkg^{-1} based on ideal bodyweight) was associated with a mortality reduction of 8.8% in established ARDS

[49], and lung protective ventilation has been shown to prevent ARDS in ICU [50]. A recent meta-analysis demonstrated reduced need for post-operative ventilator support with intra-operative use of low-tidal ventilation, although mortality or length of stay was not affected [51].

Prone ventilation has mortality benefit in more moderate to severe ARDS [52]. Clearly, this is of limited use intraoperatively. Extracorporeal membrane oxygenation is increasingly used for the most severe ARDS, however its use currently is limited to patients with a very high predicted mortality and is restricted to subspecialist centres [53].

A trial of muscle relaxants showed a reduction in adjusted mortality for ARDS at day 90, with fewer ventilated and ICU days, although overall mortality was not affected [54]. The role of steroids requires further clarification [55], but steroids are now no longer used routinely [56]. The lack of other successful therapies, including negative trials for intravenous salbutamol [57], simvastatin [58], nitric oxide [59] and exogenous surfactant [60] suggests that preventing ARDS may be more fruitful.

The risks associated with ARDS in oesophagectomy are substantial. A landmark study (using NAEC criteria) demonstrated an incidence of ALI of 23.8% and ARDS of 14.5%. Mortality in those with ARDS was 50% compared to 3.5% in those without any lung injury and there was an association with cardiorespiratory instability [8].

Methylprednisolone is used frequently in Japan to reduce complications, including ARDS, following oesophagectomy [61] but this is yet to be well supported by robust clinical trials. A meta-analysis of the use of pre-operative methylprednisolone demonstrated a reduction in, cardiovascular complications, respiratory complications, hepatic dysfunction, sepsis, anastomotic leave, length of stay and

combined organ dysfunction, but not mortality or renal dysfunction [61]. The neutrophil elastase inhibitor sivelestat is licenced in Japan and South Korea for use in ARDS, although it is not in clinical practice in the Europe or the USA and has been evaluated for established ARDS and lung protection during oesophagectomy [62]. Use of intra-operative and post-operative infusions have shown reduced risk of ALI and reduced duration of post-operative mechanical ventilation by day five (though not at day three) [62]. Given the European and North American practice of early post-operative extubation [12, 63], its applicability to current practice remains to be determined.

1.6 Inflammatory processes in the lung

Inflammation is a key component of the development of and complications related to ARDS. Pathogen and damage associated molecular patterns (PAMPS and DAMPs respectively) have been shown to initiate pro-inflammatory cytokines by alveolar macrophages, including TNF alpha and interleukins (IL) 1-beta, 8 and 10 [64, 65]. Lung epithelial cells and fibroblasts may also secrete cytokines [65]. Epithelial and endothelial barrier failure allows protein-rich extracellular fluid to flood the alveoli. Injury to type two alveolar cells reduces alveolar fluid clearance and surfactant production [65]. Subsequent failure for the epithelial layer to heal can lead to fibrosis, with increased extracellular matrix formation triggered by fibroblasts [65]. Neutrophil recruitment is very important (discussed below).

VILI, both by itself and in ARDS, is associated with inflammation [39]. Over-distension of the lung will trigger pro-inflammatory genes, a process which may be seen even in non-injurious ventilation [66], and cytokines release may occur, even without necrosis [39]. During surgery, the insult of the controlled tissue injury drives

inflammation, driven by DAMPS. DAMPs are a variety of different molecules which trigger immune modulation via Pattern Regulation Receptors (PPRs). A number of these share homology with PAMPs, which arise from exogenous pathogens. As a result, there is a molecular convergence in the immune response to the controlled damage of surgery and other insults, such as burns, pancreatitis and sepsis [67, 68].

Perioperative vulnerability of the lung to inflammation arises from a number of sources. The risks of VILI are discussed above. Anaesthesia causes a fall in the functional residual capacity, altered lung mechanics, increases alveolar stress concentrators [40], impairs ciliary clearance and airway devices overcome upper airway immunological defence mechanisms [69]. Residual anaesthesia, high inspired oxygen fraction, inadequate humidification of gases, opioids and pain may lead to reduced cough, sputum retention and atelectasis [69]. Volatile anaesthetic agents are thought to be anti-inflammatory [70] but may drive complex immunomodulatory effects [71].

1.7 Macrophages

Macrophages are a crucial cell in the innate immune system [72]. They are active against external pathogens and key immune regulators [72]. Macrophages have been broadly sub-typed into M1 (primarily targeting intracellular pathogens) and M2, which are broadly pro-resolution of inflammation [73]. M1 activity includes cytokine secretion, reactive oxygen species (ROS) formation, phagocytosis and the presentation of antigen [72] as well destroying pathogens and host tissue [74]. M2 function appears important in fungal and helminth infections, allergy and tumour pathogenesis [74]. M2 macrophages have been shown to be crucial in the

resolution of lung inflammation and the recovery of ARDS, including by clearing neutrophils and releasing anti-inflammatory cytokines [75]. The M1/M2 phenotype probably oversimplifies a much more complex array of macrophage activities [73, 76].

1.8 Neutrophils

Neutrophils are another crucial component of the innate immune system, responsible for the following functions:

- Chemotaxis towards a stimulus.

- Phagocytosis.

- Intracellular killing.

- Release of inflammatory mediators.

In addition, more recently an additional function has been recognised – the Neutrophil Extracellular Trap [77].

1.9 Phagocytosis and intracellular killing

The mechanism of phagocytosis remains incompletely understood, but its vital importance is highlighted by its conservation amongst diverse eukaryotic cells [78]. Neutrophils may target pathogens directly, but more often require the opsonisation of the targets by immunoglobulins and/or complement [79]. Neutrophils express groups of a variety of phagocytosis-triggering receptors to initiate phagocytosis [80]. Following recognition, where ligand-gated binding triggers a cascade of intracellular processes which cause disassembly and reconstruction of the actin cytoskeleton of the cell, which in turn causes the membrane bilayer to envelop the bacterium or other pathogen, forming a phagosome [78]. Up to 1000 proteins may be involved

[78]. Both active and passive function of the zipper mechanism of actin reassembly makes phagocytosis a reliable immune mechanism at the cellular level [78]. Incomplete invagination leads to partial phagosome formation, indicating phagocytosis is not a binary all-or-nothing process [78]. Once the phagosome has formed, it then fuses with intracytoplasmic granules which attack and degrade the micro-organism. Binding of ligands to neutrophil cell surface receptors upregulates reactive oxygen species formation which provides additional mechanisms to kill the target [79].

Killing of the ingested pathogen is driven by granule formation and reactive oxygen species [79]. There are a range of granules (previously classified as primary or azurophil (myeloperoxidase positive) and secondary (myeloperoxidase negative)) [79]) which results from changes in the proteins synthesised as neutrophils mature. These proteins include receptors, chemokines and other cytokines and components of the apoptosis pathway. Neutrophils express low levels of receptors under basal conditions, but these are up-regulated following stimuli [81].

Granules contain myeloperoxidase, which generates hypochlorus acid, and multiple bactericidal proteins, as well as bacterial growth inhibitors, such as lactoferrin which binds iron [79]. Lysozyme damages bacterial cell wall integrity by degrading peptidoglycan. NADPH-oxidase is a multi-component enzyme that generates superoxide anions which are highly destructive to biological tissues. Interaction with other granule components can form other toxic species, including hypochlorus acid, hydrogen peroxide, hydroxyl radicals and single ionised oxygen atoms. The NADPH-oxidase system is highly regulated and assembled specifically in phagosomes [79].

1.10 The Neutrophil Extracellular Trap

The Neutrophil Extracellular Trap (NET) was first described in 2004 [77] and may be a crucial component of ARDS pathogenesis [82]. In response to an appropriate stimulus, there is chromatin de-condensation, disintegration of the nuclear membrane, followed by the association of nuclear and cytoplasmic structures, which is then followed by cell rupture and the release of a NET [83]. Whether this process is active and physiological or a convenient consequence of cellular rupture remains debated [84]. NETosis is known to be driven by bacterial, fungal and parasitic pathogens and a range of cytokines, including TNF alpha [85] and immune complexes signalling via C5a and C5aR1 and 2 [86].

NETosis commences with reactive oxygen species causing the disintegration of neutrophil granules, with myeloperoxidase and neutrophil elastase reaching the nucleus. Histone modification by protein arginine deiminase 4 (PAD4) causes chromatin decompensation. This now unwinding DNA associates with proteins from the granules ahead of the neutrophil membrane and this structure now forms a web in the intercellular space [87].

NETs can kill and/or prevent movement of bacteria *in vivo*, including limiting the spread of bacteria from upper to lower respiratory tract and from lung to bloodstream. NETs have also been shown to have anti-fungal and anti-viral functions [87]. NETosis has also been implicated in hypercoagulability. Tissue factor secretion (vital to triggering the coagulation cascade) during NETosis has been observed and neutrophil elastase has also been shown to deactivate tissue factor inhibitors and promote factor Xa activity [85]. Markers of leukocyte and platelet function have been associated with organ failure in patients with sepsis [88]

Patients with metabolic failure of reactive oxygen species generation, who phenotypically have Chronic Granulomatous Disease, are unable to form NETs, but this can be restored in vitro in the presence of hydrogen peroxide [85]. Intriguingly, NET formation has been associated with both myeloperoxidase and neutrophil elastase [85, 89]. Neutrophil elastase is necessary for nuclear decompensation and neutrophil death is lower in the presence of neutrophil elastase inhibitor. Neutrophil elastase is necessary for NET formation and mice with neutrophil elastase knockout did not form NETs. DNase I can reduce NET formation [90].

1.11 Neutrophils, macrophages and ARDS

The neutrophil is a critical cell in ARDS. Both infection-triggered and sterile ARDS models have shown activation and mass-migration of neutrophils into the alveolar space, driven by chemokines from epithelial cells, macrophages and other neutrophils [85]. These factors can promote NETosis, whilst decreased surfactant protein levels (SPA and SPB) reduce NET clearance [85], alongside surfactant deficiency being harmful itself. Although peripheral white blood cell counts are lower in ARDS versus at-risk patients, bronchoalveloar neutrophil counts are increased in ARDS and neutrophils from septic patients damage *in vitro* endothelial layers [82].

Alveolar cell injury and increased alveolar-capillary permeability arise from the various direct and indirect mechanisms discussed above. Some animal models show reduction in ARDS with neutrophil depletion: this includes LPS, VILI, acid-induced and transfusion-associated, whereas oleic acid and hyperoxia induce ARDS phenotypes with capillary-alveolar leak even with neutrophils depleted [82]. ARDS has been described in neutropenic patients, suggesting the neutrophil is not essential for ARDS [91].

In a murine influenza ARDS model, macrophage depletion was associated with clinical illness and higher viral replication, whereas neutrophil depletion was not. Macrophage depletion led to increased neutrophil numbers and worse diffuse alveolar damage histologically. Neutrophil-depleted rats did not develop histopathological evidence of ARDS, although they did have bronchitis and peribronchial inflammation by day five. NET formation peaked on day 10, and was in areas of heavily damaged lung tissue, worst in macrophage depleted animals. Myeloperoxidase activity was higher in the macrophage depleted group. They went on to show wild-type mice had NETS in infected, consolidated areas of lung and haemorrhagic lesions, when challenged with lethal doses of influenza [92]. .

The instillation of histones (a key component of formed NETs) instilled into the lungs of mice produce epithelial damage, alveolar flooding and haemorrhage and abnormal thrombus formation in the lung's venules. Neutrophil depletion reduced histone levels [93]. Humans with ARDS showed histones were present in ARDS bronchoalveolar fluid but were barely detectable in controls [93]. In a two-hit ARDS model, lipopolysaccharide (LPS) combined with high-volume ventilation induced NETosis, but NETosis did not increase with LPS alone [90]. It may be that NETosis is driven by secondary insults and becomes more important in with multiple pathologies.

Preventing neutrophil degranulation reduced lung injury and vascular permeability in a *Streptococcus pyogenes* model [82]. Neutrophil elastase damages the endothelial cytoskeleton, targeting actin, E-cadherin and VE-cadherin, contributing to increased alveolar-capillary permeability, induces apoptosis and releasing pro-inflammatory cytokines [85]. Neutrophil elastase inhibition has been shown in animal models to have protective effects [82] and sivelestat is used clinically in

Japan to treat ARDS and prevent ARDS following oesophagectomy (discussed above). As there is yet to be an agent developed which selectively inhibits NETosis without modulating other neutrophil functions, it remains to be conclusively determined how important NETosis itself is to ARDS, or whether it represents a marker of neutrophil presence [94].

NETs can also cause microvascular thrombosis and endothelial dysfunction as well as mediating neutrophil-platelet interactions [95]. Platelets are increasingly recognised as having pro- and anti-inflammatory effects, and disordered coagulation is an important pathogenic mechanism in ARDS [94]. NETosis has been linked with transfusion-related acute lung injury (TRALI). TRALI is pathologically distinct from ARDS, in that donor anti-neutrophil antibodies (major histocompatibility complex class one) react with recipient neutrophils causing sequestration in the pulmonary vasculature. The importance of NETosis in TRALI has been confirmed in animal models and supplementary supporting evidence in humans [96].

In surgery, NETosis has been shown to form following ischaemia-reperfusion from liver resection, with larger surgical insult being associated with higher NETosis [97] and NETosis has been associated with primary graft dysfunction following lung transplant [98].

The deposition of immune complexes has also been associated with ARDS [86]. Furthermore, inhibition of complement C5a or its receptors (C5aR1 and C5aR2) can protect against the development of ARDS in mouse models [86].

1.12 TNF alpha

The Tumour Necrosis Factor (TNF) and TNF Receptor (TNFR) superfamily is highly conserved in nature and has vital functions animals as diverse as mammals, zebrafish, molluscs, arthropods and corals [99]. TNF alpha (initially called cachectin or differentiation inducing factor) plays an important role in the inflammatory response. Initially described as an agent that allowed macrophages to exert control over established tumours in mice [100], it has an array of roles in infection and the response to malignancy [101].

The TNF superfamily has 19 ligands, and 29 receptors have been described to date [99]. TNF alpha is a type two transmembrane glycoprotein consisting of three monomer units. Production largely occurs in macrophages and T lymphocytes, but can also occur in other immune (B lymphocytes, natural killer cells, neutrophils) and non-immune cells (endothelial cells, smooth muscle, cardiac muscle, fibroblasts and osteoclasts) [102].

TNF is physiologically available in soluble and membrane-bound forms. This contributes to differential effects (see below). Transmembrane TNF has a molecular weight of 75kDa, higher than would be predicted by its amino acid sequence, probably from glycosylation and phosphorylation whilst soluble TNF has a molecular weight of 55kDa [103]. Transmembrane TNF is cleaved by TNF-alpha-converting enzyme (TACE, also known as ADAM17) to a soluble 17.6kDa active unit, although other proteases can have the same effect [101, 104, 105]. TACE is a member of the adamalysin family of zinc-binding metalloproteinases and is expressed in a wide range of tissues [106]. In TACE gene knockouts, serum TNF levels fall, whilst membrane bound levels are higher [106].

1.13 Intracellular signalling from TNFR1 and TNFR2

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Intracellular signalling from TNFR1 and TNFR2 is complicated and not yet fully understood, particularly for TNFR2 [107]. The TNF superfamily's receptor signalling is broadly classified into two: the death domain receptors (which bear a death domain that can trigger apoptosis) and a second group with a TRAF (TNF receptor adaptor factor) interaction motif (TIM) domain, which are able to bind TRAF proteins (although they may also be able to signal for apoptosis through this indirect route) [108].

TNFR1 assemble as trimers prior to ligand binding, which is essential for signal transduction, as TNF alpha binding changes the orientation of these components [105, 109]. Signal transduction may result in apoptosis, necroptosis or cell survival, depending on the post-receptor modulation, which illustrates the complexity of TNF alpha's activity. TNFR1 responds to both soluble TNF and membrane-bound TNF [108]. Upon ligand-binding, two receptor signalling complexes form, with both spatial and temporal separation. Complex one activates anti-apoptotic pathways, whilst complex two (death-inducing signalling complex (DISC)) triggers pro-death processes once the receptor has been internalised [108].

Complex one consists of TNF Receptor Associated protein with Death Domain (TRADD) interacting with TNFR1 via its death domain, alongside a number of other adaptor proteins, including TRAF 2, cellular Inhibitor of Apoptosis (cIAP) 1, cIAP2 and Receptor Interacting Protein (RIP) 1. This complex in turn activates Mitogen Activated Protein Kinase MAP3K, N-terminal jun kinase (JNK) and subsequently AP-1, whilst it may also acquire LUBAC (linear ubiquitin chain assembly complex), which activates I kappa B kinase (IKK) and so upregulates NF-KB [108].

TNFR1 complexes may also be internalised [110], deubiquitinated and form the DISC [108]. This intracytosolic vesicle contains TNFR1 associated with RIP1, TRADD, FADD and caspase-8. Deletion of this death domain inhibits apoptotic signalling [110]. If NF-KB has been activated, cFLIP inhibits complex two to prevent caspase-8 activation. If NF-KB is inactive, no such process occurs and the cell will become apoptotic. If caspase-8 is inhibited or deleted, RIP1 and 3 can be phosphorylated and trigger necroptosis [111], although this cannot occur in some cell types [108].

NF-KB is released from NF-KB inhibitor-α, via IKK. NF-KB moves to the nucleus and promotes an array of genes [101] NF-KB has five family members in mammals, NF-KB1. NF-KB2, RelA, RelB and c-Rel [112]. These can promote a range of gene modulations which are responsible for the manifestations for inflammation. In ARDS, NF-KB upregulation is a crucial component of ARDS fibroproliferation [113].

Overall, TNFR1 promotes cell survival in most cell types, alongside inflammation and chemokine synthesis, whilst promoting death of infected and damaged cells, and orchestrating both organ and behavioural responses to infection, such as fever and sleep [107].

TNFR2 responds to membrane-bound TNF, whilst soluble TNF is a much less effective ligand [114]. When membrane bound TNF binds to TNFR2, receptors trimerise and initiate an intracellular signalling cascade. This interacts directly with TRAF 2, and via TRAF2, TRAF1 and 3 and cIAP 1 and 2 are activated. TRAF2 activation increases NIK, which in turn decreases IKBA, activating NFKB. TRAF3, cIAP1 and cIAP3 trigger proteolytic processing of p100, which also leads to NFKB activation. MAP3K activation also upregulates JNK [108]. In vitro models have

shown both TNFR1 and 2 signalling is needed to trigger apoptosis [115]. TNFR2 lacks a death domain, so direct apoptosis signalling is not possible [109].

It appears TRAF2 is subject to phosphorylation initiated by TNFR2, which leads to TRAF2's ubiquitination and proteasome-dependent degradation, although a number of other possible regulatory pathways have been described [108]. TRAF2 degradation via TNFR2 inhibits anti-apoptotic signalling in some situations and this implies cooperative signalling for apoptosis between TNFR1 and TNFR2 [108]. TNFR2 has been shown to protect against both ischaemic and excitotoxic effects in the central nervous system [107].

Although it has the capacity to trigger apoptosis indirectly, it is thought TNFR2 primary role in cell signalling is to trigger cell survival and differentiation [101]. In T-helper cells, TNFR2 is important for T regulator cell differentiation, proliferation and lineage stability [116].

Both TNFR1 and 2 are cleaved by TACE (like TNF alpha) and shed, which decreases cell surface expression and also allows the free receptor to bind TNF, reducing the circulating pool [104]. TNFR2 levels are down-regulated via reduced transcription and by receptor shedding. The resultant TNFR2 is able to bind TNF but signalling function is lost – acting in two ways to down-regulate TNF by reducing ligand abundance and signal transduction apparatus [103, 117].

Figure 2: differential signalling pathways for TNFR1 and TNFR2. TNFR1 is stimulated via both membrane-bound and soluble TNF, whereas TNFR2 responds to membrane-bound TNF only. TRADD allows TNFR1 to trigger both pro- and inflammatory pathways, providing complex regulatory interplay in the TNF signalling cascade.
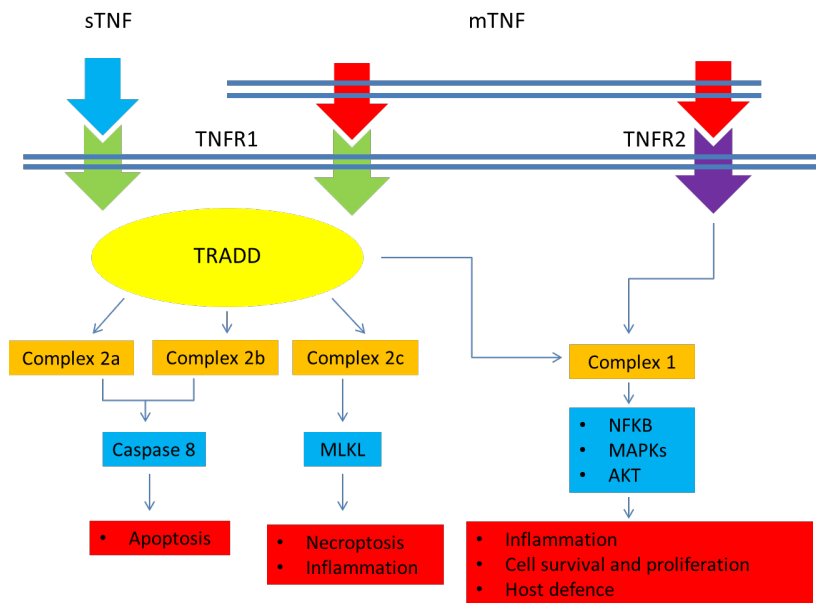
1.14 The role of TNF alpha in immunity

TNF and TNFRs are important for the effective immune response but also many of the harmful pathophysiological processes seen in sepsis [102]. TNF alpha's effects on the endothelium promote capillary leakage and neutrophil migration, pro-coagulation effects and anti-viral response in epithelial cells [118]. Both capillary leakage and local coagulation are important in the pathogenesis of ARDS [113]. Macrophages both secrete TNF and are activated by it [118]. Systemic responses include fever, hepatic modulation of acute phase reactants and haemopoietic regulation [118].

Mice deficient in TNFR1 and/or TNFR2 receptors have apparently normal development and homeostasis in unstressed conditions [119]. Mice lack normal lymphoid architecture and germinal cell formation as well as having dysfunctional immune response and tissue repair processes [105]. TNFR1 knockout mice were susceptible to *Listeria monocytogenes* infection, dying at what would be sub-lethal doses in wild-type mice. This indicates TNF alpha's importance is the systemic immune response, although liver acute phase reactants were not different between knockout and wild-type mice, indicating preservation of multiple immune pathways. TNFR2 knockout mice were comparable to wild-type controls [119]. Both TNFR1/TNFR2 and TNFR1-alone knockout mice were protected against a combined lethal LPS-D-gal (a hepatotoxin potentiated by LPS) challenge, whilst TNFR2 knockout succumbed. Resistant mice showed no symptoms over five days. Sub-lethal dosing showed increased susceptibility in TNFR2 knockouts [119]. In contrast, LPS alone was less lethal in TNFR1, TNFR2 and TNFR1/TNFR2 knockouts than controls, indicating LPS lethality is not related solely to TNF alpha. This indicates although TNFR1 is critical for defence against pathogens, its function

is complex and clearly works in parallel with other immune pathways, resulting in overall pro- or anti-inflammatory effects dependent on the context [119].

TNF increases neutrophil phagocytosis, cytotoxicity, endothelial adhesion, degranulation and the length of the respiratory burst [120]. Roughly similar levels of TNR1 and 2 have been reported on neutrophils, whereas TNFR2 predominates on monocytes [121]. Other effects include regulation of organogenesis, neuronal remyelination, cardiac remodelling, cartilage regeneration and inhibition of tumorigenesis. Pathogenic functions include inflammation induction, necroptosis, inhibition of T-regulatory cells, tissue degeneration, hypernocioception, tumourigeneisis and atherogenesis [105].

1.15 TNFR signalling *in vivo*

Transgenic mice bred with a number of modifications to TNF signalling pathways to elicit the relative importance of TNF signalling by different cell types. Mice bred with uncleavable membrane bound TNF and exposed to an LPS challenge developed acute restrictive pulmonary dysfunction, unlike completely TNF deficient mice, which did not respond. BAL neutrophil and macrophage numbers and total protein levels (an indicator of lung permeability) were similar between mTNF and wild-type mice but mTNF mice showed reduced lung myeloperoxidase activity. Partial reductions in lung inflammation were seen in mice with TNF knockout in their macrophages and neutrophils compared to wild-type, with reduced neutrophil recruitment and protein leakage. In contrast, mice with TNF knockout T cells had exacerbated modelled acute respiratory dysfunction, with significantly higher neutrophil numbers [122]. This illustrates that, even in a comparatively simple

situation of modelled LPS-induced ARDS, TNF signalling is both pro- and anti-inflammatory, depending on the source of the signal.

There is an interplay between ligand, receptor and overall activity; in mice deficient in both TNFR1 and TNFR2, TNF levels were higher following an LPS challenge than in mice lacking either receptor, whilst lowest responses were seen in wild-type mice [119]. However, hepatic responses in terms of cytokine secretion were similar in both receptor knockout mice compared to wild-type controls, again demonstrating the complexity of TNF in the orchestration of the immune response [119].

TNFR knockout was examined in mice using *Micropolyspora faeni* as a pneumonitis stimulus. In TNFR1 and 2 and TNFR1 knockouts, neutrophil accumulation was markedly reduced, whereas lymphocyte and monocyte levels were comparable across strains. In comparison, TNFR2 knockout was associated with increased neutrophil influx into the lung [119]. However, genotype did not affect neutrophil migration in response to intranasal LPS [119]. Increased TNFR2 had been detected in the bronchoalveolar lavage fluid of patients with early ARDS (before day 5 in this study) or deemed at risk of ARDS, although not in the late ARDS group (after day 21) [123].

1.16 TNF alpha in disease states and as a pharmacological target

TNF alpha has been shown to upregulate leucocyte and platelet adhesion molecules, upregulation of thrombogenic and fibrinolytic pathways, augment other inflammatory pathways and upregulation of vasodilators including inducible nitric oxide. Administration of TNF in animal models produces patterns of organ dysfunction similar to sepsis [124].

An array of different inflammatory mediators, including TNF alpha, play a role in the development of ARDS [125]. TNF alpha causes pulmonary endothelial apoptosis and promotes neutrophil sequestration from the circulation to the pulmonary tissue. It increases reactive oxygen species generation, which increases myosin light chain phosphorylation and decreases epithelial sodium channel expression, promoting loss of the epithelial barrier and increased alveolar flooding [125]. Increased vascular permeability may also be observed from loss of barrier function of epithelial cells via cytotoxic effects [115, 123]. Microtubular disassembly in pulmonary artery endothelium has been demonstrated [126, 127]. However, TNF alpha has also been shown to promote alveolar fluid clearance via increased sodium channel activation in the alveolar endothelial cells, driven by signalling via its lectin-like domain [128, 129]. TNF alpha therefore may have a role in both the generation and resolution of non-cardiogenic pulmonary oedema [129].

There has been a therapeutic revolution with the development of antibodies that bind to cytokines to modulate disease, including in rheumatology, oncology, respiratory medicine, gastroenterology and haematology [101]. TNF inhibition is effective for rheumatoid and other arthritidies, ankylosing spondylitis, psoriasis and inflammatory bowel disease [101]. TNF appears to be anti-inflammatory in systemic lupus erythematous, whilst improvements have been shown in SLE arthritis and nephritis, but many trials have been abandoned due to high adverse event rates [130]. Multiple sclerosis is made worse by TNF inhibition [101]. Even successful treatment may be complicated by opportunistic infection, latent tuberculosis reactivation, lymphomas and autoimmune disease [101, 105].This indicates the complexity of TNF in disease processes, with positive effects in some groups and negative in others.

Agents to inhibit TNF were based on binding to TNF or TNFR to prevent signal transduction. It has been proposed more sophisticated targeting may prevent such complications, including natural anti-TNF immunisation, inhibiting TNF synthesis, blocking multiple cytokines and targeting down-stream signalling molecules [131]. Another strategy would be selective TNFR inhibition or stimulation [132, 133].

Animal models with TNFR1 and TNFR1/TNFR2 knockouts using a polymicrobial intraperitoneal sepsis model have improved survival [134]. A meta-analysis of clinical trials of anti-TNF agents sepsis in humans in showed a very modest net benefit, but this has not translated to clinical practice and uncertainty remains about timing and dosage of agents [135]. Given its effects, a trial requiring in excess 10000 patients would be needed to reliably demonstrate a benefit [136].

Conventional biologics are antibodies require parenteral administration because of their size and vulnerability to enteral proteases. However, it is the binding domains which are of crucial importance. A Domain Antibody (DAB) is the smallest units of an immunoglobulin that will bind, which may be generated from the heavy or light chains of a conventional immunoglobulin [137]. GlaxoSmithKline have developed an anti-TNFR1 DAB GSK2862277, which consists of the 13kDa fragment of a conventional anti-TNFR1 molecule's variable region and bind monovalently with the TNFR1 receptor to block signalling, avoid receptor stimulation [137]. Selective TNFR1 blockade has been proposed as a possible therapeutic strategy in multiple sclerosis [105, 107] and to prevent post-operative pulmonary complications and ARDS [132].

This novel agent has some evidence to support its utility for ARDS. In mouse VILI and VILI/LPS combined, a number of experiments were conducted [132]. In a pure

VILI model, the active DAB group were protected from deterioration in lung mechanics. DAB was shown to reduce neutrophil counts in the lung and lavage fluid and also reduced Intercellular Adhesion Marker-1 (ICAM-1). BAL protein and TNF levels were higher in the untreated group. Alveolar protein deposition and neutrophil migration were attenuated in the DAB group. Repeating the experiment with the VILI/LPS model showed similar results. Monoclonal anti-TNF did not show the beneficial effects of the DAB. The benefits of the DAB over monoclonal anti-TNF may be due to better delivery and/or tissue penetration or specific TNFR1 signalling modulation [132]. A further experiment, using inhaled LPS as a model of mild ARDS in healthy human volunteers, showed reduced inflammatory indices and lower BAL neutrophil counts [133].

As DAB was administered before the injurious stimulus, it may be most useful as a preventative agent or early in ARDS. This may differ significantly from the effects of the agent in established ARDS, and indeed may alter depending on the stimulus (for example, pneumonia or non-pulmonary sepsis may very well be different from VILI). Prevention of lung injury, using pulmonary vascular permeability index (PVPI) and extravascular lung water index (EVLWI) as biomarkers, has been tested in a clinical trial, using oesophagectomy as a model of ARDS (TFR116341 EudraCT Number: 2014-000643-33).

1.17 Summary

Post-operative respiratory complications and, in particular, ARDS are serious yet potentially preventable problems that follow surgery. Patients undergoing oesophagectomy are at particular risk of post-operative pulmonary complications and ARDS. Perioperative strategies to prevent ARDS may well have a significant

role to play in the reduction in harm to patients. TNF alpha may represent a useful therapeutic target and there are existing data to support this.

The original intention of this thesis was to analyse the clinical and biomarker data from the TFR116341 trial, its translational sub-studies. It was also planned to study and compare with prior trials utilising oesophagectomy as a model of ARDS to assess its continuing utility as a model, given the changes occurring in clinical practice in this cohort [13].

The aims of this thesis were to:

1. Confirm the importance of ARDS in the context of oesophagectomy.

2. Investigate the differences observed between trials which have used oesophagectomy as a model of ARDS

3. Seek insights into the evolving challenges of recruiting patients undergoing oesophagectomy to trials of perioperative pharmacological interventions.

4. Investigate perioperative immune modulation in oesophagectomy relevant to ARDS.

5. Investigate the effects of a novel agent (GSK 2862277) developed as a potential ARDS modulator in the context of:

    a. Modulation of neutrophil function in vitro.

    b. Modulation of macrophage function in vitro.

Chapter 2.

METHODS

2.1 The impact of the acute respiratory distress syndrome on outcome after oesophagectomy

Trial participants

Between April 2008 and June 2011, 362 adult patients undergoing elective oesophagectomy were enrolled into the BALTI-Prevention trial at 12 academic hospitals in the UK. The results have been published previously [138]. The North American-European Consensus Criteria were used to define ALI/ARDS: (ALI PaO2:FIO2<40.0 kPa; ARDS PaO2:FIO2<26.7 kPa) at the time and for the design of the study [46].

Intervention and Data collection

Baseline characteristics, operative information and postoperative variables were recorded for all participants. Anaesthetists were instructed to follow a low tidal volume and fluid conservative strategy, but otherwise management was left to the individual clinician's discretion. Patients were defined as having ARDS in the presence of hypoxaemia (PaO2:FIO2 ratio less than 40.0 kPa), bilateral infiltrates on the chest x-ray and absence of clinical evidence of left atrial hypertension and categorized as having early (day 0–3), late (day 4–28) or no ARDS according to the timing of the first episode of ARDS. The categorization of ARDS was made a priori into 'Early' and 'Late', to separate 'primary ARDS' associated with the initial insult of surgery and anaesthesia from that acquired by later complications (secondary ARDS), such as anastomotic leak.

Study outcomes were ventilator free days, organ failure free days, 28 and 90 day mortality and health-related quality of life measured by Euroqol Health Outcome

Questionnaire (EQ5D) at 28 and 90 days. Ventilator-free days were as previously defined.22 Organ failure–free days were defined in a similar manner, with an organ failure–free day being a day without evidence of non-respiratory organ failure. Organ failure was defined by a Sequential Organ Failure Assessment score of four or more.24 Postoperative pneumonia was recorded if diagnosed by the attending clinicians. As patients had undergone recent upper gastrointestinal surgery, non-invasive ventilation was not used as a standard measure, but was not strictly prohibited. Levels of care were determined according to United Kingdom Department of Health definitions [139].

Statistical analysis

Linear regression of secondary outcomes comparing ARDS status was undertaken with and without adjustment for randomization. Linear regression models were then fitted for the secondary outcomes for ARDS status with an interaction term, to examine whether treatment difference depended on observed ARDS status.

Multivariate logistic regression was performed to establish a risk model for ARDS, examining all recorded potential risk factors. A forward stepwise regression model was produced using the specified baseline variables used in the univariate analysis, with P values of 0.05 and P value of 0.1 for subsequent removal from the model.

Multivariate analysis was then fitted for each stage of ARDS, to examine whether the response to different treatments was dependent on baseline characteristics. An unadjusted model was fitted, including terms for treatment allocation, baseline moderation and terms for treatment by moderator interaction. An adjusted model was also produced, containing terms for treatment, moderator and interaction with terms for age and hospital.

Safety outcomes were analysed according to ARDS status. These included respiratory, cardiovascular, surgical and other complications and sepsis. Adverse events were defined as atrial fibrillation, ventricular bigeminy, hypokalaemia and sinus tachycardia. Serious adverse events included anastomotic leak, ARDS, arrhythmia, pleural effusion, pneumonia, chyle leak, respiratory failure, inoperable tumour, pneumothorax, sepsis, surgical complications and other. Data were analysed using STATA Version 11, (StataCorp LP, College Station, Texas, USA).

2.2 ARDS following oesophagectomy: a comparison of two trials

Details of the methods of the BALTI-P trial and the associated translational sub-study have been published previously [138]. Patients were randomised to either placebo or inhaled salmeterol preoperatively and postoperatively. At two hospital sites (Queen Elizabeth Hospital Birmingham and Birmingham Heartlands Hospital, UK), patients were recruited to the translational sub-study. The VINDALOO trial protocol has been published [140]. Patients were recruited at Queen Elizabeth Hospital Birmingham and Birmingham Heartlands Hospital, UK, and randomised to either placebo or a single dose of 300 000 IU of vitamin D. In both studies, patients underwent oesophagectomy with care provided as deemed clinically appropriate by the attending surgeons and anaesthetist and followed for their hospital stay.

Databases of the outcomes from the two trials were available for analysis. Smoking status was self-reported in both trials. We collected additional data retrospectively using medical notes, intensive care unit (ICU) charts, electronic patient databases and clinical letters, which provided the preoperative drug history, data for preoperative risk scoring and intraoperative drugs used. The administration of regular medications on the morning of surgery was at the discretion of the attending

anaesthetist. In the BALTI-P sub-study, patients were excluded if they did not undergo an oesophagectomy with attempted one lung ventilation (OLV). In VINDALOO, only patients who passed the primary endpoint of oesophagectomy with OLV and postoperative PICCO readings were included (consistent with the VINDALOO trial's analysis).

Differences in the baseline characteristics and perioperative care between trials were assessed. Outcomes for both trials were determined by a clinical endpoints committee. ARDS was defined using the Berlin criteria [45] for the VINDALOO trial. The BALTI-P trial pre-dates the Berlin criteria, which could not be applied, as applied positive end-expiratory pressure was not recorded. Therefore, we defined ARDS in the BALTI-P trial participants as those with a Pao2:Fio2 (P:F) ratio of 39.9 kPa or below, bilateral chest X-ray infiltrates, absence of cardiogenic dysfunction sufficient to explain pulmonary oedema (based on the opinion of the attending clinician) and requiring invasive ventilation (ventilation with positive end-expiratory pressure of 5 cm H2O was standard care in the ICUs involved and non-invasive ventilation was contraindicated in patients following upper gastrointestinal surgery at the time both trials were undertaken).

Statistical analysis.

Continuous variables were subject to normality testing using the Kolmogorov-Smirnov test. For the patients' baseline data and univariate analysis of perioperative factors, normally distributed continuous variables were analysed with Student's t-test, non-normally distributed data with the Kruskal-Wallis test and Mann-Whitney U-test and categorical data with the $X2$ or Fisher's exact test as appropriate. Those factors that were significant ($P<0.05$) were then subject to multivariate analysis.

Multivariate analysis of ARDS status was undertaken using forward conditional multivariable binomial logistic regression of the two significant factors in the univariate analysis. Analyses of baseline and univariate data were undertaken using GraphPad Prism V.6.07 for Windows (GraphPad Software, La Jolla, California, USA). Multivariate analyses were performed using SPSS Statistics V.22.0 for Windows (Version 22.0, IBM, Armonk, New York).

2.3 TFR116341 Trial

The TFR116341 trial was approved by the West Midlands (Coventry and Warwickshire) Ethics Committee and was listed in the European Union Clinical Trials Register (EudraCT Number 2014-000643-33). Patients due to undergo oesophagectomy for cancer were randomised to receive a single dose of novel anti-TNFR1 agent GSK2862277 or placebo, and screened systematically for ARDS post-operatively.

Patients were recruited from a number of academic hospitals in the UK. A translational sub-study was run at two academic centres in Birmingham. Inclusion criteria were planned surgical transthoracic oesophagectomy, aged 18-80, capable of giving informed consent, without substantial derangements of liver function and normal QT interval. Females were eligible if they were post-menopausal, had undergone tubal ligation or hysterectomy.

Exclusion criteria were:

- a positive test for antibodies binding GSK2862277.
- pneumonia within 14 days of dosing.

- forced expiratory volume in one second under 50% predicted or resting oxygen saturation of less than 92% (in those subjects where these tests were performed).

- history of allergy to study medication.

- having received or due another investigational product within 30 days of dosing, corticosteroids ($10mgday^{-1}$ or more of prednisolone or equivalent), anti-TNF or anti-interleukin-1 60 days prior to dosing.

- history of severe systemic disease the investigator felt rendered unsuitable.

- chronic liver disease.

- alcohol intake of over 28 units for males and 14 units for female.

- positive serology for hepatitis B or C, human immunodeficiency virus infection.

- *Mycobacterium tuberculosis* infection (demonstrated by positive interferon gamma release assay Quantiferon™ test).

- live attenuated vaccination within three weeks of dosing or required before day 28.

Screening for antibodies to GSK2862277 was performed in a GSK facility in Philadelphia, USA, and therefore seven days from recruitment to surgery were required to allow time for transportation, US Federal customs procedures and testing.

Patients were randomised to receive either drug or placebo in a double-blind manner, via an eFlow Rapid™ ultrasonic nebuliser (Pari, Starnberg, Germany), one to five hours prior to the start of surgery. Once under general anaesthesia, a PICCO™ cardiac output monitor (Pulsion, Feldkirchen, Germany) was placed with

a femoral intra-arterial catheter and readings were taken before surgery commenced. At the end of surgery, further PICCO readings were taken and a bronchoscopy and bronchoalveolar lavage (BAL) was performed prior to extubation.

The primary endpoint of the study was the change in pulmonary vascular permeability index (PVPI) [141] on completion of surgery. Secondary endpoints were change in extravascular lung water index (EVLWI), adverse events, clinical laboratory safety data, ECG readings, vital signs, $PaO_2:FiO_2$ ratios, BAL fluid biomarker ratios, changes in , $PaO_2:FiO_2$ ratios, PVPI, EVWLI and sequential organ failure assessment scores on days two to four, plasma and BAL drug concentrations and derived pharmacokinetic data, incidence of the development of antibodies to GSK2862277, ARDS incidence to day 28, survival to day 28, ventilator free days, ICU and hospital length of stay, organ failure free days, haemodynamic assessments, oxygenation index and plasma biomarker changes over time.

It was estimated having 40 patients in each arm would provide adequate power, based on data from the Beta Agonists in Lung Injury – Prevention Trial (BALTI-P) [138]. Interim safety analyses were planned at 10 and 40 patients recruited. Recruitment estimates were based on experience from the BALTI-P [138] and Vitamin D to prevent acute lung injury following oesophagectomy (VINDALOO) [140] studies.

Statistical analysis.

Data was categorical and therefore described using percentages. Comparison of the number of patients who were screened, dosed, withdrew or had surgery cancelled/abandoned/changed, was made using a chi-squared test. Analysis was

undertaken using GraphPad Prism V.6.07 for Windows (GraphPad Software, La Jolla, California, USA).

2.4 Neutrophil studies

Whole blood was available from the following sources:

- Healthy young controls: volunteers under the age of 45 without pre-existing medical conditions, and healthy young volunteers (under the age of 35) recruited to the *Mechanisms for the susceptibility to bacterial infection in those with influenza* (REC Ref 16/WM/0026).

- Healthy elderly controls: patients recruited to the *Mechanisms for the susceptibility to bacterial infection in those with influenza* (REC Ref 16/WM/0026), drawn from the Healthy Elders cohort, a group of volunteers registered with the Institute of Inflammation and Ageing, University of Birmingham, who donate blood and participate in experimental work relating to ageing and immunity.

- Pre-operative and on the first day postoperatively following oesophagectomy from the TFR116341 trial (EudraCT Number 2014-000643-33).

- Patients with established critical illness recruited from *A feasibility study of early and enhanced rehabilitation in critical care and potential impact on immuno-endocrine function* (trial registry number ISRCTN90103222).

2.4.1 Neutrophil extraction

Whole blood was taken and mixed with dextran. After 45 to 60 minutes, the plasma was removed and overlaid to a gradient of 56% and 80% Percoll™ diluted in 0.9% sodium chloride and centrifuged at 220g for 20 minutes with minimal acceleration

and no brake. The overlying fluid left was aspirated and discarded, then the granulocyte layer aspirated into phosphate buffered saline. This was then centrifuged at 400g for 10 minutes, then the neutrophils re-suspended in RPMI with glutamine, penicillin and streptomycin, adjusted to give a final count of 1million per ml. 100ul samples of some extractions were centrifuged at 300rpm for 5 minutes on a Cytospin centrifuge (Shandon, Minnesota, USA) for quality control checks.

2.4.2 Neutrophil extracellular trap chemiluminescence assay (figure 3)

Neutrophils were extracted from whole blood taken in EDTA containing Vacutainer bottles (BD, Franklin Lakes, New Jersey, USA), as outlined above. Neutrophils (3ml at 1 million per ml) were exposed to 10ng/ml soluble TNF-alpha (for priming) or vehicle control for 15 minutes at 37C and 5%CO2, before being centrifuged at 400g for 10 minutes at room temperature and being re-suspended at RPMI. 100ul of neutrophils at 1 million per ml were added to 100ul of RPMI as vehicle control or PMA (final concentration 625ng/ml) as a stimulant with or without DAB GSK2862277 or dummy DAB at 10nM final concentration. Each condition was run in quadruplicate. They were incubated for three hours at 37C and 5% CO2. Sytox Green dye (20ul at 5uM in PBS) was added to each well with 200mIU of MNase. This was incubated at room temperature for 10 minutes in the dark, and then the contents of each well was moved to a micro-Eppendorf tube and centrifuged at 1500g for 10 minutes. 160ul of supernatant was added to a black 96-well plate and chemiluminescence determined on the Syngery 2 reader (BioTek, Winooski, Vermont, USA).

For experiments with DAB, either DAB, Dummy DAB or vehicle control were applied during the 15 minute period for priming (referred to as "priming phase") or during the three hour incubation (referred to as "incubation phase").

Figure 3: flow diagram illustrating Priming and Stimulation phases of NETosis assay.
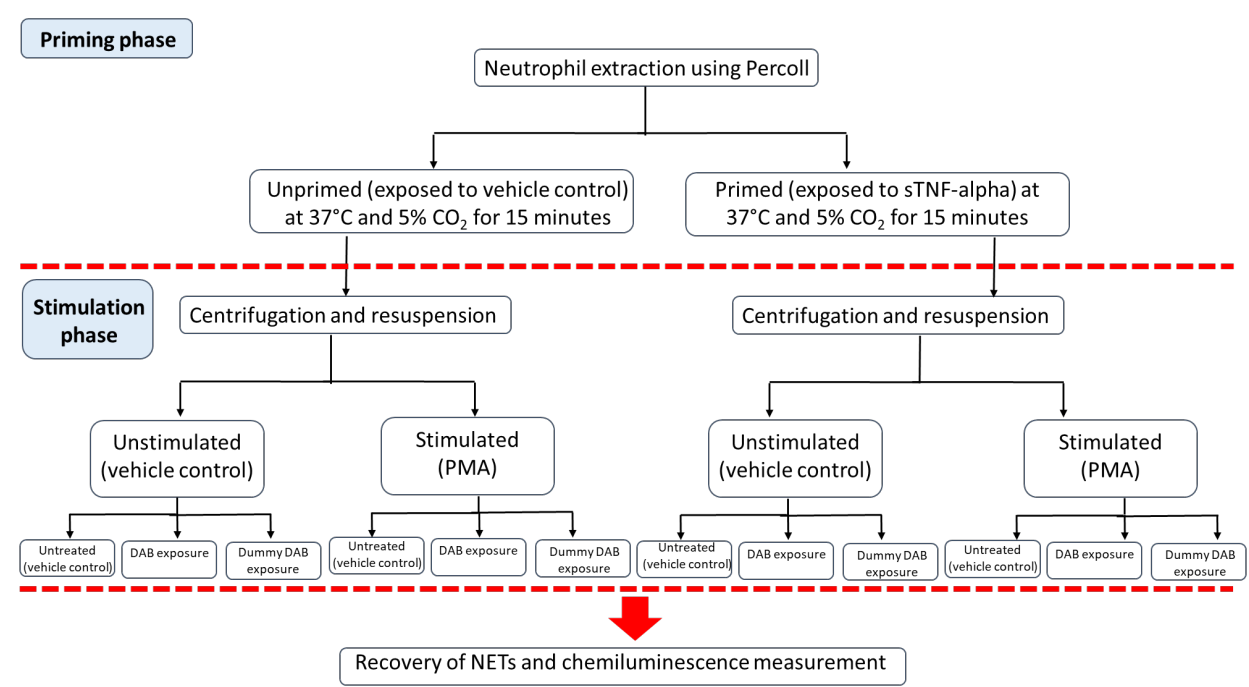
2.4.3 Phagocytosis assay

Neutrophils were extracted from whole blood taken in lithium-heparin containing Vacutainer bottles (BD, Franklin Lakes, New Jersey, USA), as outlined above.

A 96 well u-bottomed plate was prepared by instilling wells with 200ul 2% BSA-PBS then removing it. 100μL of neutrophils prepared at 1 million per ml in RPMI-GPS were added to all wells, except for the 0min time point, which were placed on ice in Eppendorfs. Gram positive (Staphylococcus aureus) and Gram negative (Escherichia coli) pHrodo bioparticles™ (Thermofisher, Paisley, United Kingdom) were added to the appropriate wells. Samples of neutrophils treated with CD16 alone, isotype control alone and untreated were also run, to identify background signal and as controls for gating the assay using the flow cytometer.

At time points (60 minutes and 30 minutes), pHrodo particles were added to wells. During the course of the assay, the plate was incubated at 37C and 5% CO_2. Once the time course was complete, the plate was placed on ice to stop further phagocytosis, and the Eppendorfs kept on ice added to the plate. It was centrifuged at 400g for 5 minutes at 4°C. The supernatant was removed by firm tapping and blotting onto paper. 100μL of 2%BSA-PBS was added to re-suspend the cells. 1uL of CD16 anti-human allophycocyanin-conjugated IgG1 antibody (ThermoFisher, Paisley, United Kingdom) was added to the CD16 control and the timed neutrophil wells. An isotype control (APC IgG1, ThermoFisher, Paisley, United Kingdom) was added to the isotype control well. These antibodies were incubated on ice for 20 minutes. Following this, a further 99μL of 2% BSA-PBS was added, then centrifuged at 400g for 5 minutes at 4°C. The supernatant was removed by firm tapping and blotting onto paper. The cells were re-suspended in 200μL 2% BSA-

PBS and transferred to flow cytometry tubes. The samples were then run on the Accuri C6 flow cytometer (BD Biosciences, San Jose, California, USA). The gating strategy was to exclude background and isotype control signals. The CD16 signal was used to identify neutrophils from the background of pHrodo particles, then the median fluorescent index and percentage of neutrophils positive for signal was obtained. These were multiplied to provide a phagocytic index.

Phrodo bioparticles are coloured with a pH-sensitive dye. When they are phagocytosed, the decrease in pH in the phagosome relative the media causes the dye to become visible when exposed to FL2 laser light on the Accuri C6.

2.4.4 Neutrophil receptor analysis

Whole blood was taken into tubes containing lithium heparin as an anticoagulant. Following a protocol from R&D Systems (Minneapolis, Minnesota, USA) 100µL blood was placed into flow cytometry tubes, to which 0.5ml 0.5% BSA in PBS was added, vortexed and centrifuged at 500g for 5 minutes at 4°C, twice. Fluorochrome-labelled antibodies were then added as required by the individual experiments and incubated at room temperature in the dark for 30 minutes (5µL anti-TNFR1, PE conjugated and anti-TNFR2, FITC conjugated, R&D Systems Minneapolis, Minnesota, USA and 5µL of CD16 anti-human allophycocyanin-conjugated IgG1 antibody from ThermoFisher, Paisley, United Kingdom, and appropriate isotype controls from the same manufacturer). 500µL of R&D Flow Cytometry Buffer (R&D Systems, Minneapolis, Minnesota, USA) was then added, vortexed, then centrifuged at 300g for 5 minutes at 4°C, the supernatant discarded and the wash repeated. 1ml BD lysis buffer was then added and incubated for 10 minutes at room temperature in the dark. This was then centrifuged at 300g for 5 minutes at 4°C,

then washed once in 500μL Flow Cytometry Buffer and centrifuged at 300g for 5 minutes at 4°C. The cells were then re-suspended in 200ul Flow Cytometry Buffer and read on the Accuri C6 BD Flow Cytometer.

Identification of neutrophils was as follows. The forward versus side scatter was used to determine populations of neutrophils and lymphocytes, as described [142]. Additionally, the CD16 marker was used to identify neutrophils from within the granulocyte cluster. Gates for TNFR1 and TNFR2 labelled antibodies were set against blank cells and isotype controls. An FMO control run against the blank was performed.

2.5 Cell-free DNA

A standard curve using calf thymus DNA (Sigma Aldrich, Gillingham, UK) serially diluted in Tris-EDTA was formed, of 200ul ranging from 0 to 250ngml^{-1}, corresponding to the lower and upper limits of detection. 100ul of plasma samples in EDTA were used, combined with 100ul Tris-EDTA. 20ul of sytox green at 20uM was added to each well and the plate incubated for 10 minutes, then the plate was read on the fluorometer.

2.6 BALTI-P and VINDALOO cytokine levels

These experiments were undertaken by the original trial teams and the data provided by Professor D Thickett for further analysis. Plasma cytokine levels and S-RAGE biomarker data were available from the BALTI-P [138] and VINDALOO [143] trials. These had been determined using Luminex and ELISA kits (R&D, Abingdon, UK), as described [138]. These were analysed to investigate further

immunological features associated with oesophagectomy. Equivalent cytokine data was not available from the TFR116341 trial due to slow trial recruitment.

2.7 THP-1 cell work

THP-1 cells are a human monocytic cell line which, when stimulated, will differentiate to a human macrophage model [144]. THP-1s for this project were provided by Dr A Scott, Institution of Inflammation and Ageing, University of Birmingham. THP-1s were held in long-term culture in RPMI 10% FCS and GPS. THP-1s were recovered from suspension by centrifugation at 500g for 5 minutes at 4°C. They were then resuspended in a known volume of RPMI 10% FCS and GPS and their concentration determined using dilution in an equal volume of trypan blue (Sigma Aldrich, Gillingham, UK) and their concentration determined using a Haemocytometer. They were constituted at 500000 cells per ml then stimulated with PMA at 100ngml^{-1}. These were plated at 500ul in 12 well or 100ul in 96 well (black with clear bottom) plates. These were incubated 37°C and 5% CO_2 for 24 hours. Media were changed at 24 hours and the THP-1s continued in culture for 1-3 days.

In order to assess the effects of DAB, those cells in 12-well plates had media removed, then 250ul added with vehicle control, DAB or Dummy DAB as appropriate (concentrations were doubled in order to account for further dilution – see below). After one hour, 250ul bronchoalveolar fluid (pooled from neutrophil-rich BAL available from the VINDALOO trial) was added and incubated for 6 hours. BAL contained TNF alpha 2.4pg/ml, IL1 beta 12.9pg/ml, IL1-ra 35.7 pg/ml, IL6 436.7 pg/ml, IL8 608.5 pg/ml, IL10 0.11pg/ml, IL17 0.68pg/ml, TNFR1 231.8pg/ml and TNFR2 375.8pg/ml. This addition of fluid led to final DAB or Dummy DAB

concentrations at 10 or 100nM. Trials were also run with 1 or 10ng of sTNF. After 6 hours, the BAL mix was removed and the media replaced (with DAB and Dummy DAB) and incubated for 18-24 hours. After 24 hours, this media was removed, centrifuged at 500g for 5 minutes and the supernatant recovered and frozen at -80°C until further analysed.

2.7.1 Cell viability assay

The THP-1 cells treated as described above were then exposed to 160ul of CellTiter 96 Aqueous One Solution (Promega Corporation, Madison, Wisconsin, USA). The plate was incubated for 2 hours then the 100ul transferred to a 96 well black plate with clear bottoms, then read on a plate reader. This was repeated at four hours.

2.7.2 DCFDA assay

In order to assess reactive oxygen species formation, a kit utilising 2',7'–dichlorofluorescin diacetate (DCFDA) (Abcam, Cambridge, UK) was used. The 96-well plate had the media changed, then DAB or Dummy DAB added into 50ul media. After one hour, 50ul of pooled BAL was added, leaving the DAB and Dummy DAB at 10nM. This was incubated for 6 hours, then the media replaced and DAB and Dummy DAB was added. This was then incubated for 18-24 hours. The media were removed and 100ul of buffer was added to each well. This was removed and 100ul DCFDA solution was added and incubated for 4 hours at 37°C in 5% CO_2 in the dark. This was then removed at a further 100ul buffer added and the plate read.

2.8 Alveolar macrophage recovery

The Midlands Lung Tissue Collaborative consents patients undergoing thoracic surgery at Heartlands Hospital, Birmingham, UK, were consented to provide tissue

from resections which was not required for histology for scientific research. These were taken for macrophage recovery.

Samples were cut from tissue after surgical resection, placed in 0.9% NaCl and stored at 4°C overnight, prior to transport to the laboratory. The lung samples was washed with 0.9% NaCl under pressure through a 16g needle until the tissue was pale and the fluid running clear. The 0.9% NaCl used for transporting was added to the wash and centrifuged at 500g for 10 minutes at 4°C. The supernatants were combined with a small variable volume of PBS and layered over Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 800g for 30 minutes at 4°C with minimum acceleration and no break. The band of macrophages was recovered into PBS, centrifuged at 500g for 5 minutes at 4°C and then re-suspended in RPMI with 10% FCS and GPS. 10ul of suspended cells were added to 10ul of trypan blue and counted on a haemocytometer, to determine the concentration of viable cells.

2.8.1 Alveolar macrophage phagocytosis assay for *E. Coli*

Phagocytosis particles were prepared by mixing 20µl DMSO to 15µg Celltracker Deep Red (Thermofisher, Paisley, United Kingdom). 125µl stock *Escherichia coli* (Thermofisher, Paisley, United Kingdom) were washed in 10mls fresh lysogeny broth (LB) broth and centrifuged at 2000g for 5 mins at room temperature. It was re-suspended in 4 ml fresh LB and add 20µl Celltracker Deep Red (with an end concentration of 5µM with *E coli* at 50 million/ml). This was then incubated in the dark at 37°C and 5% CO_2 for 45minutes. They were then had PBS added and were then centrifuged at 2000g for 5 mins at room temperature. This was then re-suspended in 2ml PBS (at a final concentration of 100 million/ml). The bacteria were then heat-killed at 65°C for 2 hours and then stored until use at 4°C.

Recovered alveolar macrophages were plated at 250 000 cells/well in a 12 well plate in 500ul of RPMI with 10% FCS and GPS and incubated for 24 hours at 37°C and 5% CO_2 . The media was changed at 24 hours and DAB or Dummy DAB added concentrations under test. They were incubated for 24 hours at 37°C and 5% CO_2.

After incubation, cytochalasin D at 5µg/ml was added to negative control well to inhibit phagocytosis followed by incubation for 30 minutes at 37°C and 5% CO_2. Media was removed then *E coli* suspension was added to give a macrophage:bacteria 1:50 ratio, alongside an *E coli* only control, then incubated for four hours at 37°C and 5% CO_2. Media were then removed and washed gently three times with PBS. Warmed trypsin for 20 minutes until cells detached. An equal volume of serum containing media was then added. Cells were transferred to flow cytometry tubes, then centrifuged at 500g for 5 minutes at room temperature, then re-suspended in R&D flow cytometry buffer, then analysed with a Fortessa flow cytometer (BD Biosciences, San Jose, California, USA).

2.8.2 Alveolar macrophage receptor identification.

Alveolar macrophages were removed from suspension in RPMI with 10% FCS and GPI. Those in culture had their media removed and cells were liberated from the plate using non-enzymatic Cell Dissociation Solution (Biological Industries, Beit-Haemek, Israel). Cells were added to round bottomed flow cytometry tubes at tube 100ul at 10^6/ml and washed in 0.5% BSA/PBS and centrifuged at 500g at 4°C for 5 minutes twice. Primary antibodies were added and incubated for 30 minutes. The cells were then washed three times in Flow Cytometry buffer (R&D, Minneapolis, Minnesota, USA), centrifuging at 300g for 5 minutes at 4°C. The cells were then re-

suspended in 200ul flow cytometry buffer and analysed on the Accuri C6 flow cytometer.

2.9 Statistical analysis for neutrophil, macrophage and plasma cytokine experiments

Data from the above experiments were first analysed for normality using the D'Agostino and Pearson normality test. For normally distributed data, summary descriptive data of mean and standard deviation were reported. Non-normal data were reported using median and interquartile range.

If data were normal, multiple groups were compared with ANOVA, then, if significant, with paired or unpaired t-tests as appropriate. Non-normal unpaired data were analysed using the Kruskal-Wallis test, then, if significant, individual groups using the Mann-Whitney U-test. Non-normal paired data were analysed using first Friedman's test, then Wilcoxon Matched Pairs test. Significance was taken as $p < 0.05$. For one experiment, an outlier was excluded using Grubb's test, prior to analysis.

Correlations between data were performed using Spearman's Rank Correlation Coefficient, as data in all analyses were non-normal.

Analyses were undertaken using GraphPad Prism V.6.07 for Windows (GraphPad Software, La Jolla, California, USA).

Chapter 3

THE IMPACT OF THE ACUTE RESPIRATORY DISTRESS SYNDROME ON OUTCOME AFTER OESOPHAGECTOMY

This chapter has been published as a paper.

P. Howells, D. Thickett, C. Knox, D. Park, F. Gao, O. Tucker, T. Whitehouse, D. McAuley, G. Perkins. The impact of the acute respiratory distress syndrome on outcome after oesophagectomy. *British Journal of Anaesthesia*, 117(3); 375-381.

3.1 Introduction

The Acute Respiratory Distress Syndrome (ARDS) frequently complicates the recovery from major surgery.[145] It is associated with high mortality[146-148] and although this has improved with time,[47] it remains an important cause of death and morbidity. Management of patients with ARDS consumes substantial healthcare resources.[149] The definitions of ARDS were recently updated in 2013, with the removal of the term acute lung injury (ALI).[45] For the purposes of this report, the term ARDS is used as the overarching term to describe the cohort of ALI and ARDS patients.

The outcome of ARDS varies according to the underlying disease process which is responsible for causing it. In a multicentre prospective observational study, an overall hospital mortality of 41.1% for ARDS was found. However, mortality was 43.6% in patients with ARDS caused by aspiration, 40.6% by pneumonia but 21.4% by severe trauma.[146] Major thoracoabdominal surgery, especially when combined with sepsis, is a common cause of ARDS with high associated mortality.[145]

Oesophagectomy carries a high risk for both mortality and morbidity. The most common complications following oesophagectomy are pulmonary.[12] Tandon, *et al*, in 2001 reported rates of ARDS of 38.3% and the mortality of patients developing severe ARDS was 50%.[150] A French study from 2012 comparing open oesophagectomy to hybrid (laparoscopic abdominal and open thoracic resection), reported major pulmonary complications in 43% of the open group and 15% in the hybrid group. Out of 280 cases, 21 cases of ARDS were reported and in six of the 12 post-operative deaths, ARDS was diagnosed. ARDS was less common in the hybrid group.[151] A large Australian study reported a respiratory complication rate

of 27.4% and increased length of hospital stay in those who developed pulmonary complications [16].

Despite a number of studies, no pharmacological treatments which directly target the underlying pathophysiological mechanisms implicated in the development of ARDS have been identified.[56] In the critical care setting, trials investigating the role of intravenous salbutamol,[57] simvastatin,[58] nitric oxide[59] and exogenous surfactant[152] in treating ARDS have all failed to demonstrate a mortality benefit. The role of steroid administration remains unclear.[55] Reductions in mortality have been demonstrated by trials of lung protective ventilation [49] and muscle relaxants.[54] Prone positioning is an effective measure in cohorts with severe ARDS.[153]

Given the limited treatments available, preventative strategies are attractive and could have substantial benefits if implemented in high risk groups, including patients undergoing oesophagectomy.[147] Valid clinical models are imperative for investigating preventative strategies.[154] Patients undergoing one-lung ventilation (OLV), such as occurs in patients undergoing oesophagectomy, provide a potentially useful model for investigating ARDS.

The aim of this study was to undertake a secondary analysis of the multi-centre Beta Agonist Lung Injury Prevention trial to characterise patients developing ARDS following elective oesophagectomy and identify risk factors for the syndrome.

3.2 Methods

See Chapter 2

3.3 Results

Of the 362 patients in the BALTI-P trial, 331 patients were included in the analysis. Patients who did not undergo surgery (n=19, 5.2%) and who withdrew consent (n=2, 0.55%) were excluded, as were patients who did not have a defined ARDS status (n=10, 2.8%). Patient age, gender, height or body weight, diagnosis (adenocarcinoma, squamous cell carcinoma, Barrett's or other), staging, chemotherapy and lung function were all similar between groups (Table 1).

Table 1: Clinical characteristics of patients undergoing oesophagectomy in the BALTI-P trial summarised by ARDS status (early less than 72 hours, late greater than 72 hours).

		Early ARDS (n=59)	Late ARDS (n=24)	Total ARDS (n=83)	No ARDS (n=248)
Age (years)	Mean (range)	63.7 (42-85)	62.3 (49-79)	63.3 (42-85)	63.2 (25-85)
Gender	Male	46 (78.0%)	18 (75.0%)	64 (77.1%)	199 (80.2%)
Ethnicity	Caucasian	59 (100%)	24 (100%)	83 (100%)	247 (99.6%)
	Missing	0	0	0	1 (0.4%)
Diagnosis	Adenocarcinoma	47 (79.7%)	16 (66.7%)	63 (75.9%)	182 (75.2%)
	Squamous cell	9 (15.3%)	6 (25.0%)	15 (18.1%)	43 (17.8%)
	Other malignant (eg mixed)	0	0	0	4 (1.7%)
	Barrett's Oesophagus	3 (5.1%)	2 (8.3%)	5 (6.0%)	13 (5.4%)
	Missing	0	0	0	6
Pre-operative chemotherapy	Yes	47 (79.7%)	18 (75.0%)	65 (78.3%)	198 (80.2%)
	No	12 (20.3%)	6 (25.0%)	18 (21.7%)	49 (19.8%)
	Not applicable	0	0	0	1
Forced Vital Capacity (litres)	Mean (SD)	4.1 (1.2)	4.3 (1.1)	4.2 (1.1)	3.9 (0.9)
	Missing	22	3	25	73
Forced Expiratory Volume in One Second (litres)	Mean (SD)	2.8 (0.9)	2.9 (0.7)	2.8 (0.8)	2.8 (0.7)
	Minimum	0.1-4.5	1.1-4	0.1-4.5	1.1-5.6
	Missing	22	4	26	75
Staging T	1	3 (5.4%)	2 (8.7%)	5 (6.3%)	12 (5.0%)
	2	11 (19.6%)	8 (34.8%)	19 (24.1%)	62 (25.9%)
	3	42 (75.0%)	13 (56.5%)	55 (69.9%)	161 (67.4%)
	4	0	0	0	4 (1.7%)
	Missing	3	1	4	9
Staging N	0	23 (41.8%)	10 (43.5%)	33 (42.3%)	88 (37.1%)
	1	32 (58.2%)	13 (56.5%)	45 (57.7%)	149 (62.9%)

	Missing	4	1	5	11
Tumour location	Cervical	1 (1.7%)	1 (4.3%)	2 (2.4%)	3 (1.2%)
	Mid oesophagus	12 (20.3%)	7 (30.4%)	19 (23.2%)	69 (28.4%)
	Oesophageal/gastric junction	46 (78.0%)	15 (65.2%)	61 (74.4%)	171 (70.4%)
	Missing	0	1	1	5
Surgical approach	Laparoscopic	16 (27.1%)	3 (12.5%)	19 (22.9%)	60 (24.4%)
	Open	43 (72.9%)	21 (87.5%)	64 (77.1%)	186 (75.6%)
	Missing	0	0	0	2
Open stage; If open surgical approach	2 Stage	34 (97.1%)	13 (81.2%)	47 (92.2%)	138 (93.2%)
	3 Stage	1 (2.9%)	3 (18.8%)	4 (7.8%)	10 (6.8%)
	Missing	8	5	13	38
Thoracotomy; If open surgical approach	Right	28 (71.8%)	16 (76.2%)	44 (73.3%)	146 (84.4%)
	Left	11 (28.2%)	5 (23.8%)	16 (26.7%)	26 (15.0%)
	Missing	4	0	4	13
	N/A	0	0	0	1 (0.6%)
ASA grade	I	1 (1.9%)	2 (8.7%)	3 (3.9%)	14 (5.9%)
	II	37 (68.5%)	19 (82.6%)	56 (72.7%)	164 (69.2%)
	III	16 (29.6%)	2 (8.7%)	18 (23.4%)	57 (24.1%)
	IV	0	0	0	2 (0.8%)
	Missing	5	1	6	11

In total, 83 patients (24.6%) developed ARDS in the first 28 days following surgery, of whom 59 (71.0%) were classified as early and 24 (29.0%) late. Overall, reduced ICU and hospital length of stay was observed for those patients without ARDS, with a longer duration for those with late versus early disease (Table 2). Specifically, there were fewer organ failure free days in the early and late ARDS groups compared to those who did not develop ARDS.

Table 2. Post-operative outcomes days 0-90 summarised by ARDS status

		Early ARDS (n=59)	Late ARDS (n=24)	No ARDS (n=248)	Statistics (95% CI) Early or late ARDS versus no ARDS
Organ failure free days	Mean (SD)	24.4 (6.2)	21 (6.8)	26.8 (3.2)	Early -2.40 (-3.60, -1.19) p<0.001 Late -5.77 (-7.55, -3.99) P<0.001
	Missing	0	0	2	
Any ventilator support on day 0-28	Yes	33 (55.9%)	21 (87.5%)	72 (29.1%)	RR = 1.62 (1.23, 2.15)
	Missing	0	0	1	
Ventilator free days	Median (IQR)	27 (18-28)	17 (10.5-23.5)	28 (27-28)	Early -5.28 (-6.81, -3.76) p<0.001 Late -10.1 (-12.4, -7.89) p<0.001
	Missing	0	0	1	
Duration of hospitalisation (days)	Mean (SD)	18.1 (7.8)	24.5 (5.3)	14.2 (6.2)	Early 3.93 (2.09, 5.77) p<0.001 Late 10.3 (7.63, 13.1) p<0.001
	Missing	0	0	3	
Duration of ITU stay (days)	Mean (SD)	12.1 (9.0)	20.2 (8.0)	7.3 (5.4)	Early 4.82 (3.00, 6.65) p<0.001 Late 12.9 (10.2, 15.6) p<0.001
Duration of ITU stay excluding deaths (days)	Mean (SD)	12.1 (9.2)	20.2 (8.0)	7.3 (5.4)	Early 4.78 (2.91, 6.64) p<0.001 Late 12.9 (10.2, 15.6) p<0.001
	Missing	2	0	2	
Duration of level 0 or 1 care (days)	Mean (SD)	8.4 (6.8)	7.8 (5.2)	10.2 (5.6)	Early-1.76 (-3.43, -0.10)p=0.04 Late 2.40 (-4.86, 0.06) p=0.06
	Missing	0	0	1	
Duration of level 2 care (days)	Mean (SD)	5.0 (3.4)	8.0 (4.2)	4.0 (3.0)	Early 0.98 (0.08, 1.88) p=0.033 Late 4.06 (2.73, 5.39) p<0.001
	Missing	0	0	1	
Duration of level 3 care (days)	Mean (SD)	5.3 (8.5)	9.5 (7.0)	0.8 (2.2)	Early 4.48 (3.21, 5.74) p<0.001 Late 8.76 (6.90, 10.6) p<0.001
	Missing	0	0	1	

EQ-5D Day 28	Mean (SD)	0.47 (0.31)	0.31 (0.42)	0.55 (0.29)	Early -0.08 (-0.18, 0.02) p=0.119 Late 0.24 (-0.39, -0.09) p=0.002
	Missing	17	8	43	
EQ-5D VAS Day 28	Mean (SD)	59.3 (18.5)	55.5 (24.1)	62.0 (16.5)	Early -2.76 (-8.60, 3.08) p=0.35 Late -6.56 (-15.70, 2.57) p=0.16
	Missing	18	9	42	
EQ-5D Day 90	Mean (SD)	0.64 (0.26)	0.54 (0.35)	0.66 (0.3)	Early -0.02 (-0.11, 0.06) p=0.63 Late -0.12 (-0.26, 0.01) p=0.07
	Missing	14	8	49	
EQ-5D VAS Day 90	Mean (SD)	65.4 (20.0)	60.3 (18.2)	68.2 (18.4)	Early -2.75 (-8.81, 3.30) p=0.37 Late -7.88 (-17.42, 1.65) p=0.11
	Missing	14	8	48	
Mortality*	Alive at 28 days	56 (94.9%)	24 (100%)	243 (99.2%)	HR = 3.73 (0.74, 18.7); p-value=0.086*
	Dead at 28 days	3 (5.1%)	0	2 (0.8%)	
	Missing	0	0	3	
Mortality*	Alive at 90 days	55 (93.2%)	22 (91.7%)	245 (99.2%)	HR = 3.36 (0.83, 13.6; p-value=0.072*
	Dead at 90 days	4 (6.8%)	1 (4.3%)	2 (0.8%)	
	Missing	0	1	1	

*Calculated using log-rank test

Patients with late ARDS had fewer ventilator-free days (median 17, interquartile range (IQR) 11-24), compared to early ARDS (median 27, IQR 18-28) and no ARDS (median 28, IQR 27-28). The duration of intensive care stay was shortest in those without ARDS (mean 7.3 days, standard deviation (SD) 5.4), longer with early ARDS (mean 12.1 days, SD 9.0) and longer still with late disease (mean 20.2 days, SD 8.0). There were no observed differences in mortality at 28 or 90 days. The findings were unchanged in the sensitivity analysis which adjusted for treatment allocation to salmeterol (Supplementary Table S1). Similarly, there were no differences in quality of life scores at 28 or 90 days (Supplementary Table S1).

Table 3 shows multivariate analysis grouped according to lung injury. Early ARDS was associated with increased age (OR 1.06 (1.00 to 1.13), p=0.05). There was an increased risk of ARDS in patients with mid-oesophageal tumours (OR 7.48 (1.62-34.5), p=0.01), whilst the risk was reduced with gastro-oesophageal tumours (OR 0.21 (0.05-0.85), p=0.03).

Analysis was undertaken adjusting for treatment allocation (salmeterol versus placebo), but this made little difference (Appendix table A2).

Table 3: Multivariate analyses of ARDS. OR: mean estimated odds ratio of the interaction term, CI: 95% confidence interval

	Early ARDS			Late ARDS			Total ARDS		
	OR (95% CI)	p	n.	OR (95% CI)	p	n	OR (95% CI)	p	n
Age (years)	1.06 (1.00, 1.13)	0.05	332	0.98 (0.90, 1.06)	0.57	331	1.04 (0.99, 1.10)	0.13	331
Gender	1.66 (0.41, 6.66)	0.46	332	0.71 (0.09, 5.35)	0.74	331	1.25 (0.37, 4.14)	0.72	331
Pre-operative chemotherapy	0.74 (0.17, 3.17)	0.68	332	7.76 (0.70, 86.42)	0.10	331	1.74 (0.51, 5.93)	0.38	331
Duration of one lung ventilation (minutes)	1.01 (0.99, 1.00)	0.25	297	1.00 (0.98, 1.01)	0.61	297	1.00 (0.99, 1.01)	0.48	297
Cumulative fluid balance at end of surgery (litres)	1.16 (0.80, 1.68)	0.45	316	1.20 (0.61, 2.35)	0.60	315	1.24 (0.88, 1.75)	0.23	315
Surgical approach	0.69 (0.19, 2.49)	0.57	329	0.21 (0.02, 2.85)	0.24	329	0.45 (0.14, 1.49)	0.19	329
Tumour: Mid oesophagus	7.48 (1.62, 34.5)	0.01	325	-	-	-	1.74 (0.54, 5.62)	0.36	325
Tumour: Gastro-oesophageal junction	0.21 (0.05, 0.85)	0.03	325	-	-	-	0.85 (0.27, 2.65)	0.77	325

Late ARDS estimates are missing due to insufficient numbers of cases in these groups for these to be calculated.

Of those patients with late ARDS, 42% were also diagnosed with pneumonia, 25% with anastomotic leak and 13% with respiratory failure, whilst other surgical complications occurred in 12.5%. In those with early ARDS, 10.2% had pneumonia and surgical complications occurred in 8.5%. There were significantly more surgical, respiratory and "other" complications ($p<0.0001$ for all), but no significant difference in sepsis between the groups. For surgical, respiratory and other non-cardiovascular complications, rates were higher in the late compared to the early ARDS groups (figure 4).

Figure 4: Safety outcomes, divided by early (before 72 hours) and late (after 72 hours) ARDS in BALTI-P participants. Higher complications were observed in patients with ARDS, with higher levels late versus early for all groups, with the exception of cardiovascular.
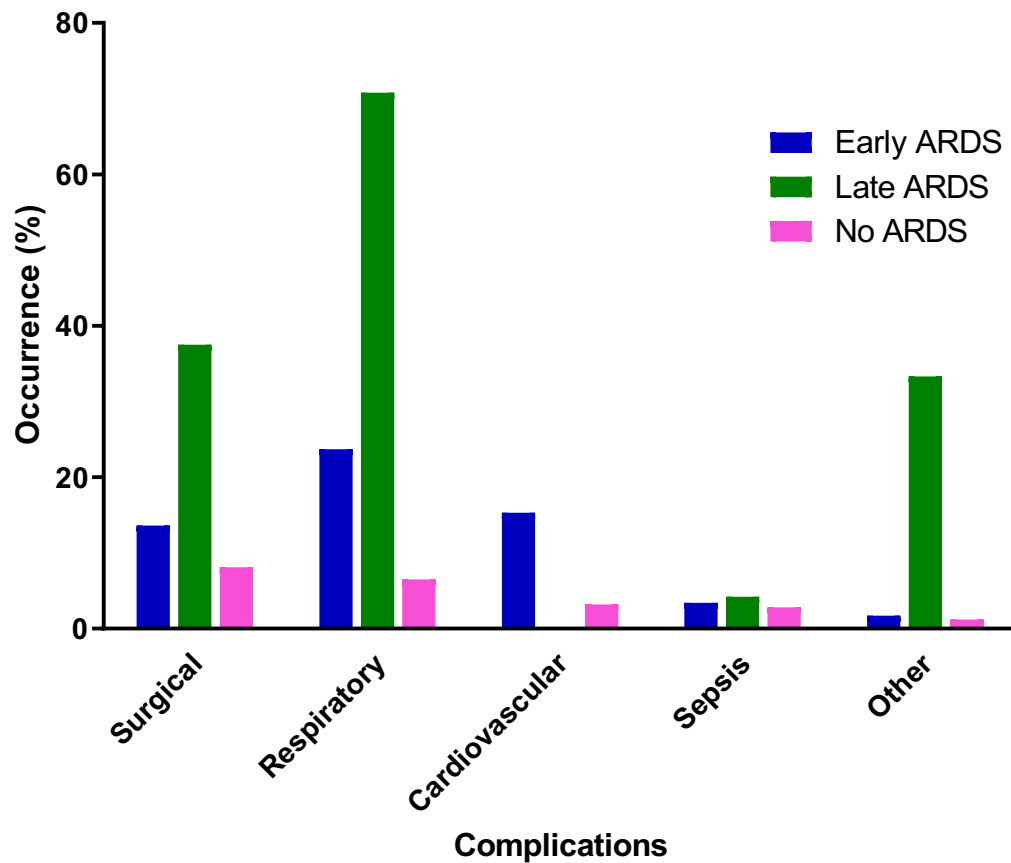
3.4 Discussion

These data demonstrate that ARDS was common following oesophagectomy surgery, with an incidence of almost 25%. We did not find differences in mortality between patients with early and late ARDS at 28 or 90 days, nor to changes in their quality of life scores. This may be due to insufficient power, especially given the study was not designed to examine this outcome and because mortality following oesophagectomy has fallen with time.[155] However, both early and late ARDS are associated with more days of organ failure, spending more days ventilated and having longer ICU and hospital stays than patients who do not develop ARDS, a finding that has been observed elsewhere.[11]

Improvements in pre-operative, intra-operative and post-operative care may all have contributed to apparent reduction in harm associated with ARDS and the reduction in the frequency and severity of ARDS observed in older cohorts.[150] Another, more recent, study has shown that post-oesophagectomy respiratory failure and ARDS were independent risk factors for in-hospital death.[156] Overall, the rates of mortality and respiratory and cardiovascular complications were similar to contemporary studies of oesophagectomy outcomes elsewhere.[157]

Scoring systems, such as the Lung Injury Prediction Score (LIPS), have been developed to identify high incidence ARDS groups *a priori* for both clinical purposes and to provide groups with high ARDS incidences for preventative trials.[154] A cohort identified using the LIPS score had an incidence of ARDS of 7%.[154, 158] The majority of ARDS detected in BALTI-P occurred in the first 72h following surgery, with a similar pattern seen in the LIPS validation cohort, which identified only 25% of ARDS on or after day 4.[158]

Oesophagectomy is attractive as a model of ARDS as the timing of the insult (surgery) is consistent and predictable. Patients can be identified, approached and consented in advance. Systemic and alveolar inflammatory changes are similar to those observed in ARDS[159] and include evidence of alveolar and endothelial damage, neutrophil infiltration and pulmonary vascular congestion.[148]

One limitation to the model is that although the ARDS incidence was high, the majority was classified as mild to moderate and this is partly reflected in the lower mortality detected in this study than others focussing on more severe patients.[153] However, the increased organ failure, increased duration of ventilated and intensive care and hospital stay all demonstrate even early onset mild to moderate ARDS has significant adverse implications for both patients and healthcare resource utilisation and it would therefore be beneficial to prevent it.

This study has identified increased age and tumour site are risk factors for early ARDS. Finding no significant risk factors for late ARDS probably reflects the small numbers in this group. The magnitude of the increased risk of ARDS associated with mid-oesophageal tumours was unexpected.

Squamous cell carcinoma (SSC) is the predominant histological subtype in cervical and mid-oesophageal tumours. A higher risk of pulmonary complications with more proximal tumours has been reported previously, with one study suggesting a relationship with increased surgical technical difficulty and recurrent laryngeal nerve injury.[160] In one small study, SCC histology was associated with more pre-operative respiratory disease and alcohol use and with more severe post-operative complications and longer ICU stays.[161] Similar rates of COPD, cardiac disease, smoking and neoadjuvant chemoradiotherapy were seen for SCC and adenocarcinomas.[161] Preoperative radiotherapy, more commonly administered

in the UK for SCC (and infrequently for adenocarcinoma), is associated with increased pulmonary complications,[162] and salvage oesophagectomy for SCC after definitive chemoradiotherapy can be technically challenging with increased post-operative morbidity. These factors may explain the higher risk of ARDS observed with mid-oesophageal tumours.

This result is also surprising given the similar ARDS incidence between the mid-oesophageal and gastro-oesophageal groups. This may be due to collinearity with other risk factors manifesting in the multivariate analysis, or a type one error. Clearly, caution must be exercised in interpreting these results and this requires validation with further studies.

It has been suggested that cumulative insults may aggregate to increase ARDS risk. McKevith and Pennefather[11] discussed the possibility that the combined 'hits' of multi-cavity surgery and OLV combine to give higher rates of ARDS when compared with other major surgery. An incidence of ARDS of 60% has been reported in patients who had undergone thoracoabdominal surgery and developed sepsis, compared to 34.6% in those with sepsis without surgery, which suggests ARDS is more likely as pathological insults aggregate.[145] We believe in this study that early ARDS was driven by factors at the time of surgery such as OLV lung injury and/or inflammation induced by the surgical insult whereas ARDS in the late group was more frequently caused by complications following surgery.

A similar concept has been proposed elsewhere, with a study of ARDS following lung resection identifying what the authors termed "primary ARDS" (i.e. due to surgery and OLV alone, without another identified cause) being observed shortly after surgery (median onset two days), whereas "secondary ARDS" (where a causal factor other than the initial surgery, such as aspiration or sepsis, was identified)

tended to occur later (median onset of 5.5 days).[163] This again suggests that accumulated insults contribute to ARDS.

There are limitations in this study. This is a retrospective observational analysis, with the potential bias that confers. Furthermore, the ongoing changes in both the epidemiology of oesophageal cancer and its management render comparisons with other, especially older, cohorts less reliable. The total number of participants may have resulted in a lack of power to identify trends, particularly mortality but this is, nevertheless, to our knowledge, the largest cohort of patients undergoing oesophagectomy who have been subject to systematic screening for ARDS. Potentially important information, such as tumour histology, use of radiotherapy, smoking status and alcohol consumption were not collected.

Both early and late ARDS are harmful for patients following oesophagectomy and increase ICU and hospital resource use. New preventative strategies to reduce the burden of perioperative ARDS would be valuable. Because of the high incidence of ARDS in patients undergoing oesophagectomy, it is a useful model for trialling such strategies and, compared to other methods for finding such cohorts, it has a number of favourable features.

Chapter 4.

ARDS FOLLOWING OESOPHAGECTOMY: A COMPARISON OF TWO TRIALS

This chapter has been published as a paper.

4.1 Introduction

Patients undergoing oesophagectomy have high rates of postoperative complications [28] including the acute respiratory distress syndrome (ARDS) [8]. We have previously shown that ARDS following oesophagectomy is associated with more non-respiratory organ failure, longer critical care and hospital stays [164], and other groups have demonstrated worse short-term and long-term outcomes associated with ARDS2 and other pulmonary complications [23]. Severe infection and cardiac dysrhythmias are common. However, this high complication rate, alongside the planned nature of surgery and the clear timing of the surgical insult [9, 13], makes oesophagectomy a potentially useful model to undertake trials to reduce perioperative complications [148].

Both the Beta Agonists in Lung Injury Trial-Prevention (BALTI-P) [159], which completed recruitment in 2011, and the Vitamin D to Prevent Acute Lung Injury Following Oesophagectomy (VINDALOO) trials, completed in 2015, [140] used oesophagectomy as a model of ARDS. We observed that the incidence of ARDS in the VINDALOO (8 out of 68, 11.8%) cohort was substantially lower than in the BALTI-P (83 out of 331, 25.1% and 14 out of 61, 23%) sub-study (see the Methods section below), independent of a pharmacological effect of the agents trialled, suggesting that there had been changes between the groups that were expected a priori to be similar.

The aims of this work were to determine which clinical features were different between the two cohorts that might explain the differences in postoperative ARDS and complications. The combined cohorts were analysed to seek further risk factors

not apparent in the individual cohorts and potential therapeutic targets for further investigation.

4.2 Methods

See Chapter 2.

4.3 Results

Table 4 shows the baseline demographic data from the BALTI-P sub-study and VINDALOO groups. Patients in VINDALOO were heavier, received a lower mean tidal volume, received more intravenous fluid, more were on beta-blockers, more received ketamine and dexamethasone and fewer remifentanil and thoracoscopic approach was more common.

Table 4. Demographic data from the BALTI-P and VINDALOO trials.

	BALTI-P (n=61)	VINDALOO (n=68)	P value
Age (years) Median IQR	64 (65-72)	67 (60-72)	0.110
Weight (kg) Median IQR	75 (60-84)	77 (68-94)	0.049
Height (cm) Median IQR	171 (167-175)	173 (168-177)	0.413
Current Smoking	16 (26.7%)	9 (13.4%)	0.075
Histology	Adenocarcinoma 13 (22.8%) Squamous 42 (73.7%) Benign 2 (3.5%)	Adenocarcinoma 58 (85.3%) Squamous 10 (14.7 %) Benign 0 (0.0%)	0.134
Hypertension n (%)	22 (40.7%)	27 (40.3%)	1.00
Ischaemic Heart Disease n (%)	4 (7.40%)	5 (7.46%)	1.00
Diabetes Mellitus	5 (9.26%)	8 (11.9%)	0.771
Chronic Lung Disease	5 (9.26%)	9 (13.4%)	0.574
Venous thromboembolic disease	3 (5.56%)	9 (13.4%)	0.342
Beta blockers n (%)	4 (7.41%)	16 (23.9%)	0.025
Aspirin n (%)	9 (16.7%)	11 (16.4%)	1.00
Dihydropyridine	8 (13.1%)	7 (10.3%)	0.784
Statin	11 (20.4%)	22 (32.8%)	0.153
Angiotensin converting enzyme inhibitor or angiotensin II receptor antagonist	11 (20.4%)	13 (19.6%)	1.00
Pre-operative haemoglobin (g/dl) Mean (SD)	121 (15)	126 (18)	0.080
Mean Tidal Volume ml/kg, (Mean (SD))	6.9 (1.9)	6.1 (1.4)	0.011
Duration of surgery	385 (318-454)	373 (321-419)	0.494

Duration of OLV (minutes) Median (IQR)	155 (130-188)	150 (130-195)	0.794
Fluid administered (ml/kg) Median (IQR)	31 (24-46)	41 (30-52)	0.012
Regional analgesia used n (%)	51 (92.7%)	55 (84.6%)	0.254
Remifentanil	13 (24.5%)	5 (8.33%)	0.022
Dexamethasone	8 (15.0%)	20 (66.7%)	0.030
Ketamine	0 (0.0%)	14 (22.2%)	P<0.0001
Thoracoscopy	10 (17.9%)	22 (35.5%)	0.039
Laparoscopy	10 (21.7%)	8 (12.7%)	0.455

Staging of malignancy was both more widely distributed and overall higher in the VINDALOO cohort (figure 5). Pre-existing Charlson Index was not different between groups (BALTI-P median 2 (IQR 2–3), VINDALOO 2 (IQR 2–3), P=0.872). Perioperative risk scores were not different between the groups (P-POSSUM Mortality (BALTI-P median 2.4 (IQR 1.9–37) vs VINDALOO 2.4 (IQR 1.5–5.4), P=0.759), P-POSSUM Morbidity (BALTI-P median 8.5 (IQR 4.6–13) vs VINDALOO 8.7 (IQR 6.3–17), P=0.141), O-POSSUM (BALTI-P median 8.5 (IQR 4.6–13) vs VINDALOO 8.7 (IQR 6.3–17), P=0.141)).

Figure 5: Percentage of patients per stage of oesophageal cancers in the BALTI-P and VINDALOO trials (***p<0.001). Patients recruited to the VINDALOO trial had overall higher staged cancers.
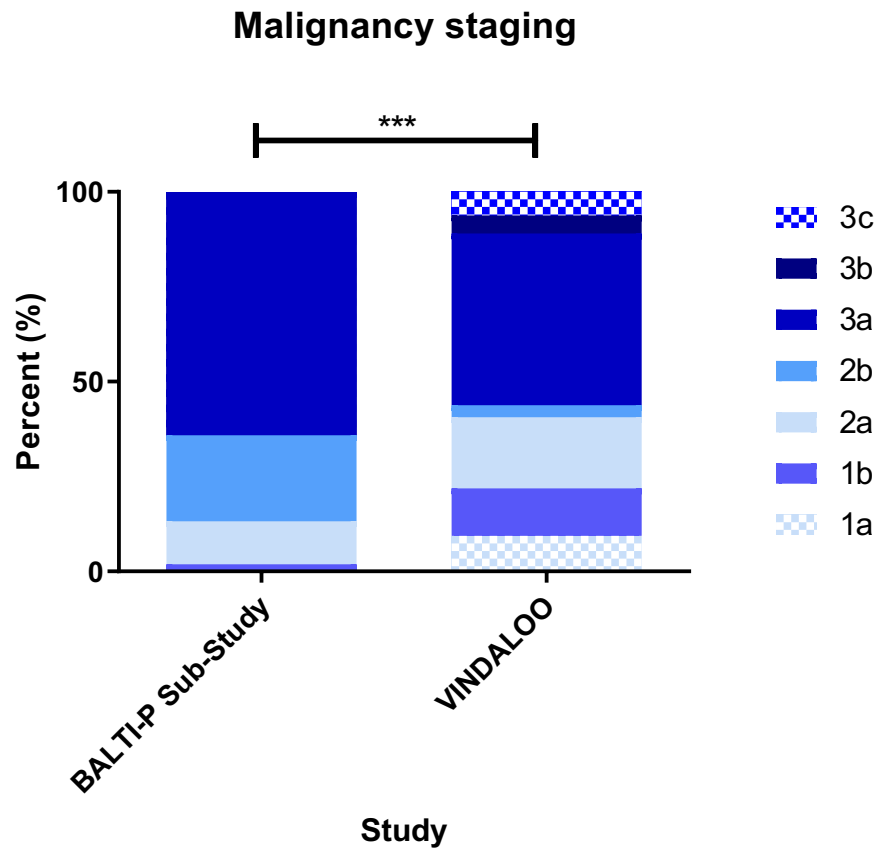
To assess risk factors further, the two cohorts were combined and assessed according to ARDS status (table 5). Univariate analysis showed that current smoking and dihydropyridine use were associated with the development of ARDS postoperatively. These variables were then subject to multivariate analysis, which showed that both active smoking (OR 3.91; 95% CI 1.33 to 11.5) and dihydropyridine use (OR 5.34; 95% CI 1.56 to 18.3) remained associated with ARDS risk.

Table 5. Comparison of patients with and without ARDS from the BALTI-P and VINDALOO combined.

Factor	No ARDS (n=108)	ARDS (n=21)	P value
Age Median (IQR)	66 (58-72)	61 (57-70)	0.367
Current Smoking n (%)	17 (16.0%)	8 (38.1%)	0.033
Histology n (%) Adenocarcinoma Squamous Cell Carcimona Benign	 85 (80.2%) 19 (17.9%) 2 (1.9%)	 15 (78.9%) 4 (21.1%)	0.776
Hypertension n (%)	40 (38.8%)	9 (50%)	0.439
Ischaemic Heart Disease n (%)	9 (8.7%)	0 (0.0%)	0.353
Diabetes Mellitus n (%)	9 (8.7%)	4 (22.2%)	0.103
Lung disease n (%)	12 (11.7%)	2 (11.1%)	1.00
Venous thromboembolic disease n (%)	11 (10.7%)	0 (0.0%)	0.367
Weight (kg) median	75 (65-88)	81 (62-93)	0.485
Height Median (IQR)	173 (167-176)	172 (169-176)	0.915
Haemoglobin Mean (SD)	125 (16)	120 (19)	0.260
Duration of OLV (minutes) Median (IQR)	150 (130-181)	170 (124-205)	0.457
Mean Tidal Volume (mlkg^{-1}) Median (IQR)	6.1 (5.4-7.7)	5.8 (5.4-6.9)	0.458
Beta-blocker n (%)	17 (16.7%)	3 (15.8%)	1.00
Dihydropyridine n (%)	9 (8.3%)	6 (28.6%)	0.0173
Benzothiazepine n (%)	3 (2.78%)	0 (0.0%)	1.00
Statin n (%)	28 (27.5%)	5 (26.3%)	1.00
Aspirin n (%)	16 (15.7%)	4 (21.1%)	0.517

Angiotensin converting enzyme inhibitor or angiotensin II receptor antagonist	20 (19.8%)	4 (21.1%)	1.00
Regional anaesthesia n (%)	11 (10.9%)	3 (15.8%)	0.464
Remifentanil n (%)	14 (14.7%)	4 (22.2%)	0.483
Ketamine	12 (12.5%)	2 (11.1%)	1.00
Thoracoscopic approach n (%)	29 (29.0%)	3 (16.7%)	0.392
Laparoscopic approach n (%)	84 (83.1%)	17 (94.4%)	0.302

The effect of these factors on length of stay as a measure of outcome was assessed, as this outcome was collected in both trials. This showed that those patients on dihydropyridines had longer hospital stays (dihydropyridine median 29 days (IQR 17–42), no dihydropyridine 13 days (IQR 10–18), P=0.0007), as did those with diabetes mellitus (diabetes median 25 (IQR 14–39) vs no diabetes 13 (IQR 10–19), P=0.023). There was no difference in length of stay related to smoking (median in never/ex-smokers 13 (IQR 10–23) vs active smokers 15 (IQR 11–20), P=0.73).

4.4 Discussion

Lower tidal volume is now well established in the management of ARDS following the landmark ARDS Clinical Network trial [49] and there is increasing evidence of its role in intraoperative ventilation [32, 165]. Tidal volumes were lower in the VINDALOO trial, which is likely to represent the increasing adoption of lung protective strategies, including lower tidal volumes, higher positive end-expiratory pressure and permissive hypercarbia [12]. Whether the reduction of 0.8 mL/kg is clinically significant is not certain, but may be in the context of OLV during oesophagectomy, where less than half the lung volume is subject to intermittent positive pressure ventilation [42]. This may have played an important role in the change in ARDS incidence. However, neither mean tidal volume nor duration of OLV were associated with a higher risk of ARDS. It may be other factors may be more revealing about the effects of ventilation on the lung, such as driving pressure [166] or mechanical power [167].

More fluid was administered to the VINDALOO cohort; this might represent a reduction in colloid and increased crystalloid administration and/or more balanced fluid use improving anastomosis perfusion [13]. Similarly, increasing the use of

thoracoscopic techniques and anaesthetic agents with immunomodulatory effects may reduce the inflammatory response to surgery and so the risk of ARDS [12].

This study has indicated that there are two major targets for reduction in postoperative ARDS: cigarette smoking and diyhdropyridines. Smoking has been previously demonstrated to be a risk factor for ARDS [25, 168], and the fewer current smokers in VINDALOO may have had a marked effect on the ARDS incidence between the two trials. Smoking has been associated with severe perioperative complications in another oesophagectomy cohort [169]. This work supports the premise of efforts to reduce smoking perioperatively [170]. Use of nicotine replacement therapy in critical care medicine is controversial, and trials in the perioperative setting are required to ensure safety as well as efficacy [171]. Evidence of the safety and effectiveness of e-cigarettes and nicotine replacement in the perioperative period also need to be confirmed by randomised trials [172].

The association between dihydropyridine calcium channel blockers and ARDS was unexpected. ARDS has been reported following dihydropyridine overdose [173]. Pulmonary oedema following administration of the dihydropyridine nimodipine has been described in the context of subarachnoid haemorrhage [174]. Potential mechanisms include worsened ventilation-perfusion mismatching due to pulmonary arterial dilatation, reduced cardiac function and pulmonary or inflammatory modulatory effect. Calcium channel blockade has been associated with immunomodulation, although mostly downregulating inflammatory processes [175-177]. It may be that dihydropyridine use is a marker of worse systemic disease and therefore perioperative risk, although we did not find an association with aspirin, beta-blockers or statins. It would be premature to recommend not using dihydropyridines in the perioperative period, but there is a need for further studies

on the effects of concurrent medications on patients undergoing surgery. Identifying the mechanisms through which dihydropyridines have this effect would also be useful.

A major problem in ARDS prevention trials is identifying a cohort with a high ARDS risk [148]. Even in the VINDALOO cohort, the ARDS incidence remains higher than that defined by the Lung Injury Prediction Score [158] and the postoperative complication incidence is very high, with the advantages of an initial insult of surgery at a specific time and a defined postoperative care pathway [164], which facilitates the conduct of efficacy trials. We believe this work demonstrates that oesophagectomy continues to be a useful model for trialling translational therapeutic and preventative strategies for critical illnesses prior to engaging in larger, more complex and expensive trials [148]. Examples include the Prevention of Postoperative Pulmonary and Cardiac Complications By Using HMG-CoA Reductase Inhibitor in Patients Undergoing Oesophagectomy (EudraCT Number: 2007-002454-37) and a trial of novel agent GSK2862277 (TFR116341 Trial EudraCT Number: 2014-000643-33).

There are several weaknesses with this investigation. This is a retrospective study and may well be underpowered for some factors, although this work was intended only to be exploratory and hypothesis generating. Much of the data we collected were retrospective and full data were not available for every patient. Additionally, some factors that may be important risk factors for both ARDS and oesophageal cancer, including alcohol consumption [25], were not recorded. There were significant differences in potentially important factors in anaesthetic management, discussed above, which potentially complicate comparisons made over time without protocolised surgical or anaesthetic management.

In conclusion, smoking has been associated with higher rates of ARDS following oesophagectomy. The association of dihydropyridines and ARDS requires validation in a larger cohort and mechanistic elucidation. Oesophagectomy continues to have a high risk of ARDS, which continues to offer a useful model for perioperative studies.

Chapter 5

THE TFR116341 TRIAL: CHALLENGES TO RECRUITMENT

5.1 Introduction

ARDS is a serious complication of major surgery which continues to have adverse consequences for patients [2]. ARDS rates are falling [47], but apart from lung protective ventilation [49], muscle relaxants [54] and the prone position [52], preventative and therapeutic strategies are lacking [56, 178]. Following oesophagectomy, ARDS is both common and associated with severe adverse outcomes [8, 23]. Reductions in ARDS incidence have been associated with lower post-operative mortality in this cohort [28].

The novel domain antibody GSK2862277 was developed by GlaxoSmithKline as a TNFR1 receptor antagonist. TNFR1 blockade has been shown to be effective in protecting the lung in a number or pre-clinical models, including ventilator induced lung injury in TNFR1 knockout mice [132], poly-microbial sepsis in mice [179] and a human model of ARDS induced in healthy volunteers with lipopolysaccharide [133]. Theoretically, GSK2862277 could be of benefit to patients with established hyperinflammatory ARDS and/or preventing post-operative pulmonary complications in those at high-risk [132, 137].

TFR116341 was a double-blind randomised placebo controlled trial to evaluate the effect of GSK2862277 on the lung, using oesophagectomy of a high-risk group (EudraCT Number: 2014-000643-33). This chapter aimed to evaluate the trial, focussing on the barriers to trial (and therefore translational sub-study) recruitment.

5.2 Methods

See Methods chapter 2.

5.3 Results

The TFR116341 trial had failed to recruit to half its target numbers nationally and was terminated for futility in August 2017. The Data Monitoring Committee remarked upon excessive baseline variability in the patients' extravascular lung water and pulmonary vascular permeability index, and inadequate pre- to post-operative change (A Bayliffe, personal communication). Details of the trial nationally (to January 2017) are shown in table 6, compared to data from the BALTI-P trial [138] and the VINDALOO trial [143].

Table 6. Recruitment into TFR116341, BALTI-P and VINDALOO, number of patients (%).

Trial	Screened	Screen Failures	Dosed	Withdrawal	Cancelled/ Abandoned/ Alternative Surgery
BALTI-P	362 (randomised)	0 (0.0%)	338 (93.4%)	2 (0.6%)	22 (6.1%)
VINDALOO	79	0 (0.0%)	68 (86.1%)	3 (3.7%)	8 (10.1%)
TFR116341	44	15 (34.1%)	29 (65.9%)	1 (2.3%)	1 (2.3%)

Figure 6. Outcomes of patients recruited to the BALTI-P, VINDALOO and TFR116341 trials (percentage of patients recruited). In the TFR116341 trial, the proportion of patients who withdrew consent or were screen failures was higher than the earlier trials. Conversely, cancelled surgery and abandoned/alternative surgery were higher in BALTI-P and VINDALOO.
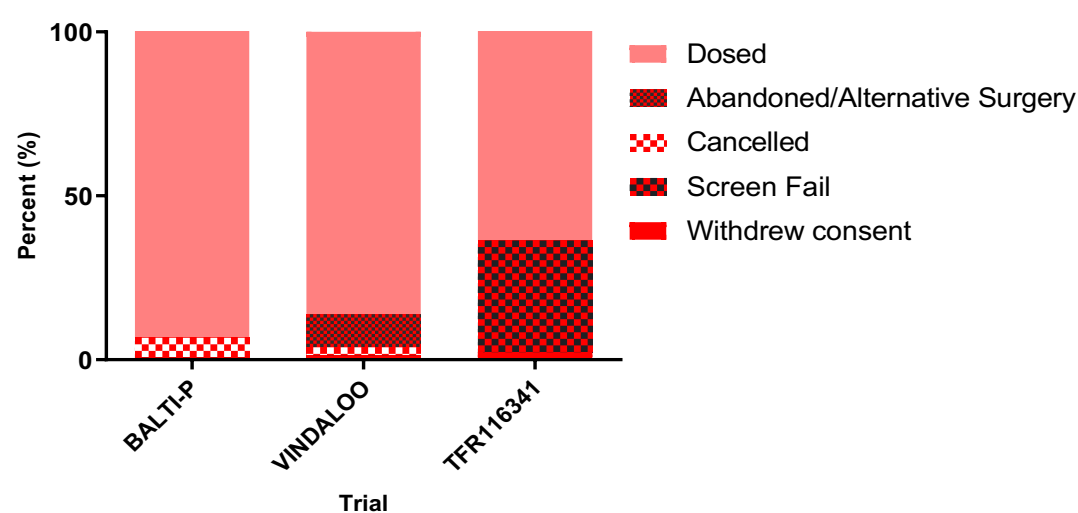
When the three trials were compared, there were significant differences in the outcome of those screened/randomised (p<0.0001) (figure 6). Four patients (9.5%) of 42 screened for ADAs were positive.

Across the two Birmingham sites, a total of 118 patients had been considered, of whom 17 had been formally screened. 12 patients had been dosed, the primary endpoint (PVPI reading at the end of surgery) had been obtained for 11. Screen failure was for positive Quantiferon™ tuberculosis test in two patients, two had anti-drug antibodies and one a prolonged QTc interval. One patient was withdrawn as he underwent transhiatal oesophagectomy, which did not require one-lung ventilation. Two patients were not screened due to exposure to concurrent experimental medications.

101 patients were considered for approach for formal screening but were not screened. Reasons for inability to approach and/or screen were collected from ongoing trial meetings and local quality assurance processes. These included:

- Exclusion criterion apparent prior to screening (premenopausal, pre-existing tuberculosis, hepatitis C, high-dose steroids, other investigational therapy) (8 (7.9%)).
- Invited to attend but did not/unable to attend (9 (8.9%)).
- Alternative medical and/or surgical therapy was subsequently pursued (9 (8.9%)).
- Surgical consultation too soon for consent and screening (20 (19.8%)).
- Approached but declined (34 (33.6%)).
- Unable to recruit due to trial suspension (17 (16.8%)).

- One patient was psychologically distressed and approach for consent was judged not to be in her best interests (1 (1.0%)).

- Not recorded (1 (1.0%)).

The majority of approached patients declined. Although not every patient was asked for or gave reasons, where they did, these were:

- Trial participation being too burdensome.

    - Psychological stress of considering the trial.

    - Additional visits to the hospital/distance to travel to the hospital too great.

    - Excess blood taken.

- Concurrent significant life events.

    - Death of partners close to surgery.

    - Requirement to close/sell a business.

    - Carer to dependent relatives.

- Patients did not want to be exposed to experimental therapy.

- Individuals perceived they were too old (despite being within the age range for inclusion).

- Strong opposition from close family members.

Recruitment in Birmingham was undertaken by a number of doctors working as investigators and research nurses, all of whom were experienced in both clinical perioperative care and in conducting clinical trials, and one experienced clinical trials coordinator with a background in health science. Additional potential barriers were discussed on a number of occasions with the trials senior investigators.

Consensus included the following factors, beyond those given by the patients above, were important:

- Short-notice scheduling of surgery was the single biggest limiting factor.

- Participation in a trial of a novel agent from a commercial pharmaceutical company was more difficult to recruit to than to drugs/vitamin supplements already in routine use, particularly when sponsored by a non-commercial entity.

- The patient information leaflet was off-putting.

- Patients, despite efforts from the research team, struggled to accurately perceive perioperative and anaesthetic risk.

- Patients were better empowered to direct their care and were more sceptical of participation than previously.

- Patients wanted explicit approval from their surgeon and/or anaesthetist, and were sometimes perplexed it was not their clinical team leading recruitment.

5.4 Discussion

Recruitment and retention of clinical trial participants is challenging and a range of factors affected TFR116341. Recruitment in cancer trials is often slower than anticipated [180] and some large and important critical care trials have taken substantial periods of time to complete [181]. Older patients are generally more likely to decline [182], which has implications for trials, such as TFR116341, targeting diseases in which increasing age is a risk factor [13]. A study of older adults in the USA found pharmacological agents were likely to discourage participation than non-pharmacological trials [183]. Patients declining consent to trial participation were also often unwilling to participate in research as to why, and

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those who did often did not disclose or did not have specific reasons [182]. Being unable to clearly identify patients' barriers makes it very difficult to optimise trial design to improve recruitment.

A "general discomfort with the research process", randomisation (instead of choice to receive a drug), inclusion of placebo and trial setting have been shown to be important deterrents to trial participation [184]. Fear of further deterioration in health status is a factor against trial participation in cancer patients, as is inadequate prior research awareness [185]. Long and jargon-filled patient information leaflets may be off-putting [186]. Patients frequently stated the burden of TFR116341 trial participation to be "too much." This reflects the overwhelming psychological demands of participation in the face of major surgery, uncertainty of the experimental drug and perhaps feelings of loss of control [184]. Major surgery is associated with higher level of anxiety in patients [187] and perception of risk is often poor [188, 189]. Declining to participate in research may be a way of relieving anxiety by exercising self-determination [190].

General education about research before an approach for a specific trial and innovative approach styles (such as in groups) may be beneficial [185]. Whether patients had previously been approached for chemotherapy or involvement in observational studies that were also running at the Birmingham centres was not recorded but patients who had already been involved in these trials seemed more receptive to TFR116341, perhaps by being more research aware.

Clinical decision making has moved from doctor-centred directive treatment plans to the shared decision making model, where integration of the patient's expectations and goals is paramount [191]. Patients are supported to choose between their

treatment options and may have implications for trial participation. The role of nurse specialists may have a profound influence, but study of this in relation to research participation is limited, as most studies have focussed on doctors [180]. For many patients participation in research offers benefits include the positive self-image driven by their altruism and education about their own health [180, 182, 191], which could be used to enhance recruitment [183]. Despite this, clinicians (rather than investigators) are remarkably reluctant to raise trial participation with eligible patients and several major trials have relied on a few clinicians for the majority of consents [180].

Protocol complexity can be a major problem for trials [192]. In TFR116341, a large number of patients could not be approached due because of inadequate time for screening for ADAs, in comparison to VINDALOO, in which there were few patients who met its less stringent exclusion criteria and patients could be enrolled at shorter notice [140]. Patients in VINDALOO were dosed at a mean of 10 days prior to surgery, with the shortest being 3 days [143], indicating that the typical time from surgical consultation to surgery has shortened since VINDALOO was completed. The higher than expected latent tuberculosis prevalence probably reflects insufficient epidemiological study [193].

The TFR116341 trial suffered slower than expected recruitment across all sites (K Hardes, personal communication). Clinician, researcher, patient and organisational factors may all played a role [180, 182]. If patients and their clinical teams [184] are willing to participate, oesophagectomy provides a predictably high incidence of severe post-operative complications [12, 13]. Future trials need to be designed to optimise recruitment and deliver the benefits of participation to patients.

Chapter 6.

PERIOPERATIVE NEUTROPHIL FUNCTION AND MODULATION BY

GSK2862277

Data from this chapter have been published as an abstract.

P Howells, D Dosanjh, D. McWilliams, E. Reeves, C. Snelson and D Thicket. Peri-operative modulation of neutrophil extracellular trap production: a translational sub-study. *Anaesthesia* (2017), 72 Suppl 2, 70.

6.1 Introduction

The neutrophil plays a major role in the pathogenesis of ARDS [65]. The modulation of neutrophil function by TNF alpha has previously been demonstrated [194] and the novel anti-TNFR1 agent GSK2862277 has been shown to reduce indices of pulmonary inflammation in a human pre-clinical ARDS model [133].

A precursor molecule to GSK2862277, namely GSK1995057, has been shown to reduce reactive oxygen species production, reduce neutrophil migration and reduced bronchoalveolar lavage neutrophil counts in primate and human models of acute respiratory distress syndrome [133]. This work with GSK1995057 indicated that pulmonary-endothelial interactions were important ARDS models for the observed reduction in pulmonary injury and via TNFR1 signalling.

Neutophils cause tissue damage through the formation of neutrophil extracellular traps (NETosis) and/or via phagocytosis being modulated [195, 196]. There is increasing evidence of their importance in the pathology of ARDS [197-203]. The aim of this chapter was to identify the effects of DAB on neutrophil function in healthy and critically unwell individuals, specifically looking at neutrophil extracellar trap production and phagocytosis. Unfortunately it was not possible to study the effects of DAB on neutrophil function *in vivo* from TFR116341, due to the small numbers recruited and the trial remaining blinded at the time of completion of this work.

The original planned research question was to compare the neutrophil function in patients having undergone oesophagectomy in those patients given GSK2862277 versus placebo. Because of poor recruitment to the trial, this was not possible.

Therefore, experiments were undertaken to attempt to ellucidate *in vitro* the effects of GSK2862277 on neutrophil function, comparing healthy young and older adults, patients undergoing oesophagectomy and patients with established critical illness.

This also provided the opportunity to study the effects of major surgery and critical illness on the neutrophil functions being investigated.

6.2 Methods.

See Chapter 2.

6.2.1 NETosis assay

For experiments with DAB, either DAB, Dummy DAB or vehicle control were applied during the 15 minute period for priming (referred to as "priming phase") or during the three hour incubation (referred to as "incubation phase"), (see section 2.4.2).

6.2.2 Phagocytosis assay

Phagocytosis was assessed as described in section 2.4.3. To investigate the effects of DAB, neutrophils were exposed to DAB, Dummy DAB or Vehicle Control for 15 minutes prior to exposure to the labelled PHRODO™ particles. This remained in their media for the duration of the incubation. In these experiments, intrinsic TNF from the neutrophils was relied upon for stimulation.

6.2.3 Additional trial data

Patients were recruited to the Critical Care Rehabilitation Trial on their fifth day of mechanical ventilation (this is Day 0, their first day of participation; Day 7 refers to their seventh day of participation and Day 14 their fourteenth).

White cell counts were available from trial samples for TFR116341 (produced by a laboratory for the sponsor) and were available from the hospital laboratory for the Critical Care Rehabiliation Trial.

Patients in the Critical Care Rehabilitation trial had their illness severity assessed by SOFA score[204], recorded at baseline and daily until ICU discharge. SOFA scores were correlated with recruitment and nearest day of discharge to week one functional neutrophil studies.

SOFA scores for TFR116341 particpants were not available at the time of the preparation of this work.

6.3 Results

Patient ages are shown in table 7.

Table 7. Baseline demographics numbers of patients contributing to neutrophil translational substudies.

Patient Group	Healthy Young	Healthy Elderly	TFR116341	Critical Care Rehabilitation Trial
Age Range (Years)	19-41	45-75	41-75	26-78

6.3.1 Neutrophil expression of TNFR-1 and TNFR2

Identification of receptor expression on neutrophils was undertaken using flow cytometry, using healthy and critical care neutrophils. Due to other samples taken for the TFR116341 and MARTINI trials, insufficient blood was available for sampling from the Healthy Elderly cohort for these experiments. There were no significant differences in TNFR1 or 2 receptor expression between healthy controls and the critical care cohort (one value excluded as outlier by Grubb's test), but high variability was observed in the critical care cohort, especially for TNFR1 (figure 7).

Figure 7: (A) Flow cytometry plot demonstrating presence of TNFR1 (pink line) and TNFR2 (black line) on neutrophils versus isotype control; median fluorescent index of TNFR1 and TNFR2 in controls and ongoing critical illness (Healthy Young n=13, Critical Care Rehabilitation Trial Day 0 n=10, Critical Care Rehabilitation Trial Day 7 n=7).

(B) No difference in the distribution of TNFR1 or TNFR 2 was detected (Kruskal-Wallis test p=0.145).

A:
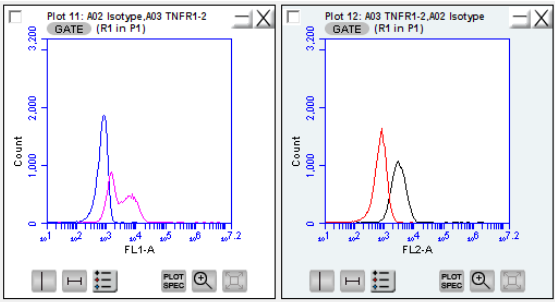
B:
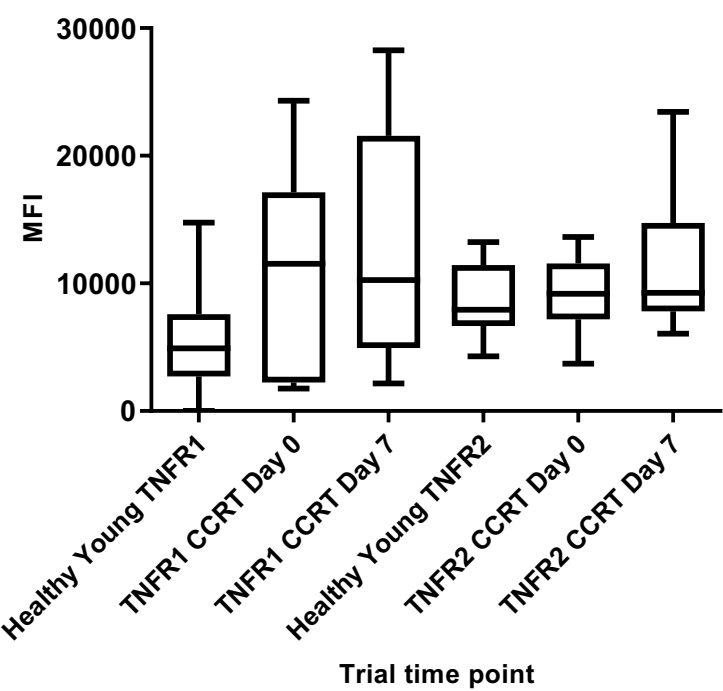
6.3.2 Neutrophil extracellular trap release is dysregulated in the perioperative period and in patients with critical illness

6.3.2.1 Baseline NETosis

Comparison of the young and elderly healthy controls, critical care rehabilitation trial and the TFR116341 trial were analysed (figure 8). The unstimulated group were significantly different (Kruskal-Wallis 0.0125). NETosis on entry to the Critical Care Rehabilitation trial was significantly lower than Young Healthy Controls (CCRT Median 6506 (IQR 5779-7701) versus Young Healthy 9376 (IQR 8415-9823) p=0.0047). Healthy elderly controls had higher NETosis that the Critical Care Rehab Group at Recruitment (Healthy Elderly Median 10666 (IQR 7660-13095) versus CCRT Median 6506 (IQR 5779-7701) p=0.0125) and Day 7 (Median 6457 (IQR 6108-9240, p=0.0462). There was no difference in pre-operative versus post-operative NETosis levels, but pre-operative and post-operative levels were higher than the baseline Critically Ill (Pre-operative Median 10312 (IQR 7478-11469), p=0.0125; Post-operative Median 9021 (IQR 5779-7701), p=0.0066).

6.3.2.2 NETosis post stimulation

The same groups with PMA stimulation were then analysed. Overall differences were significant (Kruskal-Wallis p=0.0288). NETosis was higher in Elderly (Median 40630 (IQR 34783-45456)) versus Young (Median 31855 (27462-34129)) Controls (p=0.0350). Young Controls were lower than the post-operative group (Median 49338 (IQR 39748-62827), p=0.0023).

Figure 8: NETosis in unstimulated and stimulated neutrophils (A) and with unstimulated only (B, expanded for clarity) (n=6-13), *p<0.05, **p<0.01. Baseline NETosis was suppressed in early critical illness, but was unchanged in the perioperative period. When PMA-stimulated, NETosis was much more extensive post-operatively.

A:
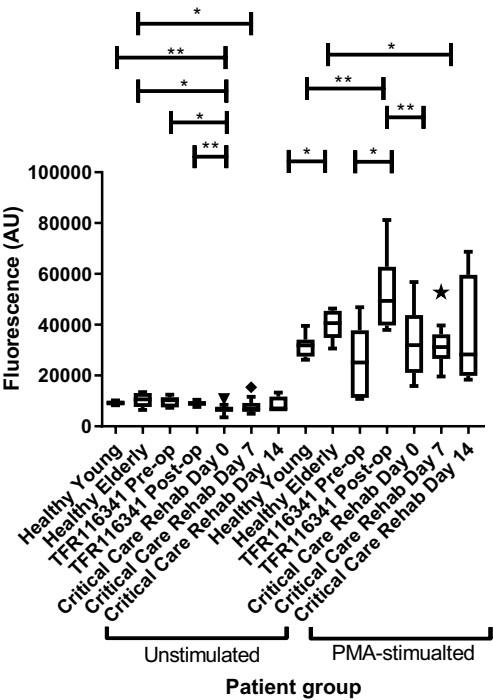
B:
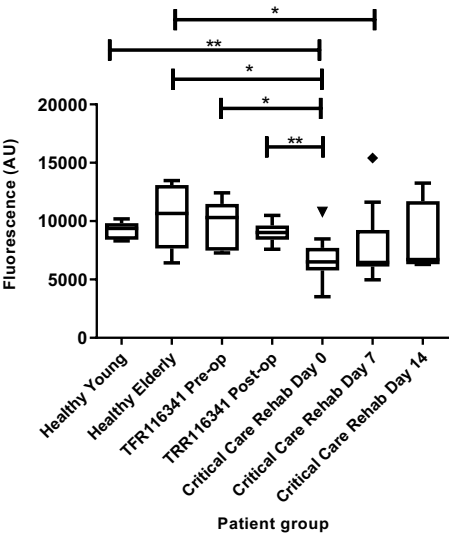
6.3.3 Correlation of NETosis and severity of critical illness

Illness severity in the Critical Care Rehabilitation group was defined by using serial SOFA scores. There was no correlation between the SOFA scores at recruitment and day 7 and concurrent NETosis (Spearman's r=0.272, p=0.365). Only the recruitment SOFA score was moderately inversely correlated with day 7 primed unstimulated NETosis (Spearman's p=-0.463, p=0.023).

6.3.4.1 Cell-free DNA in the perioperative period and in critical illness

Cell-free DNA has been used as a surrogate for NETosis in clinical studies [205, 206] and is being investigated as a potential near-patient test in a number of respiratory illnesses (D Thickett, personal communication). Samples for analysis were limited, but were available from a cohort of young healthy individuals, TFR116341 pre- and post-operatively, and Critical Care Rehabilitation Trial participants on Day Zero. Analysis of cell-free DNA showed in the TFR116341, cfDNA was lower than young healthy inviduals, at baseline (median in TFR116341 80ngml^{-1} (IQR 53-103) versus young healthy median 109 (IQR 104-122), p=0.0225) but was no different to young healthy post-operatively (median 101 (IQR 75-124), compared to control p=0.3972, compared to baseline p=0.1563). In the Rehabilitation Trial patients, there was much higher cell-free DNA in the Rehabilitation patients (median 1648ngml^{-1} (IQR 1200-1997) versus 109 (104-122), p<0.0001) (figure 9).

Figure 9: Cell-free DNA in TFR116341 (upper figure) and the Critical Care Rehabilitation Trial Day Zero of Mechanical Ventilation (lower figure) (Young healthy n=8, TFR116341 n=7, Critical Care Rehabilitation Trial n=18) *p<0.05, ****p<0.0001. These data indicate significantly lower cfDNA in the TFR116341 trial prior to surgery, but no difference in the post-operative samples. In contrast, cfDNA was substantially higher in the critically unwell.

A:
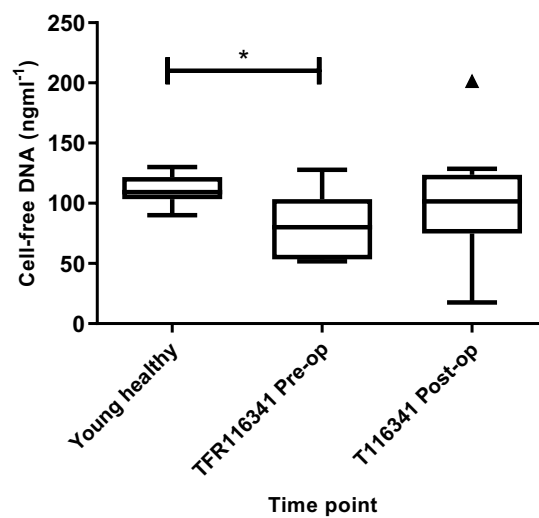
B:
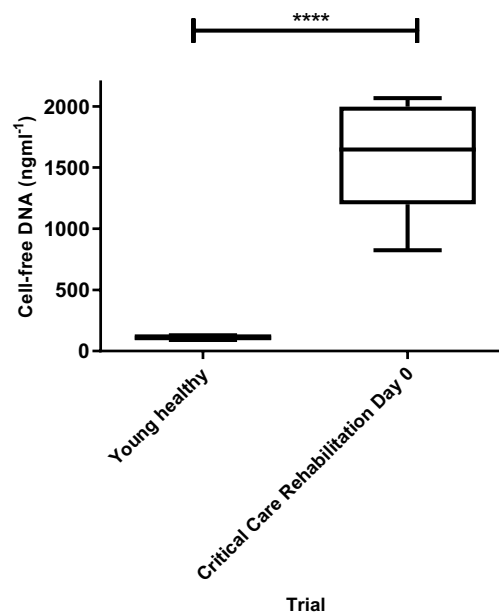
6.3.4.2 Association of cfDNA with illness severity

In the Critical Care Rehabilitation trial patients, there was no correlation between cfDNA and neutrophil, lymphocyte, monocyte or eosinophil count, nor neutrophil:lymphocyte ratio or SOFA score (table 8).

Table 8: Correlation of cfDNA and various clinically-used biomarkers and SOFA score in the Critical Care Rehabilitation Trial (Spearman's Rank Correlation Coefficient) (n=18).

Parameter	Correlation Coefficient	P-value
White cell count	0.13	0.60
Neutrophil count	0.22	0.39
Lymphocyte count	0.17	0.51
Monocyte count	-0.12	0.63
Eosinophil count	-0.10	0.69
C-reactive protein	0.35	0.27
Neutrophil:Lymphocyte ratio	-0.06	0.80
SOFA score	0.03	0.92

6.3.5 Phagocytosis in the perioperative period is not modulated

The phagocytic index for both *E coli* and *S aureus* stimuli were determined for young and elderly controls and patients from the TFR116341 and Critical Care Rehabilitation Trials (n=3-10). Regarding the PI for *E coli*, there was no significant overall difference (Kruskal-Wallis p=0.13). Similarly For *S aureus*, there was no significant overall difference difference (Kruskal-Wallis p=0.53) (figure 10).

Figure 10: Phagocytic index using *E coli* (A) and *S aureus* (B) particles. There were no significant differences in either group (Kruskal-Wallis for *E coli* p=0.13, *for S aureus* p=0.53 (Healthy Young n=7, Healthy Elderly n=6, TFR116341 n=8, Critical Care Rehabilitation Trial Day 0 n=11, Day 7 n=7, Day 19 n=3).

A:
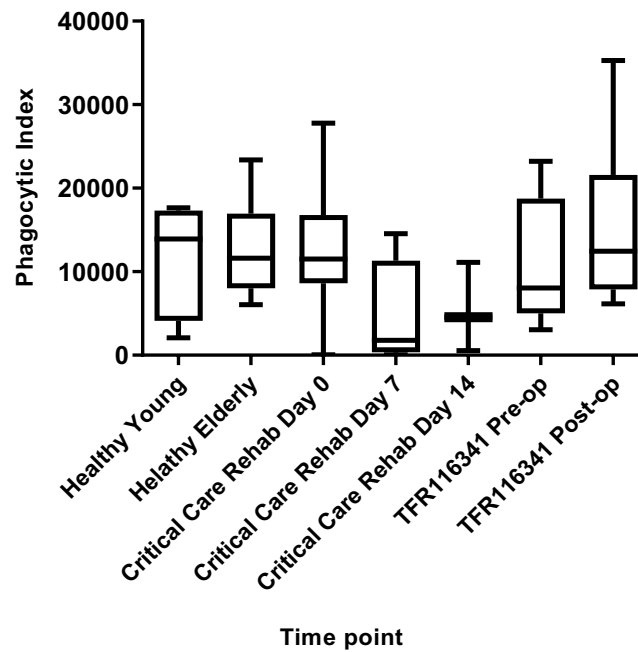
B:
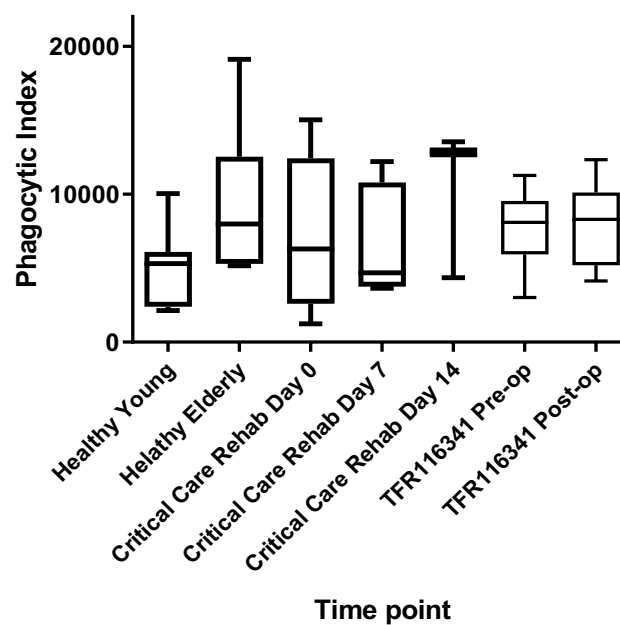
6.3.6 Phagocytosis is not related to illness severity

There was no signficant correlation between SOFA score and Phagocytic Index for participants in the Critical Care Rehabilitation Trial (Day 0, table 9; Day 7 Table 10 and SOFA Score (Day 0) in patients in the Critical Care Rehabilitation Trial) (note day 14 SOFA scores were not collected, as per the trial protocol).

Table 9: Correlation of Day 0 SOFA Score and Phagocytic Index for participants in the Critical Care Rehabilitation Trial (Spearman's Rank Correlation Coefficient) (Day 0 n=11, Day 7, n=7, Day 14 n=3).

Phagocytic stimulus	Time point	Correlation Coefficient	P-value
E coli	Day 0	0.25	0.45
E coli	Day 7	-0.06	0.93
E coli	Day 14	0.50	1.00
S aureus	Day 0	-0.41	0.21
S aureus	Day 7	0.29	0.60
S aureus	Day 14	-0.50	1.00

Table 10: Correlation of Day 7 SOFA Score and Phagocytic Index for participants in the Critical Care Rehabilitation Trial (Spearman's Rank Correlation Coefficient) (Day 0 n=11, Day 7, n=7, Day 14 n=3).

Phagocytic stimulus	Time point	Correlation Coefficient	P-value
E coli	Day 0	0.25	0.28
E coli	Day 7	-0.06	0.17
E coli	Day 14	No data available	
S aureus	Day 0	-0.22	0.57
S aureus	Day 7	0.32	1.0
S aureus	Day 14	No data available	

Note: "No data available" for the day 14 group reflects the small number of patients still alive and in-patients at this time with complete SOFA scores.

6.3.7 Modulation of NETosis by DAB and Dummy DAB

The *in-vivo* administration of DAB versus placebo on NETosis was studied by comparing those receiving GSK2862277 (n=3) to placebo (n=4) (these data were made available by GSK after completion of the trial but before its publication). There was no difference in baseline NETosis (Table 11).

Table 11: Comparison of post-operative NETosis in patients having undergone oesophagectomy, treated with GSK2862277 (n=3) or placebo (n=4).

Condition	Placebo (Median (IQR))	GSK2862277 (Median (IQR)	P-value
Unprimed unstimulated	9770 (8030-10400)	9000 (8686-9044)	0.40
Unprimed PMA-stimulated	56800 (47100-81100)	40400 (37900-54700)	0.11
Primed unstimulated	17300 (11800-18800)	16700 (11400-17500)	0.63
Primed PMA-stimulated	51600 (37700-61800)	36100 (36100-65234)	P>0.99

The effects of DAB were analysed on neutrophils from young healthy volunteers, looking at the effects on NETosis whilst applied during for three hours during the incubation phase, initially at 10nM (figure 11). Across all conditions, there were significant differences (Friedman's test p<0.0001). When each condition was analysed individually, Unprimed Unstimulated was significant (Friedman's p=0.0120), there was a significant difference between vehicle control and dummy DAB (median vehicle control 9376 (8415-9823) versus Dummy DAB 11057 (IQR 10483-13056), p=0.0313). Unprimed stimulated was significant (Friedman's 0.0120); vehicle control versus DAB was significant (vehicle control median 31673 (IQR 27156-35420) versus dummy DAB 34491 (30405-41072), p=0.0313). In the primed unstimulated group, Friedman's test was significant (p=0.0055). Individual comparison showed DAB was significantly higher than vehicle control (vehicle control median 12343 (IQR 9167-13745) versus DAB 14689 (10551-16070), p=0.0313) and Dummy DAB higher than vehicle control (median 14618 (IQR 11006-17240), p=0.0313). There were no differences in the Primed PMA conditions (Friedman's p=0.1416).

Figure 11: effect of DAB 10nM on NETosis when exposed for the incubation phase in healthy volunteers (n=6, *p<0.05). PMA predictably increased NETosis. In the unpimed group, Dummy DAB also increased NETosis compared to VC.
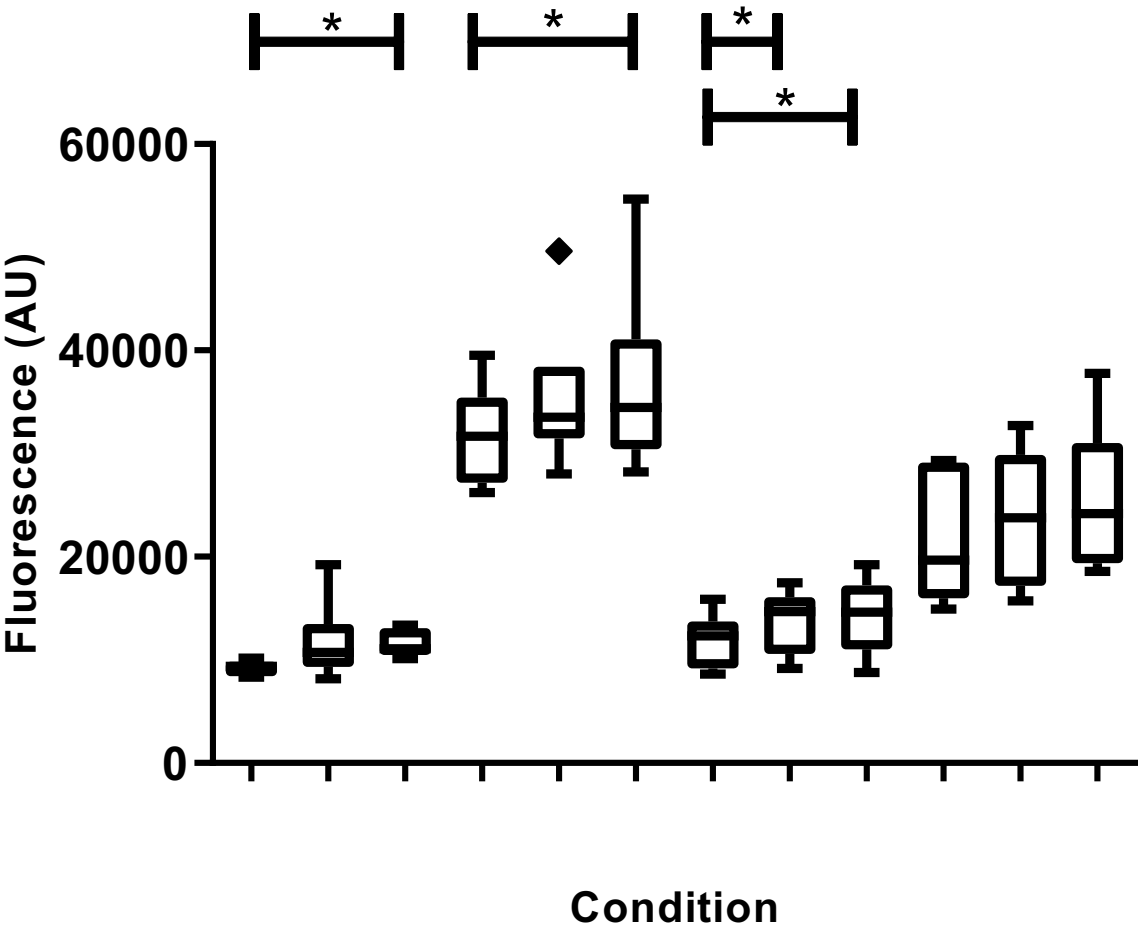
Primed	-	-	-	-	-	-	+	+	+	+	+	+
PMA	-	-	-	+	+	+	-	-	-	+	+	+
DAB	-	+	-	-	+	-	-	+	-	-	+	-
Dummy DAB	-	-	+	-	-	+	-	-	+	-	-	+

When incubated with 100nM DAB or Dummy DAB, the absolute values were significantly different (Friedman's p=0.0006) (figure 12). The unprimed unstimulated group were significantly different (Friedman's p=0.028), but there were no significant differences between groups. The unprimed stimulated groups were significantly different (Friedman's p=0.028), but there were no significant differences between individual groups. Neither of the primed groups were significantly different.

Figure 12: effect of DAB 100nM on NETosis when exposed for the incubation phase in healthy volunteers (n=5). No significant differences were observed within groups at this concentration (p>0.05 for all).
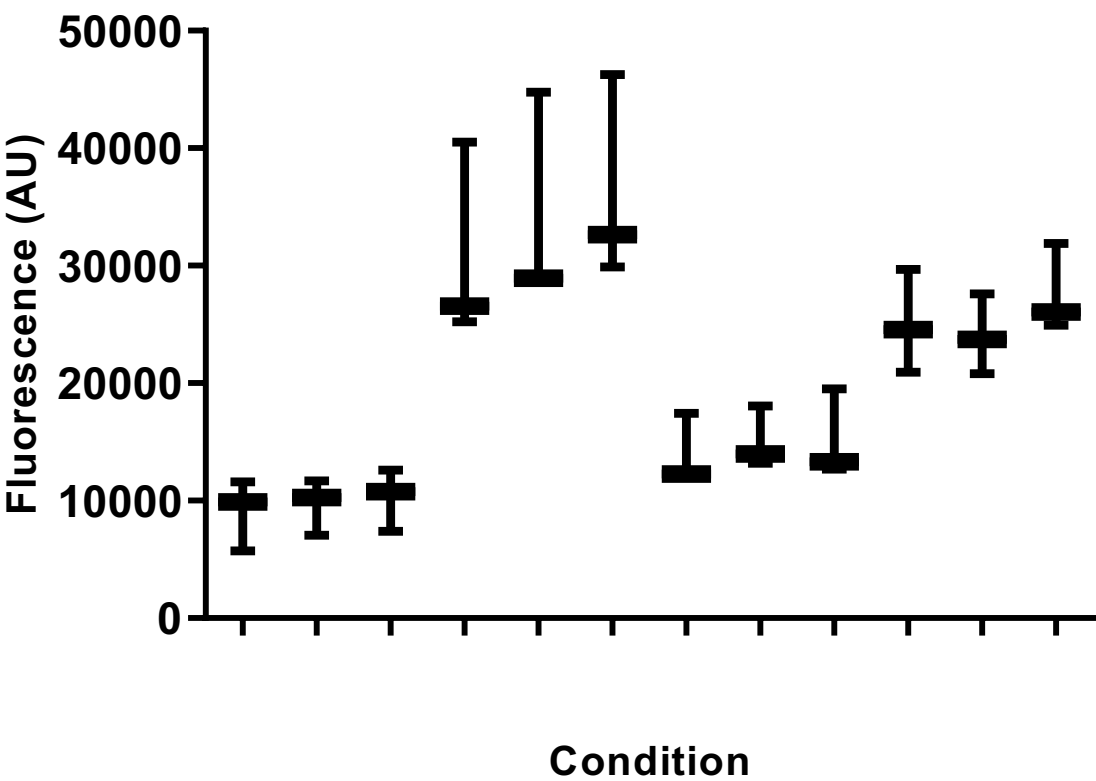
Primed	-	-	-	-	-	-	+	+	+	+	+	+
PMA	-	-	-	+	+	+	-	-	-	+	+	+
DAB	-	+	-	-	+	-	-	+	-	-	+	-
Dummy DAB	-	-	+	-	-	+	-	-	+	-	-	+

Neutrophils were primed with TNF alongside 10nM DAB, Dummy DAB or Vehicle Control (figure 13). Friedman's tests was only significant for Unprimed Unstimulated (Friedman's test $p=0.039$) but tests between individual conditions were not.

Figure 13: effect of DAB 100nM on NETosis when exposed for the priming phase in young healthy samples (n=5). PMA stimulation increased NETosis as expected. There were no significant differences within groups (p>0.05 for all).
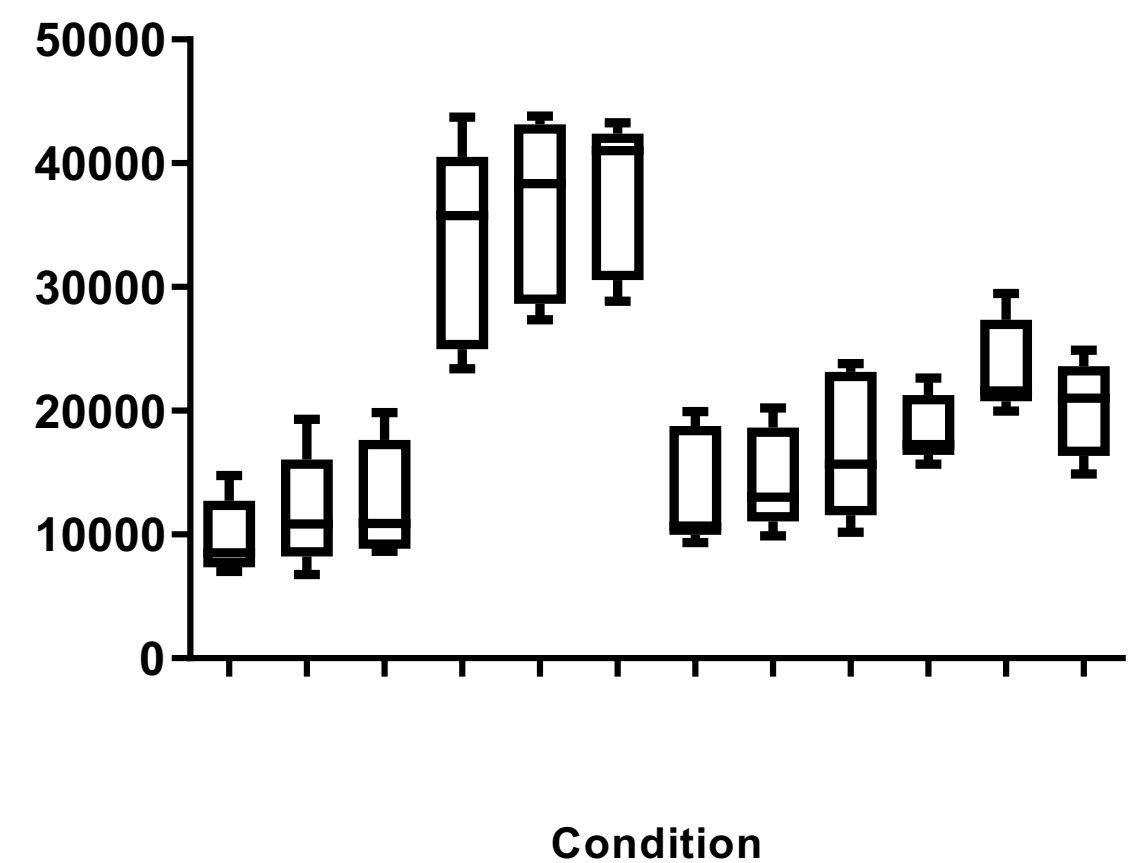
Primed	-	-	-	-	-	-	+	+	+	+	+	+
PMA	-	-	-	+	+	+	-	-	-	+	+	+
DAB	-	+	-	-	+	-	-	+	-	-	+	-
Dummy DAB	-	-	+	-	-	+	-	-	+	-	-	+

To investigate neutrophils in sick patients, samples were used from the Critical Care Rehabilitation Trial on days 0 and 7. With respect to day 0 (figure 14), the Unprimed Unstimulated group, NETosis was lower in VC than both DAB (p=0.0018) and Dummy DAB (p=0.0011), and Dummy DAB was higher than DAB (p=0.027). In the Unprimed PMA group, DAB was higher than VC (p=0.0018), Dummy was higher than VC (p=0.0058) but there was no difference between DAB and Dummy DAB. In the Primed Unstimulated group, DAB was not different to VC, however DAB was significantly lower than Dummy DAB (p=0.013) and Dummy versus control (p=0.012). Primed PMA-stimulated showed no difference between DAB and VC, however Dummy DAB was higher than VC (p=0.024) and DAB (p=0.0021).

Figure 14: the effects of DAB on neutrophils recovered from patients from the Critical Care Rehabilitation Trial Day 0 (n=11), *p<0.05, **p<0.01. PMA stimulation increased NETosis as expected. Both DAB and Dummy DAB increased NETosis compared to VC.
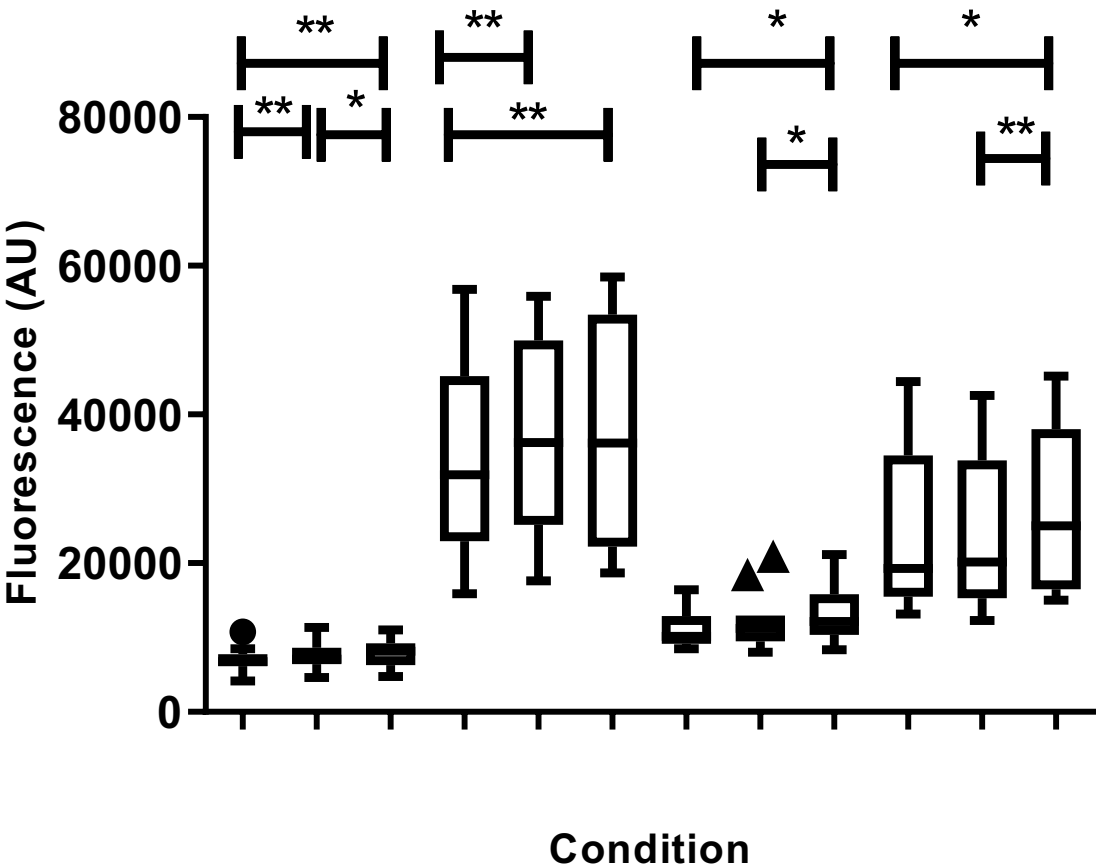
This was repeated with the Day 7 data (figure 15). Several groups showed non-normal distribution (D'Agostino & Pearson omnibus normality tests were significant). Unprimed unstimulated neutrophils were different overall (Friedman's $p<0.0001$). When compared individually, NETosis was higher in the DAB group compared to VC ($p=0.0059$), Dummy DAB was higher than DAB ($p=0.002$) and Dummy DAB higher than VC ($p=0.0020$). In the Unprimed PMA-stimulated group, VC was lower than both DAB ($p=0.0020$) and Dummy DAB ($p=0.0059$) but no difference between DAB and Dummy DAB. In the Primed Unstimulated group, overall differences were significant (Friedman's test $p<0.0001$). DAB was significantly higher than VC ($p=0.002$), as was Dummy DAB ($p=0.002$), Dummy DAB higher than DAB ($p=0.014$). For the Primed PMA-stimulated group, overall significance was found ($p=0.012$). VC was lower than DAB ($p=0.027$) and Dummy DAB ($p=0.0039$), whilst DAB versus Dummy DAB was not significant.

Figure 15: the effects of DAB on neutrophils recovered from patients from the Critical Care Rehabilitation Trial Day 7 (n=10). PMA increased NETosis as expected. DAB and Dummy DAB increased NETosis compared to controls, in the Unprimed Unstimulated and Primed Unstimulated, Dummy DAB increased NETosis significantly more than DAB.
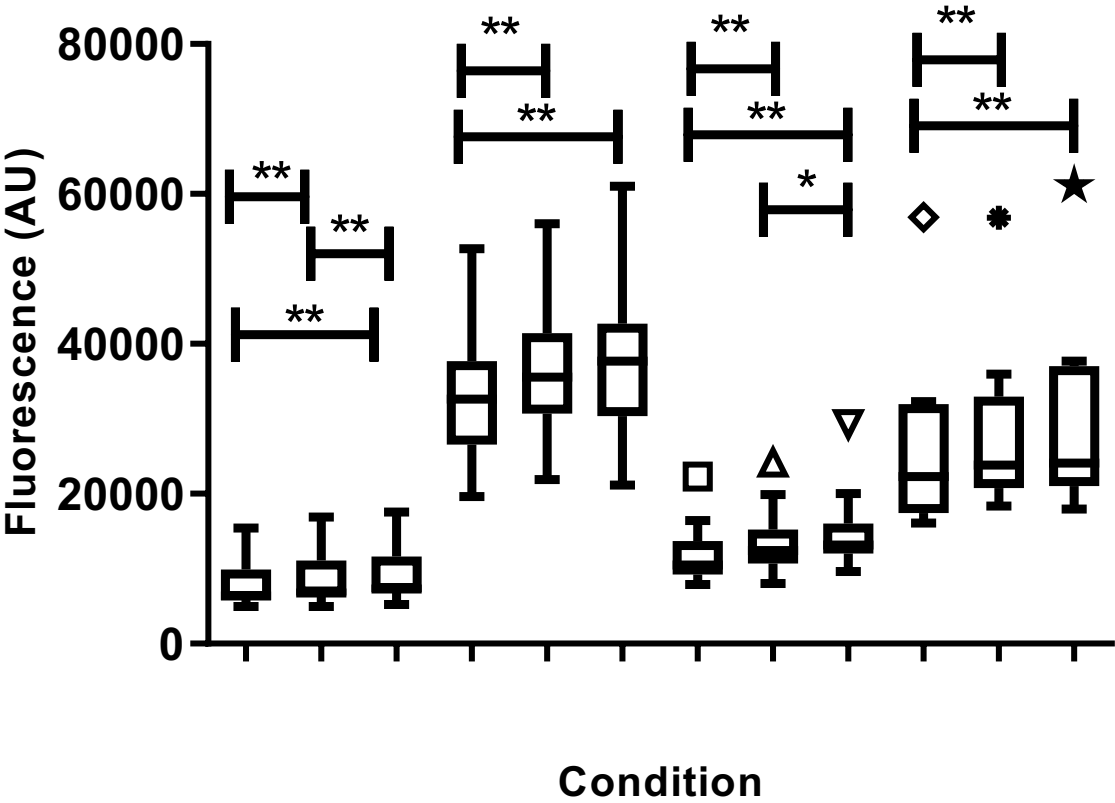
6.3.8 DAB does not modulate phagocytic activity of neutrophils

Healthy elderly controls were used to assess the effects of DAB on phagocytosis (given the results below, it was decided not to repeat these experiments in other groups). Neutrophils were exposed to DAB, Dummy DAB or vehicle control for 15 minutes prior to running the PHRODO assay. Phagocytosis experiments were run relying on intrinsic neutrophil TNF secretion. Plans to study exogenous TNF and other methods for eliciting TNFR signalling pathways were not pursued due to limited samples.

Comparison was made between vehicle control, DAB and Dummy DAB was made and there were no differences at any time point, to either *S aureus* or *E coli* (assessed by multiple Kruskal-Wallis tests). One data-point was missing, for analyses requiring complete data, last observation carried forward was used to interpolate this.

Analyses of the phagocytic index were undertaken with Friedman's test at each time point.

For those with *E coli* PHROD particles, these showed no difference (t=0min p=0.956, t=30min p=0.430, t=60min p=0.956) (figure 16 upper). Similarly, for *S aureus*, no difference was observed (for the 60 minute time point, Friedman's test 0.6425) (figure 16 lower).

Figure 16: phagocytic index of *E coli* (upper figure) and *S aureus* (lower figure) PHRODO in neutrophils exposed to DAB (n=6). No significant differences were seen between VC, DAB and Dummy DAB at any time point for either condition (p>0.05).
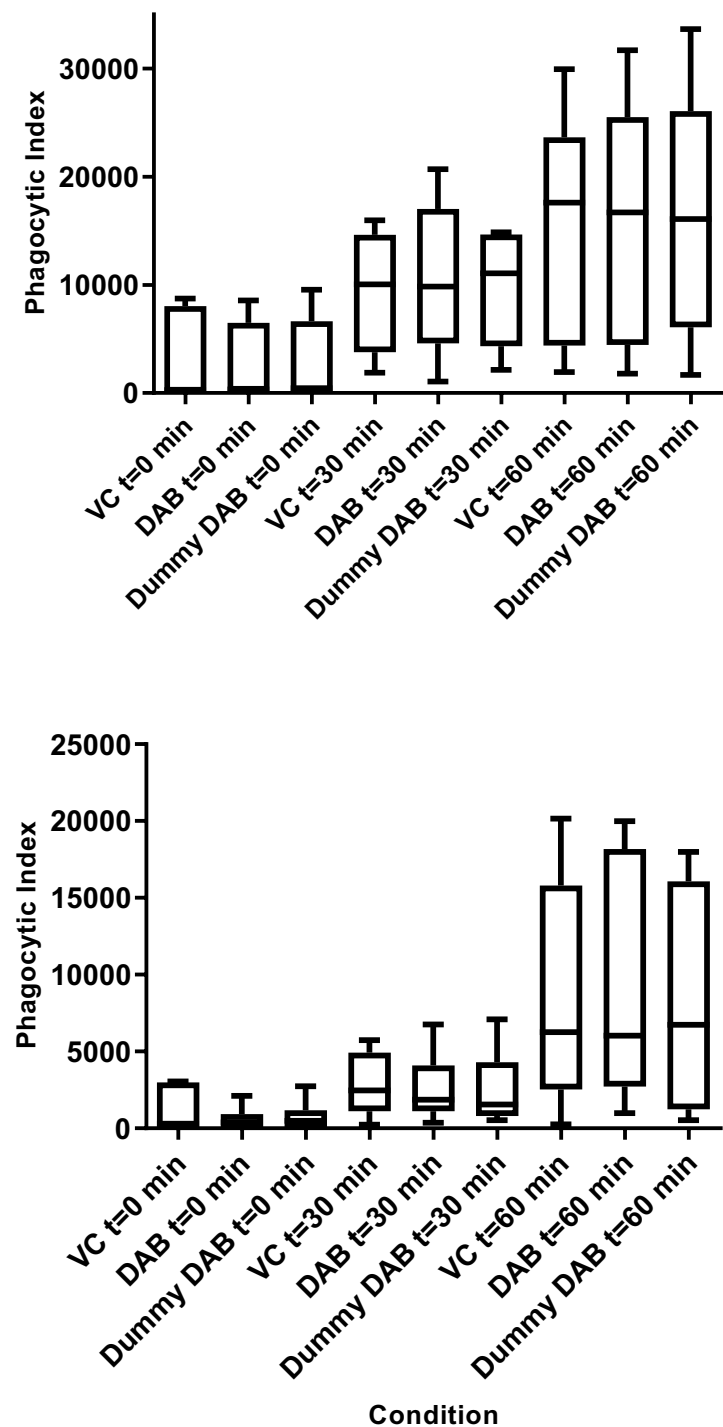
6.3.9 DAB does not alter phagocytosis *in vivo* when administered pre-operatively.

The effect of GSK2862277 was compared to placebo in the post-operative phagocytosis assays of patients exposed to drug (n=3) and control (n=4). There were no significant differences between groups, regardless of stimulus or time point (Table 12).

Table 12: Effect of GSK2862277 (n=3) versus placebo (n=4) on neutrophil phagocytosis in patients in the TFR116341 trial.

Time point (minutes)	Stimulus	PI Placebo median (IQR)	PI GSK2862277	P-value
0	*E coli*	18.0 (3.5-67.8)	117 (43.0-543)	0.11
30	*E coli*	10300 (2980-17500)	3310 (2920-6120)	0.40
60	*E coli*	20200 (7380-32200)	9660 (8710-15300)	0.40
0	*S aureus*	60.5 (5.3-183)	46.0 (27.0-81.0)	>0.99
30	*S aureus*	954 (304-2960)	1500 (989-1670)	0.63
60	*S aureus*	5050 (3840-8700)	8660 (4440-10400)	0.40

6.4 Discussion

As was shown previously [148], neutrophils expressed both TNFR1 and 2, although this chapter's experiment showed TNFR2 expressed more consistently. Given the modulation of TNF and TNFR in sepsis [207] and ARDS [208], substantially lower receptor levels were expected in sick patients. As the critical care patients had been ventilated on the ICU for five days prior to recruitment, and potentially unwell for some time prior to admission, it is possible the nadir in TNFR expression was missed or regulation of TNF pathways is primarily at the intracellular level.

This study showed no change in baseline NETosis between pre-operative and post-operative phases, but that PMA-stimulated post-operative NETosis was much higher, suggesting that surgery has a priming effect. This may be biologically desirable in providing an aggressive immune response to a second insult following surgery (such as infection or haemorrhage), or may contribute to excessive immune activity and collateral tissue damage [209]. Lower NETosis in unstimulated neutrophils in established critical illness is consistent with other features of immunoparesis previously described [207, 210].

Raised NETosis has been reported in a range of diseases [98, 196, 199, 206, 211, 212]. Currently, debate continues about whether NETs are important mediators of the pathophysiological processes of these diseases [90, 98] or whether NETosis may be a biologically useful immunological response, but its high level in critical illness may be an epiphenomenon of upregulated neutrophil activity [196].

NETosis has not only been implicated in inflammation, but also thrombosis [212], cancer metastasis [97] and acute kidney injury [206]. A comprehensive understanding of their biology remains elusive, in part due to their recent discovery

[85] but also because of the various different methodologies used for their detection, making direct comparisons more challenging [196].

Nevertheless, NETosis increasingly seems important in ARDS [197, 199, 202]. Their upregulation by infective and non-infective triggers and their potential cross-activity with the immune and coagulation system, both of which are key components of the pathophysiology of ARDS [213] adds mechanistic plausibility to clinical [202] and animal model data [92].

These experiments showed increased NETosis in response to PMA in the healthy elderly compared to young individuals. The opposite effect is reported elsewhere [194]. This may be an effect of the smaller number of subjects in this study, or perhaps the more stringent definition of healthy used in preceding investigations.

Assessment of baseline NETosis in disease has shown variable results. In a longitudinal study of burns patients, neutrophil function overall was suppressed from the day after the burn to one year (completion of the study), but NETosis was increased during septic episodes [214]. Baseline NETosis is up-regulated in the first hour after major trauma (albeit with markedly increased variability) but down-regulated from 4-48 hours, whereas PMA-stimulated NETosis was suppressed at all time-points [215]. This is one of the first reports of the hyper-acute regulation of NETosis but indicates baseline NETosis is subject to rapid changes in response to pathological stimuli.

NETosis in the perioperative period is yet to be characterised in detail. The role of neutrophil extracellular traps is being investigated in organ transplant, where neutrophil function is complex with both pro- and anti-inflammatory effects [216]. NETosis may be an important mechanism driving surgical complications, including inflammation [93, 195, 196], thrombophilia [212] and tumour metastasis [97] and,

therefore, patient harm following surgery [2]. As described above, NETosis is subject to very rapid regulation in the immune system [214], moreover NETosis and reactive oxygen species production was further modulated by exposure to a number of damage-associated molecular patterns [214]. In the perioperative phase, given the complex interaction of patients with comorbidities and pharmacological therapy, anaesthesia, the surgical insult and subsequent management [12, 13], it is conceivable a number of factors may influence perioperative neutrophil function, contributing to a complex response to the surgical insult.

Cell-free DNA has been proposed to be a proxy for NETosis [84, 206] and was associated with a risk of post-operative renal failure [206]. In the TFR116341 group, baseline cfDNA was lower than healthy controls. This may reflect these patients being post-chemotherapy and having subtle ongoing impairment in haemopoiesis or immune function. However, cell-free DNA was far higher in the Critical Care Rehabilitation Trial patients than healthy controls in the presence of lower baseline NETosis in the critically unwell, indicating it likely arises from sources besides NETosis, or NETosis is increased *in vivo* in the critically unwell [205]. These patients had heterogeneous presenting pathologies in the Critical Care Rehabilitation group, which would be expected to be associated with heterogeneous immune modulation [84, 210].

These experiments showed a modulatory effect of both DAB and Dummy DAB on neutrophils from healthy or critically unwell individuals in vitro [133]. This is likely an off-target effect of novel domain antibodies. Toll-like receptors (TLR) are present on neutrophils but their function is not yet as well-described as in other cell types [217], which is an avenue for future work. TLRs are important in ARDS [68] and they may drive NETosis [218]. The response seen to Dummy DAB could be via this

mechanism. In macrophages, the TNF and PAMP signalling pathways converge onto the MAPK-NF-κB system [72]. Potentially, an immunogenic dummy agent may inadvertently be acting in this way.

Multiple factors, including time and environmental mileau influence whether TNF signal transduction leads to increased or decreased apoptosis [217]. A much more sophisticated model may be required to ellicudate the biological effects of DAB. Most of the beneficial effects of DAB were demonstrated in animal models [137]. It may well the down-regulation of neutrophil effects by TNFR1 modulation models are driven by regulatory cells such as macrophages [82], lymphocytes [219] or endothelium [133], with potential temporal effects and/or sequence of stimuli being important [108]. Signalling related to the differential effects of soluble and membrane bound TNF may well affect the model as well [108, 220].

Modulation of phagocytosis by DAB was not demonstrated. TNF has been associated with a range of functions, but is not canonical in relation to phagocytosis, so this is perhaps not surprising [221]. Granulocyte Colony-Stimulating Factor (G-CSF), generally taken as an immune enhancing agent, has mixed effects on neutrophils, enhancing chemotaxis, phagocytosis and bacteriocidal activity but reduced scretion of TNF alpha, but increased soluable TNF-receptor and increased IL-1ra release [222]. TNF alpha was higher in poor responders to G-CSF, but patients had similar neutrophil counts, phagocytic and bacteriocidal effects [222]. Therefore, TNF alpha may not play a decisive role in regulating phagocytosis via neutrophils directly.

There are a number of limitations to this work. Insufficient recruitment to both the main and translational sub-study of TFR116341 resulted in insufficient participants for the original experiments planned, and delayed termination of the trial prevented

comparison of this to clinical and biomarker data from trial participants. This, in particular, led to the study of a simplistic model of direct neutrophil functions, especially phagocytosis, whereas regulation in an *in vivo* system may be of greater biological interest and link to the TNF alpha signalling system [201]. Limited availability of blood from participants in other trials limited which groups could contribute to which experiments, as did a limited supply of both DAB and Dummy DAB.

Attrition (by death or hospital discharge) in the Critical Care Rehabilitation Trial limited sample size in patients beyond a week of established critical illness. This is an important factor to be considered in future trial design where prolonged follow-up is considered, as loss to follow-up substantially weakens the data derived.

Dummy DAB's apparent off-target effect suggests unanticipated biological activity. In one sense, this was its role, and future work will need to ellicidate how Dummy DAB (and perhaps DAB too) exerted this effect. Unfortunately the laboratory infrastructure to determine this were not available during these studies.

Future work also needs to map *in vivo* NET activity. Clinical tissue sampling is difficult in ARDS studies [148] and so an animal model may be more appropriate. This would allow a controlled surgical stimulus and reliable sampling, including during and immediately after the ARDS and/or OLV stimulus, and post-mortem. However, the applicability of animal models to humans, the challenges accurately modelling oesophagectomy as an operation in even a large mammal and the reliability of animal ARDS models all make this a difficult undertaking [223].

There are now some established blood biomarkers [214], but as demonstrated here, cfDNA does not seem to fulfil this role, at least in this cohort. Sampling for established NETosis biomarkers in future trials may be useful.

One strategy may be to establish Dummy DAB's (and also perhaps DAB's) off-target effects using binding studies, confirming its mechanism with agonist and antagonist studies. It would be possible to study DAB's effects in a large animal model, following adminstration and a surgical stimulus, followed by analysis of multiple neutrophil functions and upstream regulators. This would permit a more focussed clinical study in humans and perhaps allow more sophisticated targeting of the drug to particular diseases and/or clinical phenotypes.

Translational substudies need to be undertaken with the challenges to recruitment considered and addressed, as discussed in Chapter 5. Planning experiments which can either utilise stored and transported samples and/or near-patient analysis, may be necessary for trials with small numbers of participants.

In conclusion, neutrophil responses to NETosis promoters are up-regulated following oeosphagectomy, whilst being depressed in patients with onging critical illness. Phagocytosis was not affected. The effect on NETosis may be important in contributing to post-operative complications following major surgery. GSK2862277 was not demonstrated to modulate phagocytosis. Inadequate replication of the biological system in the *in vitro* models limit the ability of models to ellucidate the mechanisms of effects seen in the pre-clinical models in which GSK2862277 successfully limited lung inflammation.

Chapter 7

ALVEOLAR MACROPHAGE FUNCTION AND ITS MODULATION BY

GSK2862277

7.1 Introduction

A precursor molecule of GSK2862277 has been shown to reduce indices of inflammation in a human pre-clinical LPS model of ARDS [133], and there is mechanistic work to suggest down-regulation of TNFR1 signalling may be beneficial in pre-clinical ARDS models [132, 179], but how different immune cells are modulated remains to be more fully defined.

Macrophages are an important cell in the innate immune response and ARDS [65]. They have roles as immunomodulators, secreting cytokines [224] and undertaking phagocytosis [225]. The immune response associated with ARDS involves a range of cytokines with local and systemic effects [65]. They are important in both in the inflammatory and recovery phases [65, 213], with their secretion of TNF alpha being proposed as a key cytokine in the acute phase [125]. Work in this thesis showed neutrophil NETosis and phagocytosis are not modulated by GSK2862277, whilst reactive oxygen species have previously been shown to down-regulated [148].

The original intention had been to firstly characterise cytokine profiles in the TFR116341 trial for comparison with BALTI-P and VINDALOO, secondly to recover alveolar macrophages and assess their function cytokine secretion and functional behaviour in the context of DAB administration and thirdly to assess DABs effects on recovered alveolar macrophages in a series of *in vitro* experiments. The first was not possible as data from the trial were not available at the time of preparation of this thesis. The second was not possible due to poor rates of recruitment to the TFR116341 trial. The third was hampered by limited sample availability.

The aim of this study was to characterise TNF alpha and other cytokines in the perioperative phase, using data from the BALTI-P sub-study [138] and VINDALOO trials [143], and assess the effects of GSK2862277 on macrophage function.

7.2 Methods

See Chapter 2.

7.3 Results

7.3.1 Cytokine Data from BATLI-P and VINDALOO

Results of the cytokine data are presented in Table 13 and 14. For TNF alpha, there was an overall difference in absolute levels (Kruskal-Wallis p<0.0001). When compared individually, in both BALTI-P and VINDALOO TNF alpha fell from pre- to post-operatively, but recovered to pre-operative levels on day 1. When the two trials were compared, plasma TNF alpha was significantly lower in VINDALOO at all three time points.

For TNFR1, there was an overall difference (Kruskal-Wallis p<0.0001). Levels were higher post-operatively and on day one in both trials, whilst levels were lower at each time point in VINDALOO compared to BALTI-P (p<0.0001). When fold-change was analysed, there were overall differences (Kruskal-Wallis p<0.0001). In BALTI-P, there was a fold-change rise from pre- to post-operatively, but returned to baseline on day 1, whereas in VINDALOO, pre- to post-operatively and pre- to day 1 both rose significantly (p<0.0001).

For TNFR2, overall differences were significant (Kruskal-Wallis p<0.0001). In BALTI-P, TNFR2 rose post-operatively (p=0.0017) and on day one (p<0.0001) compared to pre-operatively, with a similar pattern in VINDALOO (post-operatively

p=0.0051, day 1 p<0.0001). Levels were lower in VINDALOO at each time point compared to BALTI-P (p<0.0001). For fold change, there were significant differences overall (Kruskal-Wallis p<0.0001). In both trials, there were significant fold-rises in TNFR2 levels from baseline post-operatively and on day 1 (p<0.0001 for all). In BALTI-P, there was also a significant rise from post-operative to day 1 (p=0.0065).

IL6 levels rose in both trials post-operatively and fell but remained higher than baseline, but there was not a significant difference between the two trials, in contrast to other cytokines. IL1-ra was higher in the VINDALOO group, but fold change was greater in BALTI-P. IL 10 levels were higher in the VINDALOO group, but showed less fold-change, again indicating a more stable cytokine profile. IL8 was modestly but significantly higher in VINDALOO, but the two trials showed a similar trajectory of change over the three time points.

Soluble RAGE (a marker of respiratory type one epithelial cell damage) showed overall significant differences (p<0.0001). Interestingly, levels were higher at baseline in VINDALOO versus BALTI-P (BALTI-P median 31 (IQR 16-59) versus VINDALOO 42 (35-42), p=0.0047), but BALTI-P was much higher than VINDALOO post-operatively (BALTI-P 469 (IQR249-1016) versus VINDALOO 52 (IQR 39-72), p<0.0001), and remained elevated but less markedly on day 1 (BALTI-P 55 (IQR 35-95) versus VINDALOO 38 (IQR 30-49). When analysed for fold-change, there were significant differences overall (Kruskal-Wallis p<0.0001). In both trials, there was a fold-change rise from pre- to post-operatively, then a significant fall to day one, but remained higher on day 1 than baseline (p<0.0001 for all).

Table 13: Absolute cytokine levels in BALTI-P and VINDALOO trial participants.

Cytokine Median (IQR) pg/ml	BALTI-P absolute level pre-op	BALTI-P absolute level post-op	BALTI-P absolute level day 1	p value		VINDALOO absolute level pre-op	VINDALOO absolute level post-op	VINDALOO absolute level day 1	p value		p value B vs V
TNF alpha	10.0 (6.0-19)	3.9 (2.8-6.0)	9.3 (5.9-14.0)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 p=0.3961		13.0 (11.0-14.0)	11.0 (9.6-12)	12.0 (11.0-13.0)	Pre-post 0.0002 Pre-day 1 0.1346 Post-day 1 0.0061		Pre 0.0177 Post <0.001 Day 1 0.0025
TNFR1	1502 (1246-1807)	2398 (1722-3212)	2482 (1770-3286)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.72		404 (284-595)	941 (620-1426)	821 (533-1489)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.51		Pre <0.0001 Post <0.0001 Day 1 <0.0001
TNFR2	4474 (3458-6829)	6977 (4269-8948)	7193 (5225-10476)	Pre-post <0.0001 Pre-day 1<0.0001 Post-day 1 0.13		2807 (2107-3906)	3753 (2766-5207)	4353 (2946-5670)	Pre-post 0.005 Pre-day 1 <0.0001 Post-day 1 0.15		Pre <0.0001 Post <0.0001 Day 1 <0.0001
IL6	10.0 (2.9-39.0)	459 (233-687)	268 (166-442)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.0018		9.6 (8.5-12.0)	358 (200-619)	249 (142-448)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.0077		Pre 0.7876 Post 0.2260 Day 1 0.6325
IL8	21.0 (12.0-52.0)	52.0 (28.0-94.0)	59.0 (33.0-96.0)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001		46 (36-57)	97 (65-146)	84 (68-151)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.9625		Pre <0.0001 Post <0.0001 Day 1 <0.0001
IL10	4.4 (1.5-8.3)	32 (7.4-90)	32 (20-64)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.5239		15 (14-17)	49 (31-89)	27 (22-36)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001		Pre <0.0001 Post 0.0069 Day 1 0.2410
IL1-beta	0.16 (0.01-10.0)	0.05 (0.05-1.9)	0.04 (0.04-3.2)	Pre-post 0.5474 Pre-day 1 0.4307 Post-day 1 0.0019		7.5 (6.5-8.9)	9.0 (7.9-11.0)	8.5 (7.5-10.0)	Pre-post <0.0001 Pre-day 1 0.0003 Post-day 1 0.0846		Pre <0.0001 Post <0.0001 Day 1 <0.0001
IL1-ra	1.3 (0.01-8.2)	55 (12-276)	17 (6.9-46)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.0026		433 (327-625)	3373 (1713-4828)	1255 (898-1767)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001		Pre <0.0001 Post <0.0001 Day 1 <0.0001
S-RAGE	31 (16-59)	469 (249-1016)	55 (35-95)	Pre-post <0.0001 Pre-day 1 0.0002 Post-day 1 <0.0001		42 (35-42)	52 (39-72)	38 (30-49)	Pre-post 0.0191 Pre-day 1 0.0517 Post-day 1 0.0001		Pre 0.0047 Post <0.0001 Day 1 0.0038

Table 14: Fold change in cytokine levels in BALTI-P and VINDALOO trial participants.

Cytokine Median (IQR) ratio to pre-op	BALTI-P Post	BALTI-P Day 1	p value		VINDALOO post-op	VINDALOO day 1	p value
TNF alpha	0.39 (0.27-0.56)	0.86 (0.62-1.3)	Pre-post <0.0001 Pre-day 1 0.0025 Post-day 1 <0.0001		0.86 (0.81-0.97)	0.96 (0.88-1.0)	Pre-post <0.0001 Pre-day 1 0.029 Post-day 1 0.0014
TNFR1	1.5 (1.2-2.0)	1.0 (0.86-1.3)	Pre-post <0.0001 Pre-day 1 0.39 Post-day 1 <0.0001		2.3 (1.4-3.2)	2.0-(1.5-2.6)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.49
TNFR2	1.3 (1.1-1.6)	1.5 (1.3-1.8)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.0065		1.3 (0.99-1.5)	1.3 (1.1-1.7)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.063
IL6	50 (10-217)	26 (9.0-83)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.20		34 (20-58)	24 (13-45)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.037
IL8	2.4 (1.5-3.6)	2.5 (1.8-3.8)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.6146		2.0 (1.5-2.9)	2.0 (1.5-2.5)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.70
IL10	8.3 (2.3-22)	7.9 (3.6-24)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.64		2.8 (1.9-5.9)	1.7 (1.4-2.3)	Pre-post <0.0001 Pre-day 1<0.0001 Post-day 1 <0.0001
IL1-beta	1.7 (0.26-5.0)	2.6 (0.25-4.0)	Pre-post 0.14 Pre-day 1 0.25 Post-day 1 0.37		1.2 (1.1-1.4)	1.1 (1.0-1.3)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.12
IL1-ra	55 (12-276)	13 (3.0-549)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 0.19		8.4 (3.0-12)	2.9 (2.0-3.9)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001
S-RAGE	14 (8.4-30)	1.9 (1.0-3.3)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001		1.2 (0.95-1.5)	0.90 (0.73-0.99)	Pre-post <0.0001 Pre-day 1 <0.0001 Post-day 1 <0.0001

7.3.2 Macrophage phagocytosis and DAB

Phagocytosis was analysed using recovered alveolar macrophages (n=8). This showed an overall difference (Kruskal-Wallis p<0.0001). Cytocholasin inhibited phagocytosis as expected (1.0 versus -88 (-92 to-83%), p=0.0078). DAB 100nM promoted phagocytosis compared to baseline versus baseline (p=0.039) and Dummy DAB (DAB 42% (IQR 5.8 to 102) versus Dummy DAB 18% (IQR -18 to 24) p=0.0078). 5nM Dummy DAB reduced phagocytosis compared to baseline (-29% (IQR -48 to -11, p=0.016) and 5nM DAB was higher (Median 17% (IQR -6.1 to 24), p=0.016), (figure 17).

Figure 17: Macrophage phagocytosis as modulated by DAB, *p<0.05, **p<0.01. Cytocholasin C significantly decreased phagocytosis as expected. 100nM DAB increased phagocytosis compared to control and Dummy DAB 100nM. DAB 5nM was significantly higher than Dummy DAB 5nM, but not different from control.
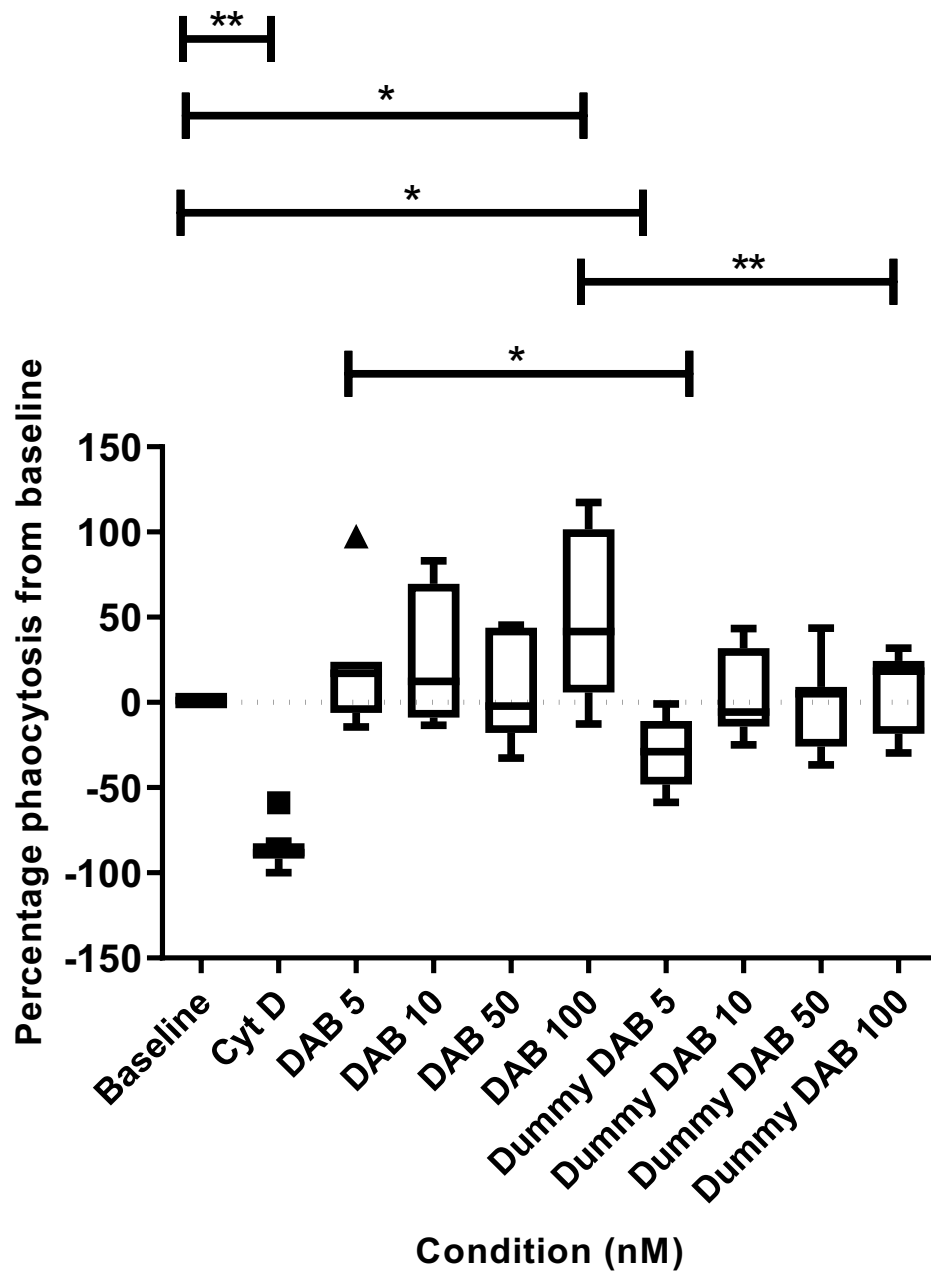
7.3.3 Macrophage survival and function

To see if effects might be mediated by macrophage death, cell survival was analysed using CellTitre™ assay utilising a macrophage-like cell line, THP-1. Adequate supplies of human macrophages were not available at the time these experiments were undertaken.

Viability was not altered by DAB (figure 18).

Figure 18: Cell survival of THP-1 derived cells, with absolute and percentage change compared to vehicle control (n=6). There were no differences at two hours absolute (p=0.29) or in percentage change (p=0.30), or at four hours absolute (p=0.24) or percentage change (p=0.24).
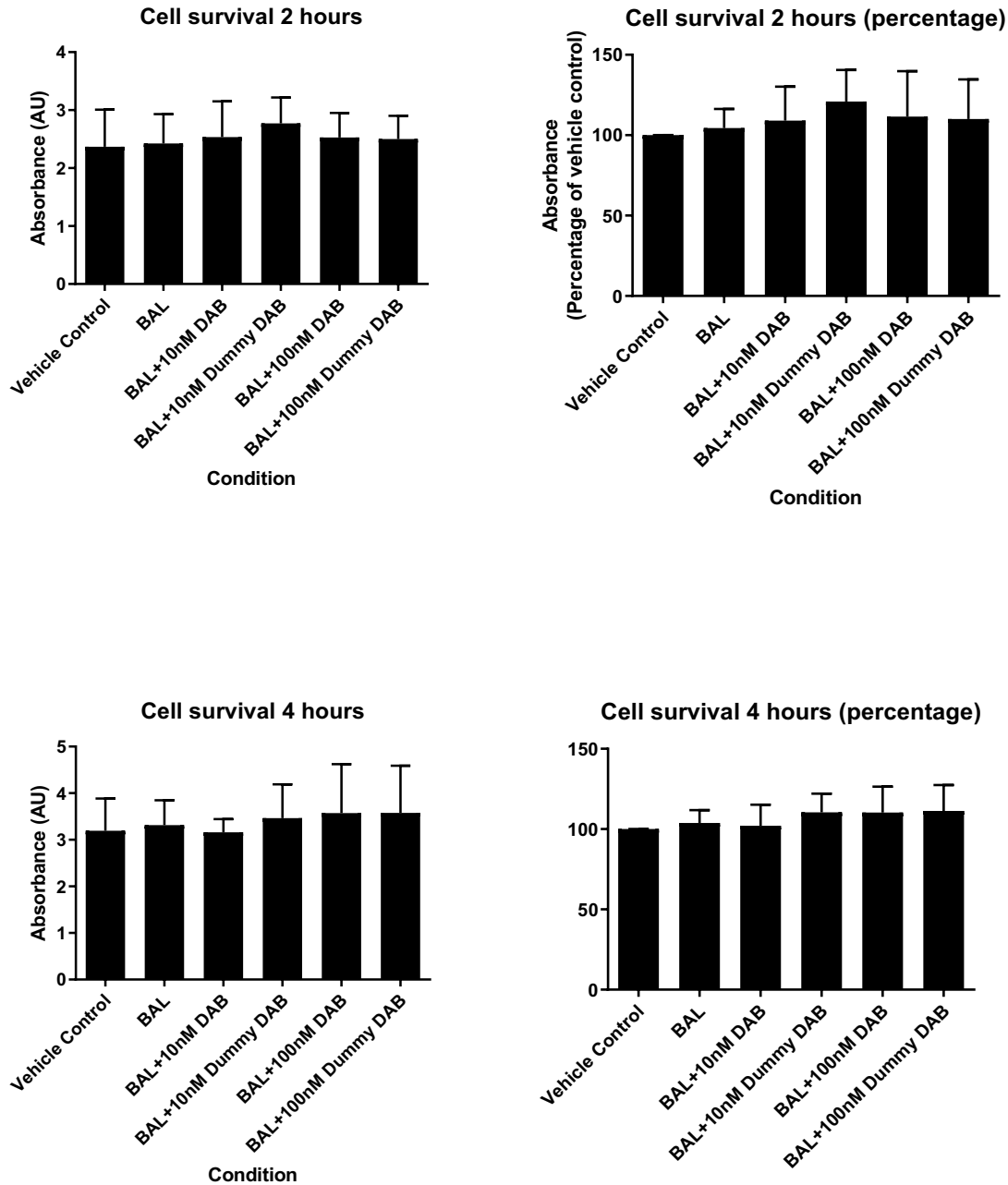
To further investigate the modulatory effects of DAB on macrophages, the effects

on THP-1 cells were analysed using the DCFDA assay to study reactive oxygen

species production. No effects at two or four hours were elucidated (figure 19).

Figure 19: Reactive oxygen species production produced by THP-1 cells at two and four hours, determined by DCFDA assay (n=6). None of the experiments had significant differences (ROS 2 hours, p=0.15, ROS 2 hours percentage p=0.11, 4 hours p=0.21, 4 hours percentage p=0.23).
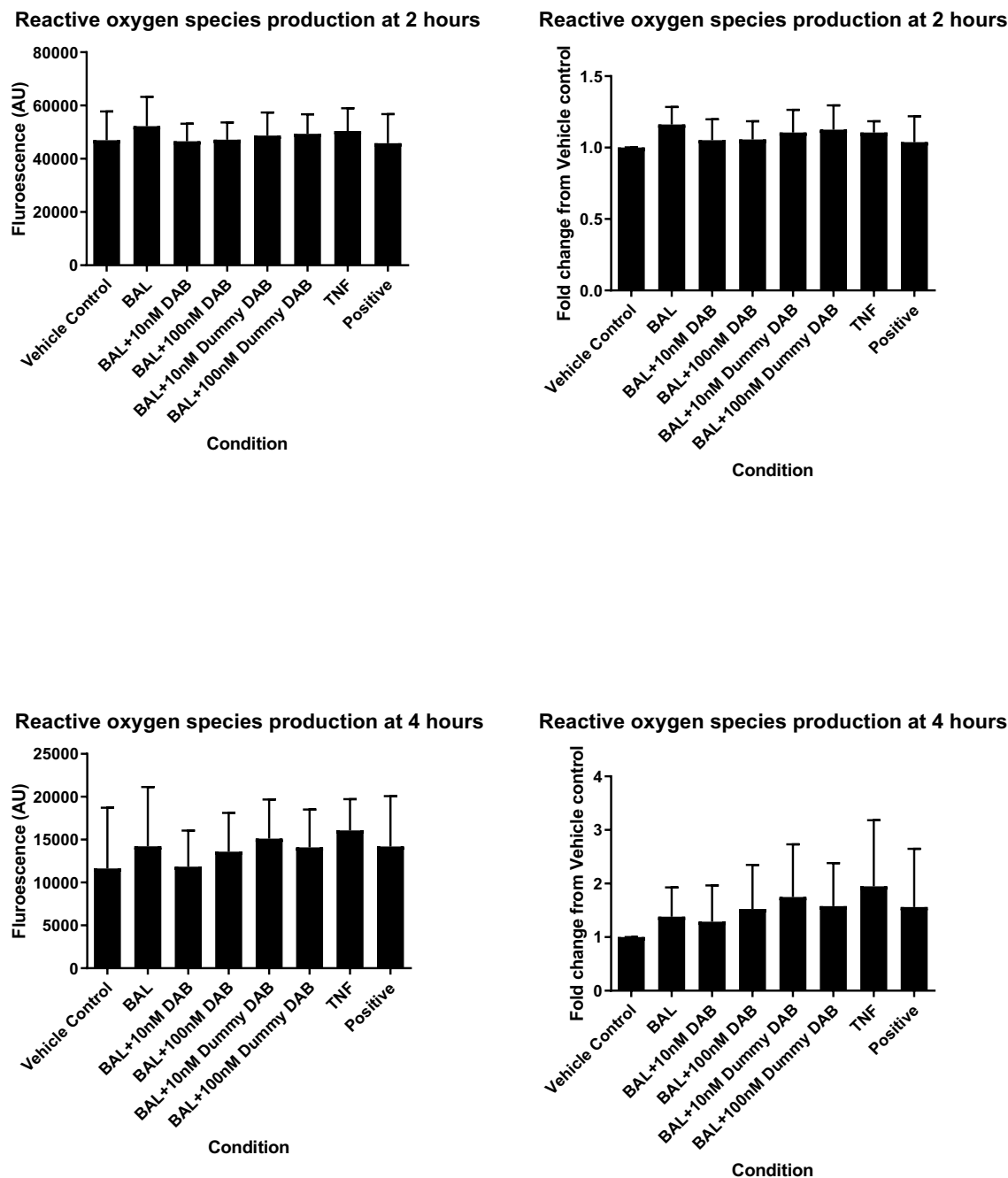
Receptor expression in alveolar macrophages was attempted with the recovered macrophages. However, repeated samples had high autofluorescence which prevented receptor analysis (figure 20).

Figure 20. Autofluorescence in recovered alveolar macrophages.
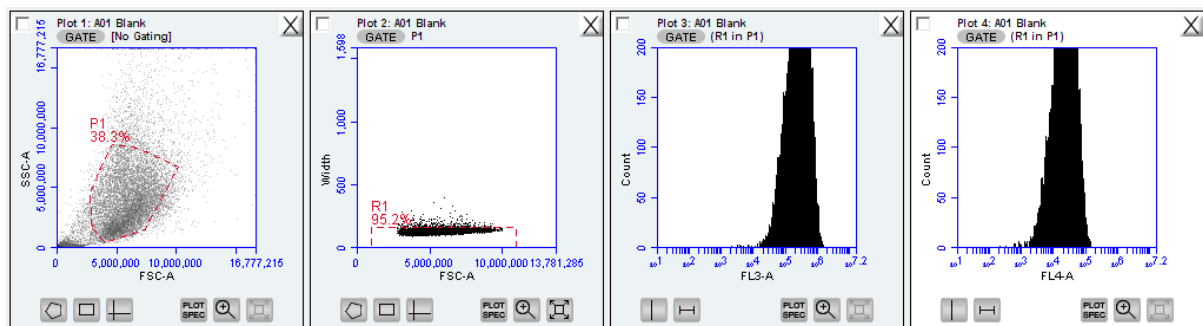
7.4 Discussion

TNF alpha is elevated at presentation in patients who present to hospital with sepsis but falls rapidly [207]. Baseline TNF levels were lower in oesophagectomy patients preoperatively (as expected) but fell further by the end of surgery, with a trend to return to baseline on the day following surgery. It may be TNF alpha's peak plasma levels occur in the early phase of the operation, or TNF alpha production is suppressed, perhaps due to volatile anaesthetic agents (although this effect in animal models is not consistent [226]). Absolute levels of TNFR1 and 2 were lower in VINDALOO, but TNFR1 fold-change was higher. There is clearly complex regulation of TNF alpha in the perioperative phase [108].

Given the reduced ARDS rates, it was surprising that TNF alpha levels were higher in the VINDALOO cohort, especially as ARDS with a hyperinflammatory phenotype has been associated with worse outcome [208]. Whether the modest absolute increase was physiologically significant is not clear. Higher levels of anti-inflammatory cytokines were observed (IL-8, IL-10 and IL-1ra), perhaps indicating better immune regulation in response to surgery. This may be because of higher levels of baseline vitamin D that were present in the VINDALOO trial [143] have modulated the immune response. Alternatively, plasma cytokine levels may not be representative of cellular level effects or regulation by downstream signal transduction [227].

DAB had modulatory effects on macrophage phagocytosis. Previous work showed modulatory effects on reactive oxygen species in neutrophils [133] but not in NETosis or phagocytosis (as described in chapter 6). This suggests that immune regulatory cells such as macrophages may more important in the effect of

GSK2862277 than neutrophils [73, 75, 76]. A re-analysis of a trial of IL-1 inhibition in sepsis has suggested a subset of patients with "macrophage activation syndrome" may have benefitted, implying the targeting of immune-regulating cells may be beneficial [228]. Some doubt must sit over the results, however, given the off-target effects of Dummy DAB observed in the experiments with neutrophils. The next stage of work to analyse this further would require agonist and antagonist panels to delineate the importance of TNF in controlling macrophage phagocytosis, but there were insufficient clinical samples or time to complete this.

Investigating macrophages, especially from current or ex-smokers, using flow cytometry was challenging due to their high autofluorescence. Given the importance of smoking in ARDS risk [25, 229], smokers' macrophages may be phenotypically different from non-smokers, but the frequency of non-smokers presenting for thoracic surgery is low and collecting adequate numbers of samples was not feasible.

Additionally, supplies of sample tissue via the MLTC were dependent on other clinical factors, including frequency and type of surgery performed, sufficient tissue not needed for histology being available and rate of procedures being performed. This limited the supplies of alveolar macrophages available for experiments and necessitated switching to THP-1 cells to allow completion of planned experiments. Clearly, these data are not directly comparable, and further work, repeating the experiments with alveolar macrophages to elucidate the effects of DAB on survival and ROS production is required.

THP-1 cells did not respond to DAB, however there were no significant effects in the positive controls of the ROS assays. It may be that these particular cells (or this

subset at high passage levels) were not sufficiently representative of alveolar macrophages to permit accurate modelling of the effects of DAB.

It was not possible to cross validate the cytokine analyses for the two trials are equivalent as they were run several years apart, although they are calibrated to the same concentrations. As discussed above, plasma levels may not accurately reflect cellular effects, down-stream signalling is important for regulation and clinical limitations on blood sampling limit the number of time points that can be analysed. Furthermore, there may be confounding by individual surgical and anaesthetic factors not captured in these data. A limitation of the perioperative model for tissue damage is the immune modulating effects of anaesthesia, which is complex and difficult to standardise for trials, [226] and a better understanding of these is of immense importance [230]. Similarly, surgical technique and duration will result in variable levels of tissue damage that again cannot be standardised [13].

The limitations of the TFR116341 trial recruitment and its implications for the originally planned work have been outlined in the introduction to this chapter. Given the signal for DAB modulating macrophage phagocytosis, and the macrophage's canonical role in ARDS [65, 213], it is clearly of importance to analyse the effect of DAB on this cell type in more detail. Further experiments need to confirm the effect of DAB, study other macrophage functions, including ROS production, and macrophage survival, and the effects on signalling to downstream immune cells. Characterisation of effects in both the initial pro-inflammatory response and the recovery phase is important, as macrophage function is different in these two periods [65].

Understanding the kinetics of the inflammatory response is important. The availability of cytokine samples from TFR116341 was provide a useful comparison with these trials. Clearly, more frequent sampling, including intra-operatively, is required and potentially this may provide a range of useful insights into the nature of the inflammatory response to surgery. It remains the case that plasma cytokine levels may not correlate well with paracrine/autocrine effects, as may be true in sepsis [207], therefore tissue sampling, perhaps from animal models, may be helpful in investigating this.

In conclusion, macrophage phagocytosis is increased by DAB GSK2862277, but modulation of ROS or cell survival was not observed in THP-1 derived macrophages. There have been changes in in an array of pro- and anti-inflammatory cytokines but not IL-6, suggesting the clinical changes seen may be reflected in immunomodulation. GSK2862277 appears to modulate the activity of a critical cell in ARDS, and further mechanistic understanding of the agent's action may better-elucidate the drug's effect *in vivo*.

Chapter 8

DISCUSSION

8.1 Overview

Given the number of patients undergoing surgery and the disproportionate burden of complications that fall on the high risk patient, reducing complications in this cohort is a global public health priority [2]. The predictability of complications, critical illness and ARDS following oesophagectomy [8, 9, 231] has made it a potentially useful resource for studying critical illness for previous trials [148].

Using samples from patients undergoing these procedures allows the study of perioperative immunological changes *in vitro* which may be important factors in the development of complications. Immune modulation in the perioperative period may have major consequences [71] via a variety of potential mechanisms [67], driving adverse outcomes.

In this thesis, oesophagectomy as a model of critical illness has been evaluated and perioperative immune modulation was investigated.

8.2 The impact of the acute respiratory distress syndrome on outcome after oesophagectomy

ARDS is associated with a number of adverse outcomes, including non-respiratory organ failure, longer ICU and hospital stay. Late ARDS, which was suspected to be more likely to be related to secondary complications such as sepsis and anastomotic leak, had worse outcomes than early ARDS. This is similar to late-onset ARDS in a general critical care cohort (which used over 48 hours from admission as a definition) [232]. Oesophagectomy was a useful ARDS model in this trial, with the risk around 25%, which permitted the effective evaluation of salmeterol in the perioperative period [138].

8.3 ARDS Following Oesophagectomy: A Comparison of Two Trials.

This study showed a fall in the rate of ARDS between the BALTI-P and VINDALOO trials, associated with a number of changes in clinical practice. They remain high compared to other surgical cohorts [2] and, indeed, populations identified as high risk [154, 158, 213]. This study also reiterated the importance of smoking as a risk factor for ARDS and post-operative complications [22, 25, 229] but also found a signal that dihydropyridine use pre-operatively was a risk factor.

This is a novel finding that requires confirmation in other, larger cohorts, cellular and animal studies, mechanistic assessment and, perhaps eventually interventional trials. It indicates there may be an array of factors that influence post-operative outcome that have yet to be investigated. Clinical trials, such as SPACE (EudraCT number 2016-004141-90) and PREVENTION HARP-2 (ISRCTN48095567), are now taking place, to study concurrent medical therapy in the perioperative period.

Although ARDS was less frequent in the VINDALOO trial, the rates of severe post-operative complications are substantial and have not fallen between these trials. Other surgical cohorts have demonstrated that post-operative pulmonary complications are associated with late deaths and increased hospital resource use [22].

The lower ARDS rate in VINDALOO is still comparable to other methods derived to date to identify high-risk cohorts for studies [154, 158]. A better understanding of specific risk factors for ARDS in oesophagectomy, such as smoking, would improve the event rate in the control group of trials, which would enhance trial power [233]. Reducing severe post-operative complications (be that specifically pulmonary or more general) may well be a pragmatic target for future trials using this model,

which, although less specific than ARDS, is of practical concern to clinicians and more patient-centred [9, 12].

8.4 Evaluation of the TFR116341 Trial

TFR116341 was a recent trial attempting to use oesophagectomy as a phase II efficacy study to evaluate GSK2862277, and provide a mechanistic translational sub-study. It was designed prior to the completion of VINDALOO [140]. Poor patient accrual was a major problem, due to exclusion criteria and the reluctance of patients to participate, which had changed from previous trials [138, 143]. This prevented the envisaged translational sub-study being possible.

All trials have recruitment hurdles, but the aggregation of several has the potential to make a trial undeliverable in a realistic time-frame [192]. Careful trial design, to optimise recruitment and obtain the best possible data, is important, for both scientific and ethical reasons [234]. Research participation can deliver individual patient benefit and patient empowerment, which could be used to aid recruitment and better engage clinical staff in future studies [180, 182, 183, 191].

8.5 TFR116341 Translational Sub-Study

This study demonstrated important differences in NETosis between established critical illness and the perioperative period. Furthermore, there are much higher levels of cfDNA in the established critical illness cohort. Clearly immune function is different in these two groups, which correlates with the known temporal patterns of inflammation in critical illness [207, 210]. How the surgical insult interacts with the various other factors in the perioperative period to lead to complications remains incompletely understood, but NETosis is at least one mechanism by which this may

occur [67, 87, 98, 206]. Baseline NETosis was not elevated, but a much larger response was elicited when stimulated. NETosis has been associated with tissue damage [92] and hypercoagulability [85], so it may be the effect of surgery is to prime neutrophils which then response in an exaggerated way to further insults, increasing vulnerability to and/or worsening post-operative complications.

Modulation of macrophage phagocytosis, but not phagocytosis by neutrophils nor NETosis, by GSK2862277 was demonstrated. This builds on Proudfoot's work showing downregulation of reactive oxygen species in neutrophils production by GSK2862277's predecessor molecule [148]. ARDS is driven by multiple interdependent signalling molecules, with both second messenger pathway and temporal sequencing having immunomodulatory effects [65, 178, 213, 227]. *In vitro* conditions and isolating individual cell experiments may not adequately model conditions *in vivo*, which may explain negative results despite efficacy in animal and pre-clinical models.

8.6 Limitations

The limitations have been discussed in each chapter. Chapters 3 and 4 are retrospective studies, limited by their size, the use of databases for analyses (with respect to chapter 4) not planned *a priori* and inconsistencies in data recording. Nevertheless, without prospective cohort studies, these data would not otherwise be available. This is turn would limit the understanding of the changes in ARDS and its implications for patients that have occurred [8, 235], which has implications for the use of oesophagectomy as an ARDS model.

8.7 Future investigations

Future work studying clinical outcomes following oesophagectomy needs to include prospective cohort studies looking the incidence of ARDS in the current era, especially utilising the Berlin Definition [45], modern anaesthetic techniques [12, 13] and therapeutic interventions [213, 236]. Concurrent systematic screening for other complications, including post-operative pulmonary complications [237] and sepsis [238] would provide more detailed insight into post-operative critical illness, and endpoints should be primarily patient-centred [239]. Ensuring adequate trial recruitment rates will also be critical for success, as discussed in Chapter 5. The identification of dihydropyridines as a potential modifiable risk factor for ARDS requires replication in other databases and mechanistic work (perhaps utilising an animal model of ARDS), prior to a randomised trial of withdraw/exchange of dihydropyridines prior to surgery versus standard care.

Animal models probably offer the most efficient mode of better-understanding the effects of DAB, which could then be sought in confirmatory human studies. The temporal effects on different cell groups, especially macrophages, as important, as they have different effects during the course of ARDS [65, 213]. Determining the mechanism of the off-target effects of Dummy DAB is also important, firstly so a true dummy negative control is available and secondly as this might reveal important mechanisms by which domain antibodies function [133].

Future trials of GSK2862277 will need to consider optimum disease targeting and careful trial design to determine clinical effectiveness. Such trials will be informed on data from TFR116341, when it becomes available.

8.8 Conclusion

Multiple mechanisms contribute to ARDS, rather than a defined canonical pathway [213, 227, 240]. Furthermore, the definition of ARDS [45] has limited correlation with pathological definitions [241], which may pollute trial cohorts. More sophisticated patient characterisation, disease phenotyping and factorial trial design offer the chance to elicit effective therapeutic strategies [56, 148, 227]. Managing variability in participants' clinical care in pragmatically-designed randomised remains a major problems for perioperative and critical care trials [242].

A deeper mechanistic understanding of the disease alongside more sophisticated models will be required to develop individualised therapeutic strategies [227]. For example, if further studies confirm priming neutrophils for NETosis is an important driver of perioperative complications, phenotyping potential NETosis would firstly identify individuals as high-risk and secondly provide an enriched cohort for testing therapeutic interventions. Further mechanistic work, especially using studies of the metabolome and complex multicellular and animal models will be needed to provide insights to develop such treatments [148, 223, 243].

Oesophagectomy remains a surgical procedure with a high-risk of ARDS and perioperative complications. Smoking appears to be a key contributing factor to this risk. Falling incidence renders oesophagectomy a less useful model for ARDS. Dihydropyridine drugs may also be important aetiological agents for perioperative ARDS. This requires repetition in other, larger cohorts and mechanistic investigation.

GSK2862277 enhances macrophage phagocytosis and better understanding of these cells in ARDS and in the perioperative period may further elucidate the beneficial effects of GSK2862277 observed in pre-clinical models. Patterns of

NETosis are different in perioperative and ongoing critical illness. NETosis is potentially an under-investigated mechanism contributing to the pathophysiology of clinical complications in these groups.

High-risk surgery is a common occurrence globally and preventing, mitigating and treating post-operative complications is an important public health challenge [2]. A better understanding of immunological function in the perioperative period is vital to this, as is the conduct of trials to optimise perioperative care [226].

APPENDIX

Supplementary data published as part of Chapter 3

Prepared by the trial statistician (Mr C Knox).

Linear regression of the secondary outcomes comparing ARDS status to no ARDS was carried out with and without adjustment for randomised treatment (table A1). Significant differences were found for all outcomes at all stages with the exception of: late ARDS and duration of level 0/1 care, early ARDS and EQ-5D score at day 28, EQ-5D VAS score at day 28 and each ARDS status, EQ-5D score at day 90 and each ARDS status and EQ-5D VAS score at day 90 and each ARDS status..

Table A1: Linear regression of secondary outcomes by ARDS status

Outcome Measure	Early ARDS (day 0-3)			Late ARDS (day 4-28)		
	Difference (95% CI)	p	No.	Difference (95% CI)	p	No.
Organ failure free days	*-2.40 (-3.60, -1.19)	<0.001	329	*-5.77 (-7.55, -3.99)	<0.001	329
	†-2.40 (-3.61, -1.19)	<0.001		†-5.72 (-7.50, -3.94)	<0.001	
Ventilator free days	*-5.28 (-6.81, -3.76)	<0.001	330	*-10.14 (-12.38, -7.89)	<0.001	330
	†-5.27 (-6.80, -3.75)	<0.001		†-10.21 (-12.46, -7.96)	<0.001	
Hospital length of stay	*3.93 (2.09, 5.77)	<0.001	328	*10.34 (7.63, 13.06)	<0.001	328
	†3.91 (2.07, 5.74)	<0.001		†10.54 (7.83, 13.25)	<0.001	
Duration of ICU stay	*4.82 (3.00, 6.65)	<0.001	331	*12.89 (10.20, 15.58)	<0.001	331
	†4.81 (2.99, 6.64)	<0.001		†12.97 (10.27, 15.67)	<0.001	
Duration of ICU stay excluding deaths	*4.78 (2.91, 6.64)	<0.001	326	*12.89 (10.20, 15.58)	<0.001	326
	†4.76 (2.89, 6.63)	<0.001		†12.97 (10.27, 15.67)	<0.001	
Duration of Level 0/1 care	*-1.76 (-3.43, -0.10)	0.038	330	*-2.40 (-4.86, 0.06)	0.055	330
	†-1.78 (-3.44, -0.12)	0.036		†-2.27 (-4.73, 0.19)	0.070	
Duration of Level 2 care	*0.98 (0.08, 1.88)	0.033	330	*4.06 (2.73, 5.39)	<0.001	330
	†0.98 (0.08, 1.86)	0.033		†4.04 (2.71, 5.38)	<0.001	
Duration of Level 3 care	*4.48 (3.21, 5.74)	<0.001	330	*8.76 (6.90, 10.63)	<0.001	330
	†4.47 (3.20, 5.73)	<0.001		†8.85 (6.98, 10.72)	<0.001	
EQ-5D Day 28	*-0.08 (-0.18, 0.02)	0.119	263	*-0.24 (-0.39, -0.09)	0.002	263
	†-0.08 (-0.18, 0.02)	0.119		†-0.24 (-0.39, -0.09)	0.002	
EQ-5D VAS Day 28	*-2.76 (-8.60, 3.08)	0.353	262	*-6.56 (-15.70, 2.57)	0.158	262
	†-2.75 (-8.60, 3.11)	0.356		†-6.62 (-15.78, 2.54)	0.156	
EQ-5D Day 90	*-0.02 (-0.11, 0.06)	0.630	260	*-0.12 (-0.26, 0.01)	0.073	260
	†-0.02 (-0.11, 0.06)	0.616		†-0.12 (-0.26, 0.01)	0.077	
EQ-5D VAS Day 90	*-2.75 (-8.81, 3.30)	0.372	261	*-7.88 (-17.42, 1.65)	0.105	261
	†-2.75 (-8.82, 3.31)	0.372		†-7.89 (-17.43, 1.68)	0.106	

*Unadjusted difference between patients with ARDS and those without

†Estimated difference between patients with ARDS and those without adjusted for treatment allocation

CI 95% confidence interval

Logistic models were fitted for each stage of ARDS with an interaction term to examine whether the response to different treatments depends on the specified baseline characteristics. An unadjusted model was fitted including a term for the treatment allocation, baseline moderator and a term for the treatment by moderator interaction. An adjusted model was also fitted containing a term for treatment, moderator and an interaction term adjusted for age and hospital. The table below shows the mean effect size for the treatment by moderator interaction effect as well as the p-value for the test of the interaction term. Statistical significance was approached in modelling of early ARDS for the interaction of treatment allocation with age, mid-oesophagus tumour type and oesophageal-gastric junction tumour type. The adjusted and unadjusted model for age and treatment suggests that for participants allocated to salmeterol that for an increase in age there is an additional increase in risk of early ARDS. The adjusted and unadjusted model for mid-oesophageal tumour and treatment suggests that for participants allocated to salmeterol who have a mid-oesophageal tumour there is an additional increase in risk of early ARDS compared to patients with other tumour types. The adjusted and unadjusted model for oesophageal/gastric junction tumour and treatment suggests that for participants allocated to salmeterol who have an oesophageal-gastric junction tumour there is an additional decrease in risk of early ARDS compared to patients with other tumour types (table A2).

Table A2: Multivariate analyses of ARDS – Interaction between treatment allocation and baseline variables

Baseline Variable	Early ARDS			Late ARDS			Total ARDS		
	OR (95% CI)	p	No.	OR (95% CI)	p	No.	OR (95% CI)	p	No.
Age (years)	*1.06 (1.00, 1.13)	0.045	332	*0.98 (0.90, 1.06)	0.568	331	*1.04 (0.99, 1.10)	0.130	331
	†1.07 (1.01, 1.14)	0.023	332	†0.97 (0.89, 1.06)	0.475	331	†1.05 (0.99, 1.10)	0.091	331
Gender	*1.66 (0.41, 6.66)	0.475	332	*0.71 (0.09, 5.35)	0.737	331	*1.25 (0.37, 4.14)	0.720	331
	†1.35 (0.32, 5.82)	0.656	332	†0.58 (0.07, 4.66)	0.609	331	†0.98 (0.28, 3.45)	0.976	331
Pre-operative chemotherapy	*0.74 (0.17, 3.17)	0.683	332	*7.76 (0.70, 86.42)	0.096	331	*1.74 (0.51, 5.93)	0.378	331
	†0.71 (0.16, 3.14)	0.647	332	†8.42 (0.70, 101.56)	0.093	331	†1.66 (0.47, 5.84)	0.430	331
American Society of Anesthesiologists' grade 2 or more	-	-	-	-	-	-	*0.34 (0.02, 4.93)	0.429	314
	-	-	-	-	-	-	†0.27 (0.02, 4.22)	0.354	314
American Society of Anesthesiologists' grade 3 or more	*1.07 (0.29, 3.95)	0.917	314	-	-	-	*2.15 (0.64, 7.27)	0.216	314
	†1.16 (0.30, 4.52)	0.831	314	-	-	-	†2.50 (0.71, 8.76)	0.152	314
Duration of one lung ventilation (minutes)	*1.01 (0.99, 1.00)	0.254	297	*1.00 (0.98, 1.01)	0.610	297	*1.00 (0.99, 1.01)	0.482	297
	†1.01 (0.99, 1.02)	0.374	297	†1.00 (0.98, 1.01)	0.561	297	†1.00 (0.99, 1.01)	0.612	297
Cumulative fluid balance at end of surgery (litres)	*1.16 (0.80, 1.68)	0.447	316	*1.20 (0.61, 2.35)	0.604	315	*1.24 (0.88, 1.75)	0.229	315
	†1.19 (0.79, 1.78)	0.406	316	†1.22 (0.60, 2.47)	0.660	315	†1.27 (0.88, 1.83)	0.263	315
Surgical approach	*0.69 (0.19, 2.49)	0.569	329	*0.21 (0.02, 2.85)	0.240	329	*0.45 (0.14, 1.49)	0.191	329
	†0.85 (0.22, 3.25)	0.756	329	†0.20 (0.01, 2.92)	0.240	329	†0.51 (0.15, 1.76)	0.289	329
Tumour: Mid oesophagus	*7.48 (1.62, 34.52)	0.010	325	-	-	-	*1.74 (0.54, 5.62)	0.356	325
	†7.50 (1.53, 36.68)	0.013	325	-	-	-	†1.76 (0.51, 6.05)	0.371	325

| Tumour: oesophageal-gastric junction | *0.21 (0.05, 0.85) | 0.029 | 325 | - | - | - | *0.85 (0.27, 2.65) | 0.773 | 325 |
| | †0.22 (0.05, 0.94) | 0.041 | 325 | - | - | - | †0.88 (0.26, 2.89) | 0.827 | 325 |

OR mean estimated odds ratio of the interaction term

*Unadjusted treatment effect

†Treatment effect adjusted for hospital and age at randomisation

Missing Late ARDS estimates are due to insufficient numbers of cases in these groups

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