Oxidative stress, redox status and surfactant metabolism in mechanically ventilated patients receiving different approaches to oxygen therapy (MecROX)

Short Title

Mechanistic evaluation of two approaches to oxygen therapy in critical care (MecROX)

Reference Numbers

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The undersigned confirm that the following protocol has been agreed and accepted and that the Chief Investigator agrees to conduct the trial in compliance with the approved protocol and will adhere to appropriate research governance frameworks and any subsequent amendments of regulations, Good Clinical Practice (GCP) guidelines, the Sponsor's Standard Operating Procedures (SOPs), and other regulatory requirements where relevant.

I agree to ensure that the confidential information contained in this document will not be used for any other purpose other than the evaluation or conduct of the clinical investigation without the prior written consent of the Sponsor.

I also confirm that I will make the findings of the trial publicly available through publication or other dissemination tools without any unnecessary delay and that an honest accurate and transparent account of the trial will be given; and that any discrepancies from the trial as planned in this protocol will be explained.

For and on behalf of the Trial Sponsor

Name: Sharon Davies-Dear

Signature…….. Date 20-Mar-2024

Chief Investigator

Name: Dr Ahilanandan Dushianthan

Signature…… Date 25-Mar-2024

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1. Study summary

Title	Mechanistic evaluation of surfactant biology and redox status during different approaches to oxygen therapy for invasively ventilated adults in intensive care (MecROX)
Study linked to the RCT	Evaluating the clinical and cost-effectiveness of a conservative approach to oxygen therapy for invasively ventilated adults in intensive care (UK-ROX)
IRAS number	320671
Internal sponsor reference	RHM CRI 0426
Study design	Prospective observational study enrolling patients within the UK-ROX RCT
Study participants	Adults (aged ≥18), mechanically ventilated needing supplementary oxygen and enrolled into UK-ROX RCT
Planned sample size	100
Study duration	30 months
Planned study period	February 2023- July 2025
Primary outcome	The difference of percentage of DPPC (PC32:0) in relation to total phosphatidylcholine (PC) composition (% of total PC in surfactant) at 48 hours between conservative and usual oxygen target groups.
Secondary outcomes	1. Surfactant index: This is a composite PC surfactant molecular index calculated from surfactant specific PC molecules (PC32:0, PC32:1 and PC30:0) and unsaturated surfactant PC34:1 at 48 hours. Surfactant index = $\frac{\{PC32{:}0+PC32{:}1+PC30{:}0\}}{PC34{:}1}$ 2. Surfactant PC concentration (urea corrected) at 48 hours. 3. Systemic oxidative stress: Total free thiols, lipid peroxides and total surfactant oxidation products at 48 hours. **Other exploratory and explanatory outcomes:** 4. Surfactant total PC and PC32:0 *methyl*-D_9choline enrichment at 48 hours. 5. Surfactant total lysoPC and lysoPC16:0 concentrations, composition and *methyl*-D_9 choline enrichment at 48 hours. 6. Surfactant oxidised PC composition and concentrations at 48 hours. 7. Whole-body redox balance by quantifying stable products of reactive oxygen species (ROS) (e.g., isoprostanes), RNS (e.g., nitrite, nitrate, nitrosation products) and RSS (e.g., total free thiols, thiosulfate, low molecular weight thiols including sulfide) at 48 hours.

	8. Exploratory outcomes: Comparison of clinical outcomes (ICU mortality, hospital mortality, 90-day mortality, ICU, and hospital length of stay) in relation to surfactant abnormalities. 9. Exploratory outcomes: Comparison of clinical outcomes (ICU mortality, hospital mortality, 90-day mortality, ICU, and hospital length of stay) in relation to specific markers of oxidative stress.
Inclusion and exclusion criteria	Inclusion criteria: 1. Enrolled in UK-ROX study 2. Age: Adult (≥18 years old) 3. Receiving mechanical ventilation for hypoxaemic respiratory failure 4. Receiving supplemental oxygen (fractional inspired concentration of oxygen (FiO_2 >0.21) at the time of enrolment. 5. Anticipated to be mechanically ventilated for minimum of 48 hours Exclusion criteria: 1. Previously randomised into UK-ROX in the last 90 days 2. Currently receiving extracorporeal membrane oxygenation (ECMO) 3. The treating clinician considers that one trial intervention arm is either indicated or contraindicated

2. Study flow charts

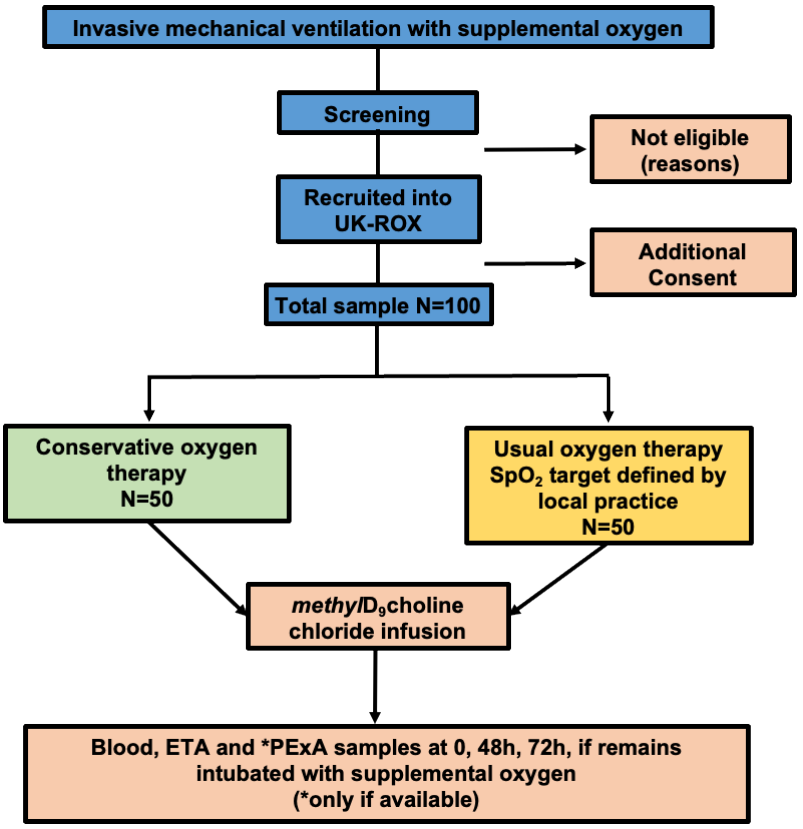
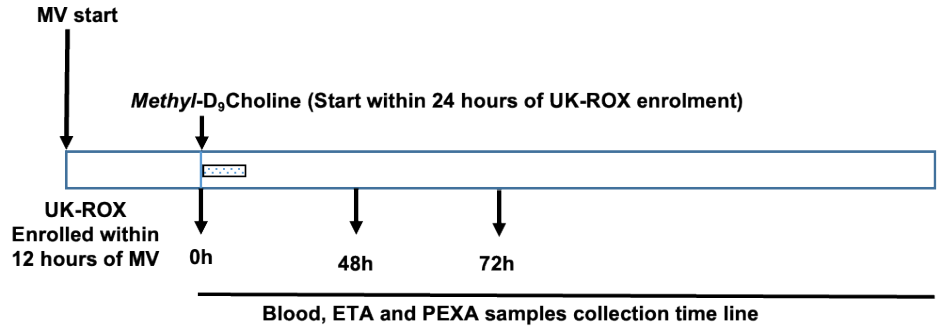


Figure 1: Study flow chart.



1. UK-ROX intervention will be started within 12 hours of mechanical ventilation
2. *Methyl*-D_9choline infusion will commence within 24 hours of UK-ROX randomisation – 3.0 mg/kg body weight over 3 hours.
3. EDTA Blood samples will be taken at 0, 48 hours, 72 hours
4. ETA samples will be taken at 0, 48 hours, 72 hours or until extubation
5. *PEXA samples will be taken at 0, 48 hours and 72 hours or until extubation.*only if available

Figure 2: Sampling schedule.

3. Lay summary

Aims: Intensive Care Patients (ICU) are often given oxygen via artificial ventilation through a breathing machine. Giving too much oxygen is harmful and can damage the lungs. We want to improve outcomes for patients by understanding how this excess oxygen causes lung damage. In this study, we aim to determine whether using a lower oxygen target in patients on a breathing machine reduces lung damage.

Background: Although oxygen is necessary, excess oxygen can be harmful especially to those requiring artificial ventilation. Usually, lungs are kept open by a detergent-like material in the lungs called "surfactant" so they can work properly. However, too much oxygen can kill the cells that make surfactant or increase surfactant breakdown. This can cause the lung to become damaged and fill with fluid instead of air and causes a patient unnecessary harm. Too much oxygen can also cause powerful chemical reactions (called oxidative stress) that can damage cells around the body, causing major organs to fail. This can lead to the worsening of a patient's condition.

Design and method used: A national research study (UK-ROX) is looking to find out whether giving less oxygen to patients in ICU will improve their survival compared to standard care (more oxygen). UK-ROX trial will not be able to assess how exactly the excess oxygen may cause harm. Therefore, this study will run in parallel with UK-ROX to look in more detail at how excess oxygen might affect lungs.

We intend to recruit 100 patients from the UK-ROX trial and conduct this detailed sub-study to determine whether surfactant and oxidative stress play a role in excess oxygen induced lung damage. Consecutive participants will be recruited from both groups.

We will take blood, lung fluid and breath samples from these participants three times during the study. We will take blood from a small catheter already in place as part of their standard ICU treatment. Lung fluid samples will be taken through a small suction tube attached to the breathing tube already in their windpipe. Breath samples will be collected via a PExA

instrument that will be connected to the ventilator outflow circuit to capture expiratory samples.

Patients on a breathing machine will usually receive sedation as part of their treatment, so they are unlikely to be able to consent. So, we will approach a nominated person (usually their next of kin) for their opinion on whether they think the patient would want to participate. We will ask patients at the earliest opportunity when they regain consciousness.

Patient and public involvement: Patient and public members helped design the UK-ROX trial and this study. These people will actively participate in the oversight of the study, contribute to the production of patient documents, and help dissemination of study findings to the public.

How will this research help? The results will help us decide how much oxygen we should give patients on a breathing machine and details of how excess oxygen can damage the lungs. It will also provide essential information to design future studies and treatments to reduce harm from too much oxygen. Possible future treatments may include changes to the amount of oxygen given, medications such as surfactants, antioxidants, or diet (to reduce oxidative stress).

Dissemination: Results will be disseminated through media, health charities, publications, and scientific conferences. We will provide evidence for any oxygen-related guidelines for doctors and nurses to provide the best possible care for ICU patients needing oxygen therapy.

4. Background and rationale

4.1. The description of main clinical study

Oxygen therapy is the commonly used medical intervention in the intensive care unit setting. Mechanically ventilated patients often require supplementary oxygen, and the desired oxygen therapeutic target levels are not fully defined. Currently, there is a large, multicentre, randomised controlled trial is underway in the UK to assess the two different oxygen targets

(Conservative vs standard) in patients requiring mechanical ventilation in the intensive care (UK-ROX)[1]. This HTA funded study of 16,500 mechanically ventilated patients across the UK is at recruitment stage. UK-ROX aims to evaluate the clinical and cost-effectiveness of a conservative approach to oxygen therapy to achieve a low oxygen saturation target [SpO_2 90±2%] compared with the standard target, determined by local practices, on 90-day all-cause mortality in ventilated patients requiring oxygen. The primary aim of this sub-study (MecROX) is to provide a mechanistic evaluation of systemic, alveolar redox status and dynamic surfactant biology following different oxygen therapeutic strategies in mechanically ventilated patients enrolled into the UK-ROX study. This is an observational sub-study embedded within the UK-ROX and all patients will be co-enrolled with UK-ROX interventional study.

4.2. Need for Research

Acute unanticipated mechanical ventilation is associated with significant morbidity and mortality. Pre-COVID-19 data from the Intensive Care National Audit and Research Centre (ICNARC) case-mix programme (CMP) for England, Wales, and Northern Ireland between 2015-2019 describe nearly 300,000 patients required mechanical ventilation with a mortality of around 33% and annual bed-occupancy burden of nearly 400,000 ICU bed-days in 2019. This burden has increased with the COVID-19 pandemic: nearly 50% of COVID-19 patients on ICU were managed with advanced respiratory support with an increase in mortality to around 40-50%[2,3]. Mechanical ventilation is required for a range of hypoxic states from mild acute hypoxic respiratory failure to severe acute respiratory distress syndrome (ARDS), where impaired gas exchange results in severe hypoxaemia (reduced arterial partial pressure of oxygen) with a need for prolonged mechanical ventilation and hospitalisation. Oxygen therapy to correct hypoxaemia is an almost ubiquitous adjunct to mechanically ventilated patients and has saved millions of lives, particularly during the worldwide COVID-19 pandemic. However, whilst it's lifesaving, oxygen therapy is not without harms. A recent systematic review found that supplementary oxygen in patients with normal oxygen saturation increases mortality and another larger international study is currently underway to compare different levels of oxygen administration in critically ill patients[4,5].

The dose and duration of oxygen therapy vary between patients and optimal oxygen targets are not fully defined. Whilst hypoxaemia may cause harm, alveolar hyperoxia and systemic hyperoxaemia may also worsen organ injury. Pulmonary and systemic oxygen toxicity is well described in animal models of normobaric hyperoxia and oxygen dose-toxicity relationships in humans are poorly defined and likely to vary between individuals[6]. It remains unclear whether alveolar hyperoxia and systemic hyperoxaemia accelerates pre-existing lung injury in mechanically ventilated patients predisposing them to develop ARDS, or through accelerating established pathology. The balance between competing harm from hyperoxia and hypoxaemia with organ dysfunction is clinically important but difficult to quantify. Moreover, the potential mechanisms of oxygen toxicity remain poorly understood, with most studies making inferences from hyperoxic animal models of uninjured lungs, rather than using in-vivo human data from critically- ill patients.

4.3. Existing evidence and scientific rationale

4.3.1. Mechanisms of hyperoxia induced organ damage

Potential mechanisms of harm from direct pulmonary oxygen toxicity or indirect systemic oxidative stress are poorly defined and phenotyped. Exploration of the mechanisms of hyperoxia induced organ damage with quantification of redox markers of lipid and protein oxidative injury and characterisation of the initiation, propagation, and persistence of pro-inflammatory cascades may provide a focused approach for key modifiers to minimise organ damage. Nearly all ventilated patients require supplementary oxygen, but the contribution to subsequent lung injury of pulmonary oxygen toxicity or systemic oxidative stress is unclear. In pathological states, the balance between oxidative stress and native antioxidants is altered leading to the inability to remove toxic molecules resulting in changes in the redox signalling and modulation of secondary messengers causing mitochondrial dysfunction and cellular biogenetic failure[7]. Consequently**,** the main feature of hyperoxia demonstrated in *in-vivo* models and isolated cell cultures is cell death through apoptosis or necrosis[8,9].

4.3.2. Hyperoxia and alveolar and systemic redox biology

Oxidative stress occurs due to imbalance between oxidants and antioxidants. Unopposed reactive oxygen species (ROS) contribute to compromised cellular function with predisposition of oxidative damage to deoxyribonucleic acid (DNA) material, lipids, and proteins[10]. Cellular pathways leading to hyperoxia induced organ damage are complex. There is disruption of normal physiological homeostatic balance with increased highly reactive mitochondrial ROS such as superoxide anion (O_2^-) hydrogen peroxide (H_2O), hydroxyl radical (OH^-) and peroxynitrite anion ($ONOO^-$). Further imbalances in antioxidant mechanisms' including the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase and small defence molecules such as glutathione, ascorbic acid, and vitamin E, may result in direct mitochondrial and cellular damage[10]. In physiological states, the balance of oxidant and antioxidant is tightly regulated and alterations in this equilibrium can lead to a proinflammatory state with influx of inflammatory cells, activation of cytokine cascades and increased vascular permeability[11].

Beyond ROS, there are other small nitrogen (RNS) and sulfur based reactive molecules (RSS). The conceptual framework of the "reactive species interactome (RSI)" consists of the complex chemical interaction between these reactive species and their downstream intracellular targets and metabolites[12]. Direct quantification of ROS/RNS/RSS in biological samples is not straightforward due to short half-lives and limited available analytical techniques. Hence measurements of precursors such as free sulfhydryl groups of cysteine in proteins and low molecular weight free thiols [cysteine, glutathione, homocysteine, and related species] may provide an assessment of global systemic redox state with less laborious analytical methods. Additional targets of RSI include stable end products of sulfur (hydrosufides, persufides, polysulfides, thiosulfate, and sulfate) nitrogen (ammonium, nitrite, nitrate, and nitroso and nitrosyl species) and oxygen (peroxide, lipid peroxidation products such as F_2-isoprostanes, malondialdehyde, protein carbonyl groups, total antioxidant capacity and individual redox couples)[13]. In summary, unopposed ROS, RNS and RSS reactive species lead to compromised cellular function with predisposition of oxidative damage to DNA material, lipids, and proteins. Consequently, hyperoxic states may lead to measurable increase in alveolar and

systemic oxidative stress which has never been quantified longitudinally in mechanically ventilated patients.

4.3.3. Hyperoxia and alveolar surfactant biology

The contribution of different levels of oxygen therapy to surfactant metabolism alterations in mechanically ventilated patients is an important research question that has not previously been addressed. Pulmonary surfactant is essential for maintenance of alveolar patency and consists primarily of phospholipids of which 80-85% is phosphatidylcholine (PC), with desaturated dipalmitoyl-PC (DPPC) accounting for 40-60%[14]. Compromised alveolar surface tension may be due to insufficient surfactant synthesis/secretion, increased breakdown (either by hydrolysis or oxidation) or surfactant inactivation/inhibition by biophysical inhibitors[15]. Surfactant is synthesised by alveolar type II (AT-II) cells and surfactant synthesis may be compromised during hyperoxia due to apoptosis of epithelial cells. Lungs are the primary target for direct oxygen toxicity and animal studies have consistently demonstrated that lungs exposed to high concentrations of oxygen exhibited quantitative and qualitative alterations in surfactant composition and function[16].

Local alveolar hyperoxia may also cause peroxidation of surfactant phospholipids and proteins, leading to reduced surface activity and a poorly compliant lung, perpetuating systemic hypoxemia[17]. Moreover, hyperoxia is associated with alterations in the alveolar surfactant phospholipid pool size, reductions in functionally active surfactant components and morphological changes in alveolar type-II cells[18,19]. Clinically, exposure to sub-lethal doses of oxygen results in decreased lung compliance, increased pulmonary leak, and inflammation with neutrophil migration. Pathologically, there is hyaline membrane formation, alveolar septal oedema, fibrosis, and diffuse hyperplasia of alveolar epithelial cells[20]. These pathological changes mimic neonatal respiratory distress syndrome due to primary surfactant deficiency implying that surfactant deficiency may contribute to hyperoxic lung injury. However, the precise balance between surfactant synthesis, secretion and catabolism has never been quantified by human *in-vivo* studies in relation to oxygen therapy and targets. Therapeutically, exogenous surfactant supplementation may have the potential to minimise

alveolar oxygen toxicity, by improving surface tension and reducing oxidative damage through its antioxidant properties[21].

5. Hypothesis, aim, objectives and outcomes

We hypothesise that both hyperoxia and hyperoxemia increase alveolar and systemic oxidative stress and adversely impact surfactant metabolism. Specifically, in mechanically ventilated patients: (**i**) Administration of high inspired oxygen concentrations will contribute to increased alveolar and systemic oxidative stress; **(ii)** increased alveolar and systemic oxidative stress will result in adverse changes in surfactant metabolism. We will characterise these metabolic phenotypes according to surfactant metabolism, alveolar and systemic oxidative stress. Stratification of these phenotypes may help to identify select groups that may benefit from targeted exogenous surfactant replacement, personalised therapeutic oxygen therapy and/or co-administration of targeted candidate therapeutic agents such as antioxidants to minimise surfactant inhibition and breakdown (**Figure 1**).

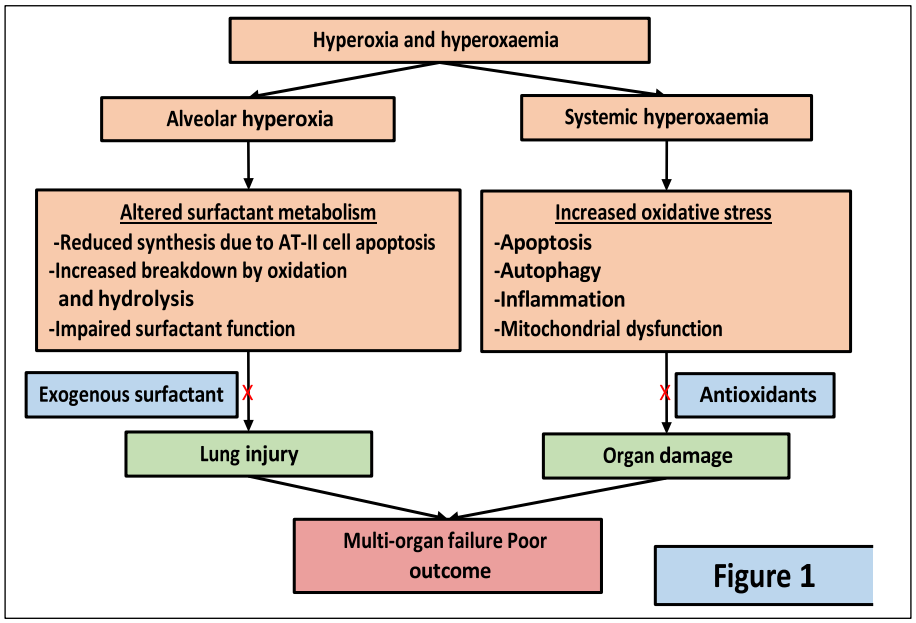


Figure 1: Hyperoxia and hyperoxaemia induced organ damage.

The primary aim of this study is to characterise in-depth the alveolar surfactant biology, oxidative stress and whole-body redox status in mechanically ventilated patients needing various oxygen supplementation strategies (**sub-group from UK-ROX,** a multi-centre randomised controlled trial).

The specific objectives

Objective 1: Quantify dynamic surfactant phospholipid composition, synthesis, and oxidative catabolism *in-vivo* and compare between the conservative and usual oxygen therapy group in mechanically ventilated patients.

Objective 2: Quantify lung and systemic oxidative stress and redox status by measuring the 'reactive species interactome' and compare between the conservative and usual oxygen therapy group mechanically ventilated patients.

Objective 3: Exploratory assessment surfactant phenotype, lung and systemic oxidative stress and redox status, in relation to clinical correlates of oxygenation, ventilation, and clinical outcomes.

Outcome measures

Primary Outcome: The difference of percentage of DPPC (PC32:0) in relation to total phosphatidylcholine composition (% of total PC in surfactant) at 48 hours between conservative and usual oxygen target groups.

Secondary outcomes

1. Surfactant index: This is a composite PC surfactant molecular index calculated from surfactant specific PC molecules (PC32:0, PC32:1 and PC30:0) and unsaturated surfactant PC34:1. This index will give a composite measure of surfactant PC alterations, which will provide a measure of surfactant PC status for the two different targets after 48 hours of oxygen therapy. *This outcome is a measure of surfactant specific PC composition.*

   Surfactant index = $\frac{\{PC32{:}0+PC32{:}1+PC30{:}0\}}{PC34{:}1}$

2. Surfactant phosphatidylcholine concentration (urea corrected) at 48 hours. *This outcome is a measure of endogenous surfactant level.*

3. Systemic oxidative stress: Total free thiols, lipid peroxides and total surfactant oxidation products*. This outcome will measure whole-body oxidative stress.*

Secondary explanatory outcomes

1. Surfactant total phosphatidylcholine and PC32:0 *methyl*-D_9choline enrichment at 48 hours. *Measure of endogenous surfactant synthesis. This will measure the surfactant PC synthesis via the CDP-Choline pathway.*
2. Surfactant total lysoPC and lysoPC16:0 concentrations, composition and *methyl*-D_9 choline enrichment at 48 hours. *This outcome is a measure of endogenous surfactant breakdown. This will help to assess dynamic surfactant PC breakdown through hydrolysis.*
3. Surfactant oxidised PC composition and concentrations at 48 hours. *Measure of endogenous surfactant breakdown. This will help to assess dynamic surfactant breakdown by oxidation.*
4. Whole- body redox balance by quantifying stable products of ROS (e.g., isoprostanes), RNS (e.g., nitrite, nitrate, nitrosation products) and RSS (e.g., total free thiols, thiosulfate, low molecular weight thiols including sulfide) at 48 hours from tracheal aspirates and plasma. *Measure of lung and systemic redox status.*

Secondary exploratory outcomes

1. Exploratory outcomes: Comparison of clinical outcomes (ICU mortality, hospital mortality, 90-day mortality, ICU, and hospital length of stay) in relation to surfactant abnormalities.
2. Exploratory outcomes: Comparison of clinical outcomes (ICU mortality, hospital mortality, 90-day mortality, ICU, and hospital length of stay) in relation to specific markers of oxidative stress.

6. Methods

6.1. Design and co-enrolment with UK-ROX

This study will co-enrol patients from UK-ROX study using both intervention and control groups. Participants will be enrolled within 24 hours of UK-ROX randomisation.

6.2. Population

The study population will consist of adults (≥18 years old), mechanically ventilated in ICU already enrolled into the UK-ROX study.

6.2.1. Inclusion criteria

1. Enrolled in UK-ROX study
2. Aged ≥ 18 years
3. Receiving invasive mechanical ventilation in the ICU for hypoxaemic respiratory failure
4. Receiving supplemental oxygen (fractional inspired concentration of oxygen (FiO_2>0.21) at the time of enrolment)
5. Anticipated to be mechanically ventilated for minimum of 48 hours

6.2.2. Exclusion criteria

1. Currently receiving extra corporeal membrane oxygenation (ECMO)
2. The treating clinician considers that one UK-ROX trial intervention arm is either indicated or contraindicated

6.3. Screening

Potential study participants of mechanically ventilated, hospitalised patients aged ≥18 already enrolled into the UK-ROX study will be identified according to the inclusion and exclusion criteria in the ICU by the research team.

6.4. Randomisation

All study participants will be already in the UK-ROX trial randomised 1:1 according to the UK-ROX study protocol to receive either conservative oxygen therapy (intervention) or usual oxygen therapy (control) using a central telephone or web-based randomisation service. Consecutive patients from each group will be enrolled into the MecROX study.

6.5. Consent procedures

Patients are already enrolled into the UK-ROX study by informed differed consent/personal consultee or a nominated consultee opinion. Patients enrolled into the UK-ROX will be approached to consider to be co-enrolled into the MecROX study. However, patients on a ventilator are often sedated and unable to provide an informed consent, additional consent will be sort from the personnel consultee prior to participation into the MecROX study. When the patient does not have capacity to consent (see above), the study information sheet will be made available to a personal consultee for to seek assent to participate in the study. The personal consultee will be fully informed of all aspects of the study and the potential risks. The following will be emphasised:

- Participation in the study is entirely voluntary.
- Refusal to participate involves no penalty or loss of medical benefits.
- The patient may withdraw from the study at any time.
- The personal legal representative is free to ask questions at any time to allow him or her to understand the purpose of the study and the procedures involved.
- There may be no direct benefit from participation.
- The aims of the study and all tests to be carried out will be explained. The personal legal representative will be given the opportunity to ask about details of the trial and will then have time to consider whether to participate or not.
- The risks of participating in the study will be fully explained.

6.5.1. If the personnel consultee is present with patient at point of assent

If the personal consultee decides that they wish for the patient to participate, and they have accompanied the patient, they will sign and date the personal consultee declaration form and will be given a copy. The original personal consultee declaration form will be stored as source documentation. These forms will also be signed and dated by the Investigator.

6.5.2. If the personnel consultee is not present with patient at point of assent

In cases where it is not possible for a personal legal representative to be present in the hospital due to local visitor restrictions, the study can be discussed with the patient's personal

consultee over the telephone. This call will be undertaken by an experienced member of the research team with knowledge of intensive care. The telephone consultation should be witnessed by another member of staff. The personal consultee information sheet may be sent by email or fax. Following the call, the conversation and outcome will be documented in the patient's medical notes and

a) if the personal consultee *is unable to sign* a declaration form, a personnel consultee telephone declaration form will be completed and signed by the trial professional taking assent and an independent professional witness.
   Or
b) if the personal consultee *can sign* a declaration form a copy will be sent (via email or online declaration form) to sign. Once the signed form is returned, the trial professional taking assent (delegated by PI to take consent/assent) will countersign the form (the date and time may differ from the original verbal assent call).

The original form will be stored as source documentation, a copy will be given to the personnel consultee and a copy of this will be placed in the medical and trial notes. Details of assent procedure will be documented in the patient's medical notes.

Patients maybe enrolled into the study once verbal assent has been provided and prior to a signed personnel consultee declaration form being received, to prevent delays with initiating the study.

6.5.3. If there is no available personnel consultee at point of assent

If it is not possible to approach a personal consultee for assent, we will not recruit the patient into this study.

Personal consultee will be given sufficient time to review the information provided and ask questions before signing the assent / declaration form. Only members of the research team

who have undergone the appropriate GCP training will be able to seek agreement from potential patients' personal or professional consultee.

6.5.4. Enrolled patients regaining capacity (consent)

Once participants have regained capacity, they will be asked to consent to their continued participation in the study. The study rationale and conduct will be explained to them outlining any interventions which have already been undertaken as well as those that are still required. Details of the informed consent discussion will be recorded in the patient's medical record. This will include the date consent was given, with the name of the study and the version number of the Participant Information Sheet and Informed Consent Form.

Throughout the trial the patient or personal consultee should have the opportunity to ask questions about the trial and any new information that may be relevant to the patient's continued participation should be shared with them in a timely fashion. On occasion, it may be necessary to re-consent the patient or personal consultee for example if new information becomes available or an amendment is made to the protocol that might affect the patient's participation in the trial. In this case the process above will be followed and the patient's right to withdraw from the trial respected.

6.5.5. Refusal or withdrawals of consent

Withdrawal from the study will only occur if the patient or personal legal representative withdraws consent or if a safety event in the opinion of the investigators warrants withdrawal.

6.6. Interventions

The interventions are outlined in the UK-ROX trial protocol (https://www.icnarc.org/Our-Research/Studies/Uk-Rox/Information-For-Sites/Document-Pack). The intervention group will receive conservative oxygen therapy with an oxygen saturation target [SpO2 of 90 (± 2) %] and control group will receive usual oxygen therapy defined by the local practice (see UK-ROX protocol for further details). Additional blood, endotracheal aspirate (ETA), and airway

samples for particles of exhaled air **(**PExA) measurements will be taken for mechanistic analysis.

Once patients are enrolled into the MecROX study, patients will be infused with *methyl*-D_9 choline chloride as described below (section 6.6.1). Serial blood and bronchial samples will subsequently be collected at time points 0 (just before or immediately after the *methyl*-D_9-choline infusion), 48 and 72 hours after the *methyl*-D_9 choline chloride infusion. Measurements on all samples will include dynamics surfactant phospholipids, surfactant proteins and proteolytic fragments, lysophospholipids products of phospholipid hydrolysis, oxidised phospholipids, and metabolites of "Reactive Species Interactome".

6.6.1. Infusion of methyl-D_9-choline chloride

Choline is an essential nutrient grouped within the vitamin B complex and is required as part of a normal healthy diet. Choline is a crucial component of the lung surfactant. Labelling naturally occurring choline (*methyl*-D_9 choline chloride) with a non-toxic, non-radioactive, stable (does not breakdown) isotope (different weight) of hydrogen helps measure the rate of surfactant synthesis and breakdown in different diseases. This has been used successfully in several clinical studies of healthy adult volunteers and in adult and neonates' patients with lung problems without any know side-effects[22,23]. *Methyl*-D_9-choline chloride will be dissolved in water at 10 mg/ml and infused at a dose of 3 mg/kg actual body weight (maximum dose of 300mg) over 3 hours, will enable dynamic assessment of surfactant synthesis and turnover. *Methyl*-D_9-choline incorporation into lung surfactant dipalmitoyl phosphatidylcholine (DPPC), the major surface-active component, will measure surfactant phospholipid synthesis. It will answer the mechanistic question:

- Are there alterations in surfactant synthesis and turnover during differing oxygen therapy targets?

The MHRA have confirmed that *methyl*-D_9-choline chloride is not an IMP. A metabolic pathway for *methyl*-D_9-choline incorporation into phospholipids is shown below, together with an example of the diagnostic mass spectrometry scans used for detection and quantification of unlabelled and deuterium-labelled PC.

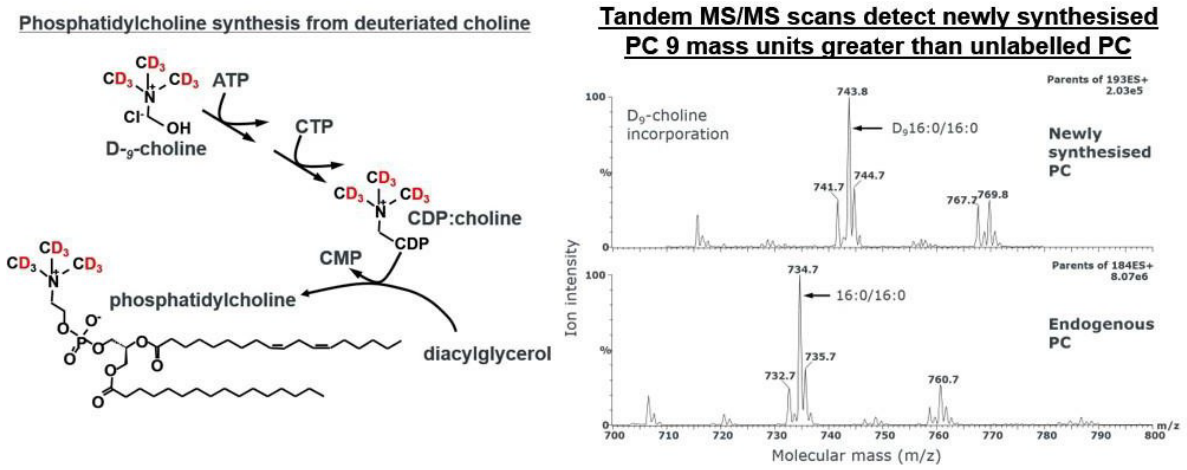


Figure 2: Surfactant phosphatidylcholine synthesis via CDP-Choline pathway and spectra from mass spectrometry analysis.

6.6.2. Sample collection

All samples to be taken from the time of *methyl*-D_9 choline infusion for a combination mechanistic study are:

6.6.2.1. Blood Sample Collection

EDTA (10 ml) blood samples will be taken just before or immediately during the *methyl*-D_9-choline infusion (0 hours) and at 48 and 72 hours after the *methyl*-D_9-choline infusion. These sample will be taken only if the patient is still hospitalised. While in ICU, these samples are usually taken from pre-existing venous or arterial access lines. Blood samples (10 ml) will be collected into EDTA vacutainers. Samples will be stored at +4°C until transfer to the laboratory for processing.

6.6.2.2. Closed suction endotracheal aspirate samples (ETA)

Endotracheal aspirate (ETA) is a commonly performed procedure for secretion clearance in the intensive care unit. ETA samples can be used to assess airway surfactant composition and metabolism. ETA samples will be taken just before or immediately after the *methyl*-D_9-choline infusion (0 hours) and at 48 and 72 hours after the *methyl*-D_9-choline infusion. The ETA samples will consist of blind suctioning by a cannula introduced through a port into the endotracheal tube. A volume of 20-50 ml saline will be administered, with an estimated recovery of 8-10 ml. This procedure will not interrupt the ventilator circuit and will not result in desaturation and

usually performed during physiotherapy sessions for mucus clearance. ETA samples will be taken into pre-labelled tissue culture (Falcon) tubes and stored at +4°C in the ICU sample fridge until transfer to the laboratory for processing. Transfer to the laboratory should be as rapid as possible, with a goal of under 2 hrs.

6.6.2.3. Particles of exhaled air (PExA)

Small airway samples are the gold-standard for surfactant measurements. However, sampling from small airway is often very difficult as bronchoscopy and lavage is the only option. While safe and a routine procedure, bronchoscopy is an invasive method for airway sampling which require medical expertise and have potential for desaturations during the procedure. Moreover, repeated sampling will likely to require preoxygenation with high inspired oxygen which will interfere with trial interventions. To avoid this, PExA from patients will be measured by PExA device (Gothenberg, Sweden)[24]. This will enable repeated small airway sampling for surfactant assessments without the use of bronchoscopy. This PExA device contains an optical particle counter (OPC) connected to an impactor for collection of samples at a diameter range of 0.41-4.55 µm. The PExA instrument will be connected to the ventilator outflow circuit to capture expiratory samples. The samples will be taken for an hour, starting just before or immediately after the *methyl*-D_9-choline infusion (0 hours) and at 48 and 72 hours after the *methyl*-D_9-choline infusion. The device will measure number of particles (count) and total accumulated mass (ng) of particles which will be collected onto a membrane for further biochemical analysis. As PExA is only available at UHS, this will be only done at University Hospital Southampton site.

6.6.3. Electrospray ionisation mass spectrometry (ESI-MS/MS) phospholipid measurements

Phospholipids, lysophospholipids and oxidised phospholipids molecular species will be analysed in tracheal fluid samples and from PExA. Lipid extracts will be analysed by direct infusion ESI-MS/MS. Lysophosphatidylcholine products of PLA_2-mediated hydrolysis of surfactant phospholipid will be determined by diagnostic precursor scans, together with molecular species compositions of intact surfactant phospholipid. Oxidation of unsaturated phospholipid initially generates higher mass peroxides, which then undergo chemical

degradation to form truncated lower mass lipid aldehydes and hydroxyls. Intact and high and low mass oxidised phospholipids will be determined by specific MRM scans using electrospray ionization tandem mass spectrometry (ESI-MS/MS). An estimate of surfactant concentration will be determined by urea dilution analysis in parallel samples of bronchial fluid and plasma samples.

6.6.4. Quantification of redox biology

Systemic and alveolar redox read out will be measured from blood and bronchial fluid. We will analyse relative proportion of stable NO metabolites such as nitrite, nitrate and nitrosation products, total free thiols as well as low-molecular weight thiols critical to intracellular glutathione homeostasis and the maintenance of intracellular redox status. Local and systemic redox/oxidative stress status will employ an array of analytical platforms including ELISA, gas-phase chemiluminescence, HPLC, IC-MS and LC-ESI-MS/MS. We will specifically quantify 8-isoprostanes, malondialdehyde (TBARS), nitrite, nitrate, total nitroso species, thiosulfate, sulfate, total free thiols, free and bound low molecular weight thiols (including cysteine, homocysteine, glutathione), sulfide and polysulfide species as those analytes have revealed a particularly high sensitivity and specificity to perturbation by oxidative stress.

7. Storage and analysis of clinical samples

All samples will be collected into pre-labelled containers supplied in kits for individual participants by the clinical trial team, will be double bagged and transferred in a closed container between the ICU and the processing laboratory. Samples will be processed and stored according to the MecROX specific Laboratory Manual.

7.1. Sample processing and storage

Three types of samples will be collected from consenting patients for analysis within the MecROX trial:

- ETA from all patients enrolled
- Peripheral blood from all patients enrolled
- PExA from a subset of patients enrolled at UHS only

All samples will be sent, processed and stored at a locally agreed laboratory prior to release for research within [details of the MASS Spec lab].

Samples will be sent to the University of Southampton for long term storage upon request before being released to analysing laboratories.

All sites will keep a record of all samples processed, stored and shipped.

A detailed Laboratory Manual will be provided to sites which will include details regarding sample preparation, handling, interim storage and shipment.

In the event of enrolment of COVID-19 patients, the virus will be destroyed where practicable in the samples for analysis. This will be achieved for the lipid analyses during extraction by adding samples to methanol, by UV irradiation for the surfactant protein analysis and by fixation for the inflammatory cell characterisation. All procedures on samples with live virus will be undertaken in category 2 or 3 facilities as appropriate and related SOPs will be supplied. All analyses to be undertaken by laboratories at Southampton.

7.2. Sample analysis

7.2.1. Surfactant lipid composition and dynamic turnover (University of Southampton)

Mass spectrometric analysis of molecular species compositions of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) in small volume ETA samples. Breakdown products of surfactant phospholipids including lysophospholipids (hydrolysis) and oxidised phospholipids (oxidation) will be analysed over the study period according to local laboratory procedures. Moreover, analysis of sequential samples (48 hrs and 72 hrs) may provide an indication of exogenous surfactant synthesis and catabolism. Infusion of stable isotope-labelled choline (*methyl*-D_9-choline chloride) IV will provide a measure of surfactant PC synthesis and hence function of ATII cells. This would be incorporated into the phospholipid analysis. The concentration of *methyl*-D_9-PC molecular species will determine if surfactant synthesis is altered PC during different oxygen therapy targets.

7.2.2. Surfactant protein composition over time (University Hospital London)

The level of the immunomodulatory surfactant protein D (SP-D) in serum has shown to be a promising biomarker for lung inflammation (and hence low levels in ETA), and we will quantify the level of SP-D in both ETA and plasma using our established ELISA. We will also quantify the level of C-reactive protein in ETA as a biomarker for blood contamination of surfactant together with total protein concentration. We have seen that an inflammatory response to infections can results in specific degradation dependent on the infectious agent. We will analyse the degradation of SP-D and its sister protein SP-A using SDS-PAGE and Western Blot analysis to evaluate if different oxygen targets result in degradation.

7.2.3. Oxidative Stress/Redox Measurements (University Hospital Southampton)

The extent of local and systemic oxidative stress with associated modifications of surfactant composition, alterations in cell signalling (due to interference with nitric oxide and hydrogen sulfide-related cell function) and shifts in redox status following the increased production of reactive oxygen species will be characterised in aliquots of pulmonary secretions and blood. Oxidative stress, nitric oxide, hydrogen sulfide and other redox related metabolites and products of 'reactive species' interactions will be quantified by an array of analytical platforms including ELISA, gas-phase chemiluminescence, HPLC, IC-MS, and LC-MS/MS to determine the following readouts: 8-isoprostanes, malondialdehyde (TBARS), nitrite, nitrate, total nitroso species, thiosulfate, sulfate, total free thiols, free and bound low-molecular weight thiols (including cysteine, homocysteine, glutathione), sulfide and polysulfide species according to local standard operating procedures.

8. Data collection

All UK-ROX trial data collection will be enhanced within the CMP research platform. Additional data-collection will be obtained beyond the standard data collection by the UK-ROX clinical trial. They will include baseline data on vital sign measurement and ventilation parameters will be recorded from data already generated in the intensive care unit. These values will be obtained closest to prior to the time of study initiation. All participant data collected will be entered onto a secure electronic data entry system. The option of entry first onto paper CRFs will be available to the sites. The site PIs will oversee and be responsible for data collection,

quality, and recording. Collection of data can be delegated (as per the Delegation Log) by the site PIs to qualified members of the research team.

All trial data collection will be nested within the CMP 'Research Platform', enabling data collection to be incorporated within the routine CMP data collection processes, streamlining data linkage. For all patients, UK-ROX will nest within the routine data collection for the CMP, including:

1. Patient demographics
2. Age, gender, ethnicity, height, weight, predicted body weight
3. Date/time of hospital admission
4. Date/time of ICU admission
5. Date/time oxygen treatment started
6. Date/time of mechanical ventilation
7. Date/time of UK-ROX randomisation
8. Diagnosis on admission
9. Co-morbidities
    a. Hypertension
    b. Diabetes
    c. Chronic kidney disease
    d. Chronic respiratory diseases (inc. Asthma, COPD)
    e. Chronic cardiac disease
    f. Cardiovascular disease (inc. Stroke/TIA, STEMI/NSTEMI/CABG/Heart failure)
    g. Cancer (active or treatment within a year)
    h. Chronic liver disease (inc. ALD, NASH, cirrhosis, liver failure)
    i. Immunosuppression
    j. Other medical history or co-morbidities
10. APACHE 2 and SOFA score on admission
11. Clinical frailty score
12. Ventilation and oxygen indices

Ventilator measurements at enrolment and daily until 96 hours or extubation, whichever happens first.

Mode (mandatory, spontaneous, mixed), CPAP/PEEP, inspiratory positive airway pressure, respiratory rate, tidal volumes, compliance, mean airway pressure, peak airway pressure, plateau pressure, I: E ratio, use of pulmonary vasodilators), supine or prone.

Mean oxygenation prior to 48 hours before intubation, at enrolment and daily until 96 hours or extubation whichever happens first.

PaO_2, FiO_2, SpO_2, pH, $PaCO_2$, lactate.

All relevant data will be obtained from ICU clinical database.

9. Statistics and data analysis

9.1. Sample size calculations

There is no previous index of surfactant damage documented in patients or animal models of acute hyperoxic lung injury. From ARDS and healthy volunteer studies, estimates of the mean (SD) for DPPC (PC32:0) composition are ARDS patients 35.6% (SD 12.1%) and healthy controls 53.1% (SD 4.3%)[14,22]. Excess exposure will likely have alterations in DPPC composition from healthy volunteers but not significantly similar to the ARDS patients, so we estimate that the standard deviation will be between 4.3 and 12.1. With the higher estimate of SD, a sample size of 90 patients will achieve 90% power to detect a difference of 8.4% with a significance level of 0.05 using a two-sided two-sample t-test. Using the lower estimate, a sample size of 90 patients will achieve 90% power to detect a difference of 3.0% between groups with a significance level of 0.05 using a two-sided two-sample t-test. Allowing for an estimated 10% drop out we will recruit 100 patients.

Variable	Timepoint	Group	Mean (SD)	Minimum absolute difference which can be detected at 90% power, N=90
DPPC/PC32:0	48	ARDS	35.6 (12.1)	8.4
	48	Healthy control	53.1 (4.3)	3.0

Table 1: Surfactant DPPC compositions a from healthy volunteers and ARDS patients used for the power calculations.

9.2. Mechanistic markers analysis

The research hypothesis is that there is a difference in DPPC (PC32:0) fractional concentration between the conservative and usual oxygen groups. The primary endpoint is percentage DPPC (PC32:0) relative to total PC composition (% of total PC in surfactant) at 48 hours. For the primary analysis, we will use a multiple regression model adjusted for baseline to investigate the difference between the conservative and usual oxygen groups. If data are not normally distributed, we will investigate whether a log transformation improves Normality. We will also perform an adjusted analysis to address clinical heterogeneity, using multiple regression, with up to 10 variables including the following baseline variables: Age, gender, BMI, percentage of PC32:0 in relation to total PC composition at admission, clinical conditions (e.g., sepsis, pneumonia) and concentration of inspired oxygen required at admission. We will also perform descriptive subgroup analysis for group differences and present them as box and whisker plots and scatter plots. As samples are collected from time points T=0, T=48 and T=72 hours as a secondary analysis, we will also investigate the use of mixed models to look at the difference between groups over time.

For the secondary outcomes of surfactant index; surfactant PC32:0, PC32:1, PC30:0; surfactant total phosphatidylcholine and PC32:0 *methyl*-D_9choline enrichment; surfactant total lysoPC and lysoPC16:0 concentrations, composition and *methyl*-D_9 choline enrichment and surfactant oxidised PC composition and concentrations (all measured at 48 hours), we will investigate the difference between groups using a two-sample t-test or Wilcoxon- Mann-Whitney test depending on the Normality of data. If appropriate, adjusted analyses will also be performed as specified for the primary endpoint. The above will be repeated for the analysis of individual redox/oxidative stress markers listed above.

For the exploratory clinical outcomes of ICU mortality, ICU+ hospital mortality and all mortality censored at 90 days, we will use Cox regression with time-varying covariates to investigate the effect of surfactant abnormalities and specific markers of oxidative stress on

survival. For ICU and hospital length of stay, we will use cause specific COX regression models to account for the fact that patients may die before being discharged. As an exploratory analysis, we will illustrate the relationship between surfactant markers and oxidative stress markers using scatter plots and will quantify these relationships using regression models.

10. Ethical and regulatory considerations

The chief investigator will ensure that this study is conducted according to the principles of the Good Clinical Practice set out by the current revision of the Declaration of Helsinki 2013.

10.1. Assessment and management of risk

10.1.1. Serious adverse events (SAEs)

All adverse events related to the UK-ROX study intervention will be reported according to the UK-ROX protocol. An adverse event is any untoward medical occurrence associated with the use of *methyl*-D_9 choline chloride or blood, TA and PExA sampling or any other element of the study protocol. For each adverse event, start date, stop date, causality, severity, action taken, and outcome will be documented in the subject file. Should any investigators involved become aware of safety issues they should contact the chief investigator (CI). An SAE/SUSAR form should be completed for all SAEs and SUSARS and emailed to UHS as the sponsor via researchsafety@uhs.nhs.uk.

10.1.2. Medical issues

Should acute medical concerns arise as part of the study, the investigator will discuss with the CI and arrange clinical review depending on the nature of the problem and subject stability.

10.1.3. Safeguarding issues

Should concerns of a safeguarding nature arise, the principal investigator will discuss with the CI. If further advice is needed, then this will be discussed with the hospital safeguarding team as appropriate and further action taken as required.

10.1.4. Right to withdraw

The participant or the personnel/professional consultee must remain free to withdraw at any time from the trial without giving reasons and without prejudicing his/her further treatment and must be provided with a contact point where he/she may obtain further information about the study. Subject withdrawal of consent from the trial must be explicitly documented in the source documents.

10.2. Research Ethics Committee (REC)

10.2.1. Regulatory Review & Compliance

This study is approved by the Sponsor, University Hospital Southampton NHS Foundation Trust and will be subject to UK Health Research Authority (NHS) approval. The sponsor will ensure that the trial protocol, patient information sheet, consent form, GP letter and submitted supporting documents have been approved by the appropriate regulatory body, Health Research Authority (HRA), main REC and that local permission has been obtained prior to any subject recruitment.

All substantial amendments and non-substantial amendments (as determined by the sponsor) will not be implemented until HRA/REC have provided the relevant authorisations. The NHS R&D departments will also be informed of any substantial amendments and non-substantial amendments. Relevant approvals must be obtained before any substantial amendment and non-substantial amendments may be implemented at sites.

All correspondence with the HRA and the REC will be retained in the Trial Master File and the Investigator Site File (maintained by the site).

An annual progress report (APR) will be submitted to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the trial is declared ended.

Within 90 days after the end of the study (as defined in section 7.10), the CI/Sponsor will ensure that the HRA and the main REC are notified that the trial has finished. If the study is terminated prematurely, those reports will be made within 15 days after the end of the trial. The CI will supply the Sponsor with a summary report of the clinical trial, which will then be submitted to the main REC within 1 year after the end of the trial. All results will be published on a publicly accessible database.

10.3. Peer review

This study has gone through scientific peer review by two independent internal and external reviewers with satisfactory/compliant review process.

10.4. Protocol compliance

Any protocol deviation will be reported to the CI and sponsor via researchsafety@uhs.nhs.uk. Any recurrent protocol deviation will be investigated by the CI and sponsor.

10.5. Data protection and patient confidentiality

To maintain confidentiality, all assessment forms, laboratory specimens, reports and other records will be identified on research systems by a coded number and initials only. All paper records will be stored in a locked filing cabinet. Anonymised data will be stored on University of Southampton servers for analysis. Patient identifiable data will be stored NHS password-protected computers. All Investigators and study site staff involved with this study must comply with the requirements of the General Data Protection Regulations 2018 regarding the collection, storage, processing, and disclosure of personal information and will uphold the Act's core principles. Access to collated patient data will be restricted to those clinicians treating the patients. Computers used to collate the data will have limited access measures via usernames and passwords. Published results will not contain any personal data that could allow identification of individual patients.

All data will be collected as part of the UK-ROX study. Data entered onto the secure electronic data entry system will undergo validation checks for completeness, accuracy, and consistency of data. Queries on incomplete, inaccurate, or inconsistent data will be sent to the local research team at participating sites for resolution. Security of the electronic data entry system is maintained through usernames and individual permissions approved centrally by the ICNARC CTU. Central back-up procedures are in place. Storage and handling of confidential trial data and documents will be in accordance with the Data Protection Act. ICNARC is registered under the Data Protection Act (Registration number: Z6289325).

10.6. Indemnity

The sponsor of this study is University Hospital Southampton NHS Foundation Trust. For NHS sponsored research HSG (96) 48 reference no.2 refers. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS Indemnity covers NHS staff, medical academic staff with honorary contracts, and those conducting the trial. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim.

10.7. Access to the final study dataset

Access to the final data set will comply with institutional policies and the UK strategy for open data availability.

11. Monitoring and auditing

The UK-ROX interventional study will be monitored in accordance with the UK-ROX protocol. The MecROX will be monitored and may be participant to monitoring and audit by University Hospital Southampton NHS Foundation Trust, under their remit as sponsor and other regulatory bodies to ensure adherence to ICH GCP, UK Policy Framework for Health and Social Care Research, applicable contracts/agreements, and national regulations. All study related documents will be made available on request for monitoring and audit by UHS, the relevant REC or other licensing bodies.

12. Study closure

12.1. End of study

The end of the study is defined as last patient and last sampling time point.

12.2. Archiving

Archiving will be authorised by the Sponsor following submission of the end of study report.

Location and duration of record retention for:

- Essential documents: Patient case notes will be stored and maintained according to standard rules and procedures. Pathology results are stored and maintained according to standard procedures.
- Study data will be held for minimum of 5 years

Destruction of essential documents will require authorisation from the Sponsor.

13. Study Deliverability

The two proposed centres (University Hospital Southampton and University Hospitals Plymouth) have already enrolled 122 and 73 patients into the UK-ROX RCT over periods of 12 months and eight months, respectively. This is an accrual rate will enable enrolment of the planned 100 patients within the anticipated study time frame from both centres (2-3 per week). If the recruitment rate is slower than anticipated, we will seek approval to include additional centres. From the Data Monitoring Committee's assessment, it is unlikely that the UK-ROX study will close prematurely. We have pre-existing infrastructure to perform all these anticipated mechanistic analyses.

14. Study dissemination

14.1. Dissemination policy

The results of the study will not be made available to the research participants at this stage. However, the study will be written up for publication in a peer-reviewed scientific journal and we will share the overall finding of the study to all the participants who took part in the study. Participants will not be identifiable in the project report or publications.

14.2. Authorship eligibility guidelines and any intended use of professional writers

The investigators involved in the study will be eligible for authorship. There is no intention to use professional writers.

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16. Appendix 1 Research Timetable

Study duration (30 Months)	09/22 - 12/22			01/23 - 03/23			04/23 - 06/23			07/23 - 09/23			1023 - 12/23			01/24 - 03/24			04/24 - 06/24			07/24 - 09/24			10/24 - 12/24			01/25 - 03/25			04/25 - 06/25			Study End
	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	
Research Activity																																		
Regulatory approvals																																		
Deliverable 1																																		
Patient recruitment																																		
Sample collection																																		
Deliverable 2																																		
Surfactant analysis																																		
Oxidative stress analysis																																		
Deliverable 3																																		
Data synthesis																																		
Publication plan																																		
Dissemination of results																																		

The regulatory approvals will be sought from October 2022- December 2022. We will start recruitment from January 2022 for a period of 18 months. Although this start up time seems ambitious, we are keen to start co-enrolling patients into the Mex-ROX study as soon as possible to avoid missing potential patients, particularly during the winter months. We have the confirmation from the Research and Development Department that they will support patient recruitment while we are waiting for the final funding contract to complete. Collected samples will be stored and analysis will commence from July 2024 for subsequent 12 months. Data synthesis, publication plan and dissemination of results will be anticipated in the last 6 months of the study (see Gantt Chart). This study is based on UK-ROX RCT and there are currently no proposed contractual STOP/GO decision points for this study and any modifications will be guided by this primary study's TSC and DSMB review processes.

17. Revision History

Protocol date and version	Summary of significant changes
V1.0 07-Jul-2022	New Production
V2.0 05-Oct-2022	Considered NIHR EME Grant committee review • Inclusion criteria modified as "Receiving invasive mechanical ventilation in the ICU for hypoxaemic respiratory failure"
V3.0 26-Oct-2022	Inclusion of data collection details
V4.0 07-Dec-2022	Considered REC committee's review to modify the consent process to exclude professional consultee involvement.
V5.0 09-FEB-2023	• Update planned study period dates • Minor typological corrections and abbreviation updates through out • Clarified that PExA samples are only collected when the PExA device is available. • Clarified MecROX participants will be recruited from both UK-ROX groups. • Replaced legal representative declaration form with personal consultee declaration form • Clarification that MecROX patients, not UK-ROX patients, are infused with methyl-D9 choline • Removed all 24 hour, 5 day and 10 day samples time points • Removed the requirement to collect Transfix blood samples • Updated tracheal aspirates (TA) with endotracheal aspirates (ETA) • Removed specific sample processing and storage details and instead refer to specifics in Laboratory Manual • Removed details of sample analysis, replaced with reference to local standard operating procedures • Introduced a Revision History
V6.0 04-JAN-2024	• Clarification that 0 hour samples taken before or during the methyl-D_9-choline infusion.

	• Clarification that methyl-D_9-choline infusion is based on actual body weight with a maximum dose for obese patients.
V7.0 15-MAR-2024	• Reduction of anticipated ventilation period in the inclusion criteria from 72 hours to 48 hours. • University of Southampton logo updated.