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PHOSGENE INDUCED ACUTE LUNG INJURY

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ABSTRACT

FACULTY OF MEDICINE

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PHOSGENE INDUCED ACUTE LUNG INJURY

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Phosgene is an extremely toxic vapour used to devastating effect during World War 1. The ease with which it can be manufactured means that it remains a high threat compound as a terrorist weapon and its extensive use in the chemical and pharmaceutical industry as a Toxic Industrial Chemical (TIC). It predominantly affects the lungs, at the alveolar level, causing an acute lung injury with extensive non-cardiogenic pulmonary oedema.

Signs and symptoms manifest after a latent phase, largely dependent upon inhaled dose, to the point where they can occur at 24h post exposure. In a mass release, it will be impossible to identify intoxicated individuals until they become symptomatic or hypoxaemic. Additionally, the logistic burden of providing supplemental oxygen and transporting large numbers of patients to hospitals may be too great.

If a reliable biomarker of phosgene toxicity could be identified, then this burden is reduced. Furthermore, a commercial off the shelf device that provides Continuous Positive Airway Pressure (CPAP) to affected patients could safely extend transfer times while minimising oxygen toxicity.

Defence Scientific and Technology Laboratory Porton Down is the only establishment in the World capable of evaluating the response of large animals to highly toxic inhaled compounds. In this study we used an established pig model to evaluate the effects of a Positive End Expiratory Pressure after exposure to phosgene. The survival and physiological data show that it is extraordinarily effective.

Access to some plasma and bronchoalveolar lavage samples made during the experiments was granted and an analysis of lipid content was made using electrospray ionisation tandem mass spectrometry. This has not been done to this level of detail before.

This novel work has shown that CPAP is extraordinarily effective at improving survival, physiological markers of critical illness and modulating surfactant composition.

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DECLARATION OF AUTHORSHIP

I declare that this thesis and the work presented in it is my own and has been generated by me as the result of my own original research. I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University.

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.

3. Where I have consulted the published work of others, this is always clearly attributed.

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5. I have acknowledged all main sources of help.

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

7. Parts of this work have been published and are at the Appendix:

Graham S, Fairhall S, Rutter S, Auton P, Rendell R, Smith A, *et al*. Continuous positive airway pressure: An early intervention to prevent phosgene-induced acute lung injury. *Toxicol Lett*. 2018; **293**:120-6.

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My ex wife and daughters whose enduring tolerance and encouragement have supported me. My partner, daughter and son, whose patience, kindness and forbearance underpinned the final stages of this endeavour, and without whom it would not have been completed.

For my father Charles Christopher Nicholson Roberts MA MB BChir DRCOG

who valued curiosity

1941- 2013

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ALI	Acute lung injury
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BFA	Brefeldin A
BHT	Butylated hydroxytoluene
°C	Degrees Celsius
CD	Cluster of differentiation
CDP	Cytidine diphosphate
CG	Phosgene
CMP	Cytidine monophosphate
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
COTS	Commercial off the shelf
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CPAP	Continuous positive airway pressure
CTP	Cytidine triphosphate
CVP	Central venous pressure

CXR	Chest X-ray film
CYP	Cytochrome P450
DCM	Dichloromethane
DPPC	Dipalmitoyl phosphatidylcholine
DSTL	Defence Science and Technology Laboratory
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
ESI-MS/MS spectrometry	Electrospray ionisation tandem mass
eV	Electronvolt
EVLW	Extravascular lung water
F_IO_2	Fraction of inspired oxygen
FRC	Functional residual capacity (of lung)
GEDV	Global end diastolic volume
GPL	Glycerophospholipid
GM-CSF factor	Granulocyte- macrophage colony-stimulating
HDL	High density lipoprotein
HMD	Hyaline membrane disease (NRDS)
HPLC	High performance liquid chromatography
HPV	Hypoxic pulmonary vasoconstriction
ITBV	Intrathoracic blood volume
ITTV	Intrathoracic thermal volume

kPa	Kilopascal (1000 Newtons per metre^2)
kV	Kilovolt
LB	Lamellar bodies
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharides, endotoxins
LWW:BW	Lung wet weight to bodyweight ratio
LWW:DW	Lung wet weight to dry weight ratio
MAP	Mean arterial pressure
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NATO	North Atlantic Treaty Organisation
NLR	Neutrophil/ lymphocyte ratio
NRDS	Neonatal respiratory distress syndrome
NTP	Normal temperature and pressure: 101.325kPa | 293.15K (20°C)
PA	Phosphatidic acid
P_aO_2	Partial pressure of arterial oxygen
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEEP	Positive end expiratory pressure

PEMT	Phosphatidylethanolamine methyl transferase
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PiCCO	Pulse contour continuous cardiac output
PLA_2	Phospholipase A_2
PPE	Personal protective equipment
PS	Phosphatidylserine
PTE	Pulmonary thromboembolism
PUFA	Polyunsaturated fatty acid
PVR	Pulmonary vascular resistance
rpm	Revolutions per minute
S_aO_2	Arterial oxygen saturation
$S_{cv}O_2$	Central venous oxygen saturation
SD	Standard deviation
SEM	Standard error of the mean
SM	Sphingomyelin
SP-A to D	Surfactant proteins A to D
SpO_2	Pulse oximetry
SV	Stroke volume
S_vO_2	Mixed venous oxygen saturation
SVR	Systemic vascular resistance
SVV	Stroke volume variation

TAG	Triacylglycerol
TIC	Toxic industrial chemical
TQMS	Triple quadrupole mass spectrometry
TTPs	Tactics, techniques and procedures
V	Volt
VLDL	Very low density lipoprotein
V_T	Tidal volume
WBC	White blood cell

1 - INTRODUCTION

1.1 Phosgene

1.1.1 Phosgene Properties and Uses

Phosgene is a highly reactive compound with the formula $COCl_2$. It is 3.5 times denser than air, with a boiling point of 7.4 °C and a critical temperature of 182 °C (1), it exists as a vapour at normal temperature and pressure (NTP). Phosgene was first synthesised by John Davy by exposing equal volumes of carbon monoxide and chlorine to sunshine for 15min, he noted that the mixture contracted to half the original volume and the chlorine colour disappeared to form a colourless gas. He named it phosgene, from the Greek φως, phos = light and γινομαι, gene = to produce (2), born of light. Its odour was recklessly described thus:

> "*Thrown into the atmosphere, it did not fume. Its odour was different from that of chlorine, something like that which one might imagine would result from the smell of chlorine combined with that of ammonia, yet more intolerable and suffocating than chlorine itself, and affecting the eyes in a peculiar manner, producing a rapid flow of tears and occasioning painful sensations.*"

The odour has been variously described as of green corn or musty hay (3, 4) or as new mown hay.

Jackson described four chief methods for producing phosgene (5):

- The photochemical combination of carbon monoxide and chlorine
- The oxidation of chlorinated hydrocarbons with chromic acid
- The interaction of sulphur trioxide or oleum with chlorinated hydrocarbons
- The combination of carbon monoxide and chlorine in the presence of a solid catalyst

It is also produced in the thermal decomposition of certain chlorinated hydrocarbons (6, 7).

Phosgene is widely used commercially in the production of many chemical compounds. It reacts with a multitude of nitrogen, oxygen, sulphur and carbon centres, as well as with a variety of other inorganic compounds, by acylation, chlorination, decarboxylation and dehydration. It is an important chemical intermediate in many manufacturing processes (8). The majority of phosgene is used in the production of isocyanates for the synthesis of polyurethanes, other uses are in production of polycarbonates, and chloroformates used to make pharmaceuticals and pesticides (9).

1.1.2 Phosgene in World War 1

Gas attacks in World War 1 began on 22 April 1915 with the release of chlorine from vast arrays of cylinders by the Germans. Phosgene was used to devastating effect during World War 1. The first recorded use was at Ypres 19 December 1915 in combination with chlorine. Allied intelligence was such that respirators had been developed containing cotton impregnated with sodium phenolate and later sodium phenolate in combination with hexamethylenetetramine (3). However the troops at Ypres on that occasion were inadequately protected. The Allies had adopted gas warfare in response to repeated German gas attacks by the end of September 1915 and this included the development of personal protective equipment (PPE) and tactics, techniques and procedures (TTPs). Thus the gas attacks became less effective as time went on (3, 10). Because of its relatively high boiling point of 7.4°C, phosgene could not be used alone in cylinders and was usually combined with chlorine in a ratio of 1:3 or 1:1. It could be used as a sole agent in large projected shells, sometimes adsorbed into pumice.

Figure 1. A typical cylinder release gas attack. It was possible to use 3000 cylinders across a front of 3000 yards (10). https://commons.wikimedia.org/wiki/File:Poison_gas_attack.jpg

The Germans had an established industrial use for phosgene in the manufacture of dyes (3). Chlorine had established peacetime uses but carbon monoxide had not. It became necessary for large quantities of carbon monoxide to be produced in order to manufacture phosgene. The Germans and Americans favoured reacting carbon dioxide with carbon; usually charcoal but the French and British used incomplete combustion of coke. All sides combined chlorine and carbon monoxide passing over a carbon catalyst and then dried before storage (3).

Accurate meteorology was vital for the deployment of phosgene. Aside from the obvious wind direction and chance of change, the presence of water in the atmosphere would greatly diminish the effectiveness of phosgene. Phosgene reacts with water to produce carbon dioxide and hydrochloric acid:

$$\mathrm{COCl_2 + H_2O \longrightarrow CO_2 + 2HCl}$$

This reaction was exploited in early PPE by keeping it moist, in addition to the countermeasures discussed above. The last gas cloud attack occurred on 8 August 1916 and attention was focused on firing shells, including ones containing phosgene (10). In April 1917 the British introduced the projector which could fire a drum of pressurised liquid into enemy territory where it would rupture and leak large quantities of vapour (10).

Vedder describes the symptoms of phosgene poisoning as quite different to other agents. Even at high concentrations the inhalation does not cause irritation of the upper airways. Immediately following exposure the victim is largely asymptomatic. Some soldiers reported a change in the taste of cigarettes to an unpleasant flavour, often reminiscent of rotten eggs (11, 12). Later, and often after exertion victims develop dyspnoea which is often followed by cyanosis and death (13). Many authors subdivide cases of phosgene induced pulmonary oedema into two groups (13-15):

Venous engorgement with cyanosis. Congested plum to blue face with tachypnoea and increased respiratory excursion, these patients may be coughing up large volumes of pulmonary oedema. Heart rate is typically 100 beats per minute.

Grey pallor. These patients are collapsed with ashen, grey lips. Their breathing is rapid and shallow and pulse may be up to 140 beats per minute. Cough is often absent.

Most phosgene casualties are in group 2 and those in group 1 can progress to group 2. Severe cases in either group exhibit extreme anxiety, reduced level of consciousness or delirium (14). Survivors often have no recollection of their illness, even those who held normal conversations at the Casualty Clearing Station (15).

The physiologist John Scott Haldane witnessed first hand the effects of phosgene and delivered a lecture on the subject at the Royal Army Medical College 8 October 1919 (15). He described multiple stupefied casualties in respiratory distress, with deeply cyanosed plum-coloured lips and distended neck veins. One of the casualties died whilst he was visiting a clearing station and a post mortem was immediately carried out by a Dr McNee. “The lungs were voluminous and much congested. Albuminous liquid could be squeezed from them in abundance. The bronchi and alveoli were inflamed, and a great deal of emphysema was present.” He concluded that the cyanosis was due to anoxaemia and the distended neck veins secondary to an increase in pulmonary vascular resistance and distension of the right side of the heart.

He noticed the characteristic dose response to phosgene, whereby a large inhalation causes immediate severe signs and symptoms, often resulting in death. At lower doses; exposure to a low concentration for a prolonged period or a relatively high concentration for a short duration, the signs and symptoms are delayed by hours and often precipitated by exertion. He goes on to describe how the administration air with supplemental oxygen titrated to effect, can reverse the cyanosis. It did not, however reverse the tachypnoea which he surmised was driven by arterial carbon dioxide content. Oxygen was recommended in a dose of 2 $lmin^{-1}$, up to 5 $lmin^{-1}$ in severe cases (16). Both Haldane and Galwey recognised the toxicity of oxygen in higher concentrations and Haldane's Oxygen Apparatus could deliver oxygen with entrained air up to 10$lmin^{-1}$ (17) although the logistic burden must have been considerable. Venesection was another treatment that proved effective in cases where venous congestion was prominent (16). Galwey states that the intervention is to reduce right heart strain and is achieved by the removal of 650ml blood over 20min. This removal reduces capillary pressure, causing uptake of interstitial fluid from the peripheries, possibly including the lungs and bringing the haematocrit closer to normal. He thought that the rise in haematocrit phosgene causes could be ameliorated by the intravenous injection of saline.

Vedder recognised that it was difficult to determine the pathology of phosgene in the human because, when weaponised it was commonly mixed with chlorine. However he reported that lung weights were increased, with the right lung typically weighing 1000g and the left 875g and often cover the heart when the chest is opened (13). The lungs were grossly abnormal with dark discolouration and exuding frothy serous fluid when sectioned, and although full of this frothy serous fluid, the upper airways exhibited little or no inflammatory change. Fluid from brown discoloured lung was acidic when tested with litmus paper and the right heart was generally dilated and often with petechial haemorrhages beneath the endocardium. Many of the organs show venous congestion. On microscopic examination of the alveoli he noticed that they are full of fluid with desquamated alveolar epithelium, white

cells and red cells. Fibrin was observed crossing the alveoli and the capillary beds. He concluded that this was the basis for obstruction of the pulmonary circulation and right heart strain and therefore, a mechanism for generalised venous engorgement. "*Thus the important acute changes caused by phosgene poisoning are practically limited to the lungs.*" (13)

Further inflammatory changes are noted with disease progression, with patches of bronchopneumonia. The majority of cases recover if they survive the first 48h (13).

By the end of World War 1 there were 180,983 British 'gas casualties' including 6,062 deaths. These official figures are a gross underestimate; records begin in 1916 and they do not include the missing or captured (18). These are victims of chlorine, phosgene and mustard. Of the three, total phosgene production was the smallest, yet it was responsible for more devastation, completely out of proportion to the other agents.

1.1.3 Early Evaluations of the Pathophysiology of Phosgene

It has long been recognised that phosgene acts primarily at the alveolar level (19). Hill showed in a series of elegant experiments that the poisoned lung, homogenised, extracted with Ringer's solution and injected into a healthy animal caused no adverse effect. Likewise the oedema fluid of poisoned animals injected into healthy subjects and plasma through which phosgene has been bubbled produced no adverse effects. He went a stage further and showed that by isolating a cat lung *in vivo* using a bronchial blocker, and then allowing the anaesthetised animal to breathe phosgene, only the exposed lung suffered the effects. Cyanosis and cardiovascular variables returned to baseline once the animal breathed air on removal of the blocker (19). Thus Hill demonstrated that phosgene acts only locally with no *direct* systemic effects. It was well known that the loss of oedema fluid was significant to cause a rise in haematocrit and Hill suggested that this could be corrected by the intravenous infusion of gum saline. Gum saline is a solution of gum acacia of 6- 7% in 0.9% saline and acts as a colloid (20). This was rejected

as a therapy on the grounds that it may worsen pulmonary oedema. Hill was unable to explain the selective alveolar damage and wrongly assumed that this damage was caused by hydrochloric acid formed by the reaction of phosgene with water.

Barcroft summarised his work on the pathophysiology of phosgene poisoning in a lecture given at the Royal Army Medical College in 1919. His work was based upon animal experiments conducted at the Royal Engineers' Experimental Ground at Porton (21). His chamber experiments were the first large scale exposures to known concentrations of phosgene vapour. Histological examination showed that increased lung damage occurs with increasing doses, as expected. Macroscopic changes were not uniform and the microscopic changes included damage to alveolar epithelium causing oedema formation and capillary changes including thrombosis. Although hypoxic pulmonary vasoconstriction was first recognised in 1894 (22) it was not eponymised as the Euler-Liljestrand mechanism until 1946 (23, 24). Barcroft observed normoxic goats with lungs four times the normal weight. Rather than using hypoxic pulmonary vasoconstriction as the explanation Barcroft first assumed that that oedematous alveoli were not perfused as a result of capillary thrombi (21). After developing an in vitro model to test the effects of raised pulmonary vascular resistance he went on to test his hypothesis in vivo. By introducing a needle into the right ventricle of a goat, it was possible to measure and trace the pressure transmitted via the needle.

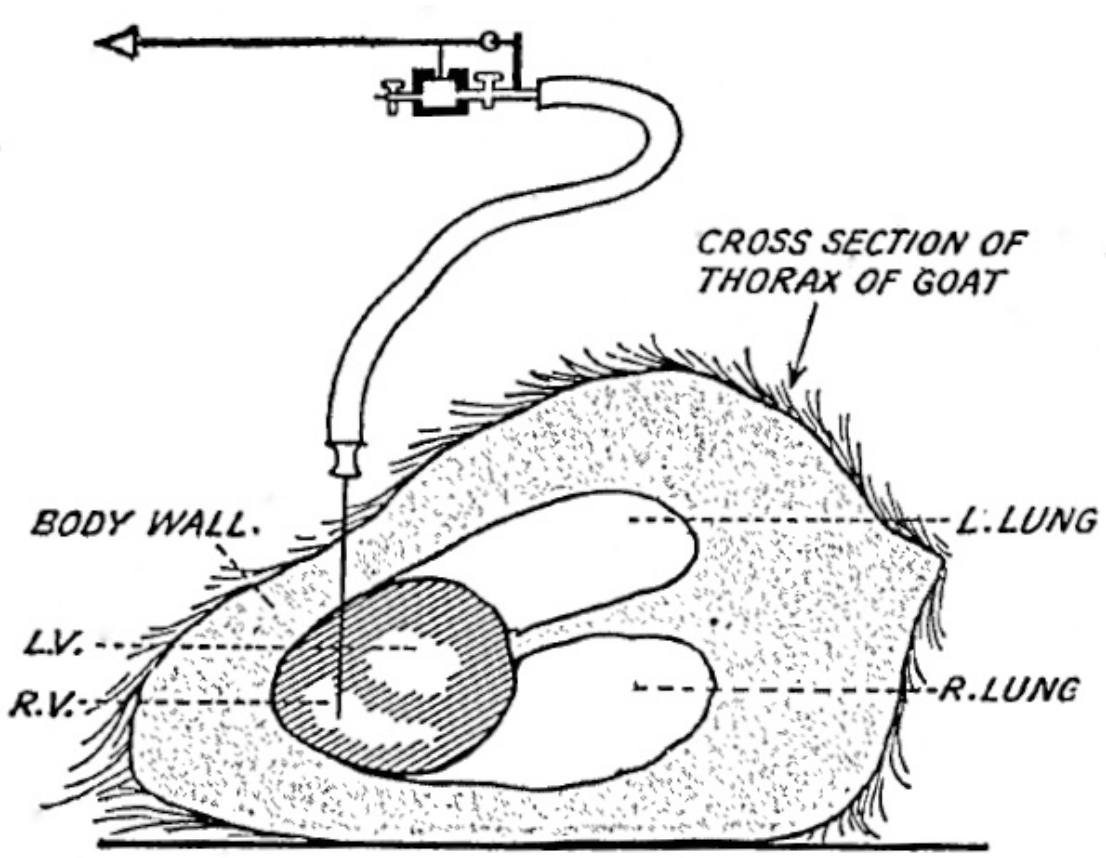

Figure 2. Schematic produced by Barcroft illustrating the method of recording intracardiac pressure by direct puncture developed in conjunction with Boycott (21).

These goats were then exposed to phosgene and he observed the typical findings illustrated in Figure 3.

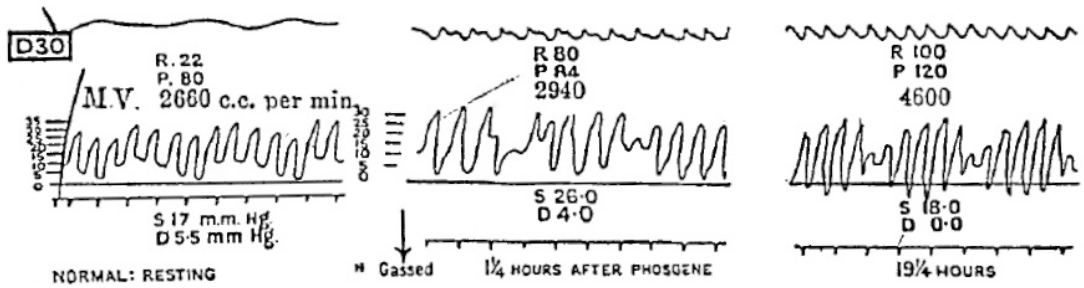

Figure 3. Tracings of respiratory rhythm above right ventricular pressure pre and post exposure to phosgene (1.25 and 19.25h post exposure). R= respiratory rate | P= pulse rate | MV= minute volume. The y-axis is right ventricular pressure measured in cmH_2O. S= mean systolic pressure | D= mean diastolic pressure expressed in mmHg. The bottom scale is time, measured in seconds.

Soon after the animals were exposed to phosgene, respiratory rate and right ventricular pressure increase, in line with the anticipated increase in pulmonary vascular resistance. Later in the experiments the right ventricular systolic pressure normalises, however the diastolic pressures are much lower, reflecting hypovolaemia. Given that the pulse pressures remain similar, post exposure, the increased pulmonary vascular resistance is likely to persist. Vedder noted that a man's lungs could contain up to 2kg of oedema (13) yet Barcroft paid little attention to the effects of hypovolaemia in his analysis and made no measurement of hypoxaemia to correlate with minute volume, linking minute volume with right ventricular systolic pressure. He went on to show that in experiments of oil or starch embolism, there is a transient rise in right ventricular pressure but minute volume would either increase or decrease. To further evaluate the relationship between minute volume and right ventricular pressure he showed that right ventricular pressure falls during an increase in minute volume in the exercising goat.

Whilst he recognised that there is a vasomotor component to phosgene lung injury Barcroft summarised that the pulmonary capillaries are compressed by oedema fluid in the alveoli. On exertion these capillaries dilate under vasomotor influences, creating a large physiological shunt that worsens hypoxaemia. He demonstrated this in a series of experiments using oximetry of blood in phosgene exposed goats at rest and during exercise, unfortunately not linking the data to right ventricular pressure. The importance of avoiding exertion in suspected acute phosgene cases was emphasised in the 1939 Medical Manual of Chemical Warfare (25).

1.1.4 Summary

The classical descriptions of early and late effects were made during World War 1. Their importance to the management of a large number of casualties regained importance in the build up to the Second World War (25), and persists to this day. Barcroft's correct observation of increased pulmonary vascular resistance was not underpinned by the hypoxic pulmonary vasoconstriction mechanism to minimise shunt. Haldane had correctly deduced that areas of consolidated lung in pneumonia are not greatly perfused as these patients are not always cyanosed (17). However the sudden collapse of patients yet to experience severe intoxication is reasonably explained by Barcroft's hypothesis that it is pulmonary vasodilation known to occur on exertion as the causative factor.

Many have classified the casualties into groups 1 (venous engorgement with cyanosis) and 2 (grey pallor) and this warrants further discussion. It was known that hypoxia and increased arterial carbon dioxide content will increase respiratory rate and there was considerable debate over the relative contributions made by hypoxia and carbon dioxide, including the thought that elevated carbon dioxide contributed to the venous engorgement by contributing to vasodilatation. Arterial carbon dioxide was not measured and elevated carbon dioxide seems unlikely when considering the alveolar air equation:

$$P_AO_2 = P_IO_2 - \frac{P_ACO_2}{R}$$

Alveolar oxygen is calculated by the partial pressure of inspired oxygen, note that it is less than atmospheric after humidification, with the partial pressure of alveolar carbon dioxide subtracted and divided by the Respiratory Quotient (R). Thus, alveolar oxygen can be increased by reducing the alveolar carbon dioxide, most simply by hyperventilation. In fact the explanation may be even simpler. Group 1 casualties are yet to experience hypovolaemia secondary to fluid losses from extreme pulmonary oedema; their veins are engorged by increased pulmonary vascular resistance and they appear cyanosed from hypoxaemia. Group 2 casualties have significant fluid losses as evidenced by their rapid heart rates and pallor from vasoconstriction and appear grey because they are cyanosed. It has been stated that patient's disease may progress from group 1 to 2 and this is likely due to circulatory fluid loss. For all his astonishing work, Barcroft neglected to note the reduced and often transiently negative diastolic pressures measured in severely intoxicated goats.

Upper airway sparing and Hill's work elegantly demonstrated that phosgene is decomposed by lungs, limiting its effect to that organ system. The cardiac effects follow from that.

In this section we have seen the maleficent adoption of chemical warfare by German forces in World War 1 and the Allied response. I have not dwelt in detail on the personal protective equipment (PPE) and other protective techniques employed, however the Allies quickly developed effective PPE. The Allied combination of superior PPE and the rapid development of their own chemical arsenal and delivery methods meant that towards the end, the Germans had been hoisted by their own petard by launching a chemical war. Many of the senior commanders in World War 2 had, at least indirect experience of chemical warfare, its hideous nature discouraged them and politicians from bringing it to bear a second time.

1.2 Pulmonary Surfactant

1.2.1 Introduction

There are approximately 5 x10^8 alveoli in healthy adult lungs, each one 300μm in diameter (26). This structure is inherently unstable and would quickly collapse without an extraordinary lining to diminish surface tension. The science of lung surface forces began with Von Neergaard's work published in 1929 (27). He noticed that the compliance of isolated pig and dog lung preparations change dramatically when inflated with liquid compared with air, shown in Figure 4. By displacing air, saline abolishes the forces of surface tension without affecting the elasticity of the tissues. Neergaard concluded that surface tension plays a greater role in lung recoil than tissue elasticity. When lungs are allowed to deflate and with the axes swapped to better illustrate lung compliance (change in volume due to applied pressure) they exhibit hysteresis, Figure 5.

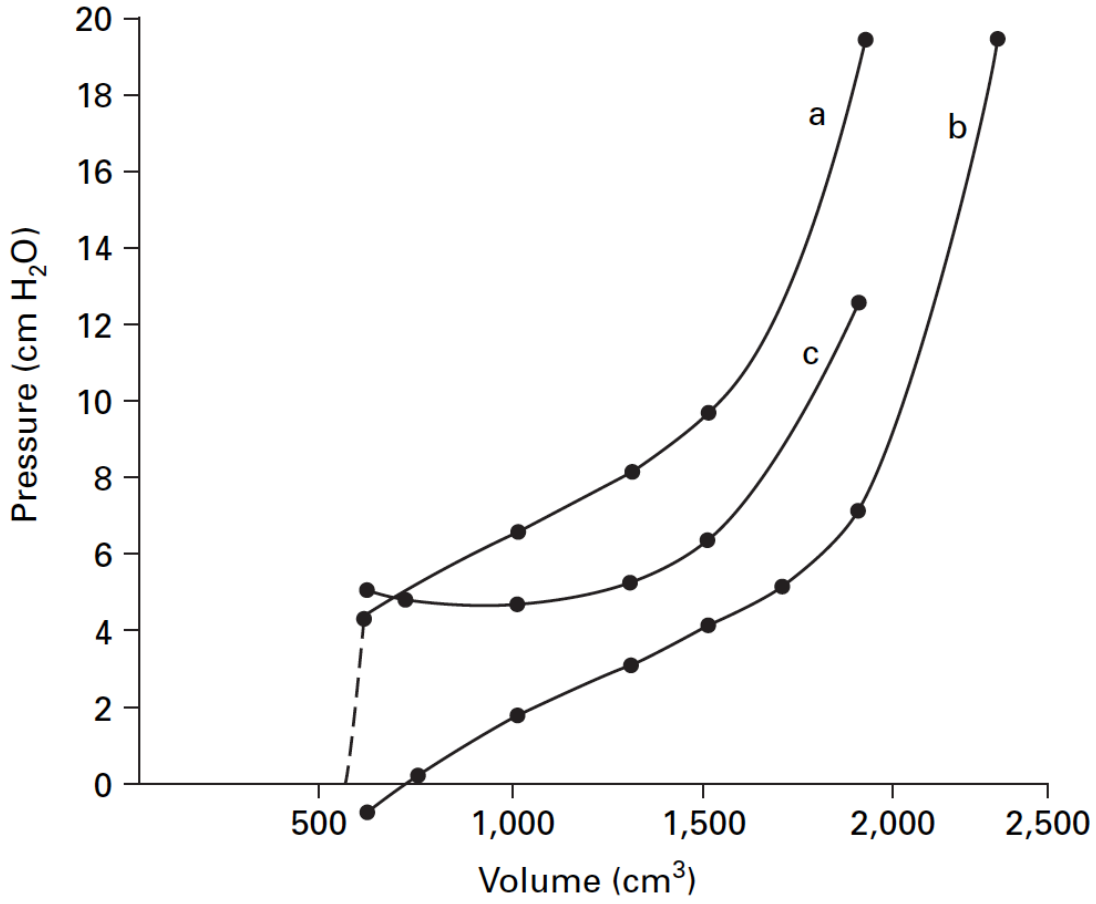

Figure 4. Von Neergaard's data can be used to assess lung compliance ($\Delta V/\Delta P$) in isolated pig lung. a= total lung recoil with air filling. b= tissue elasticity after eliminating surface tension by liquid filling. c= retractile force due to surface tension. Translated by Obladen (28).

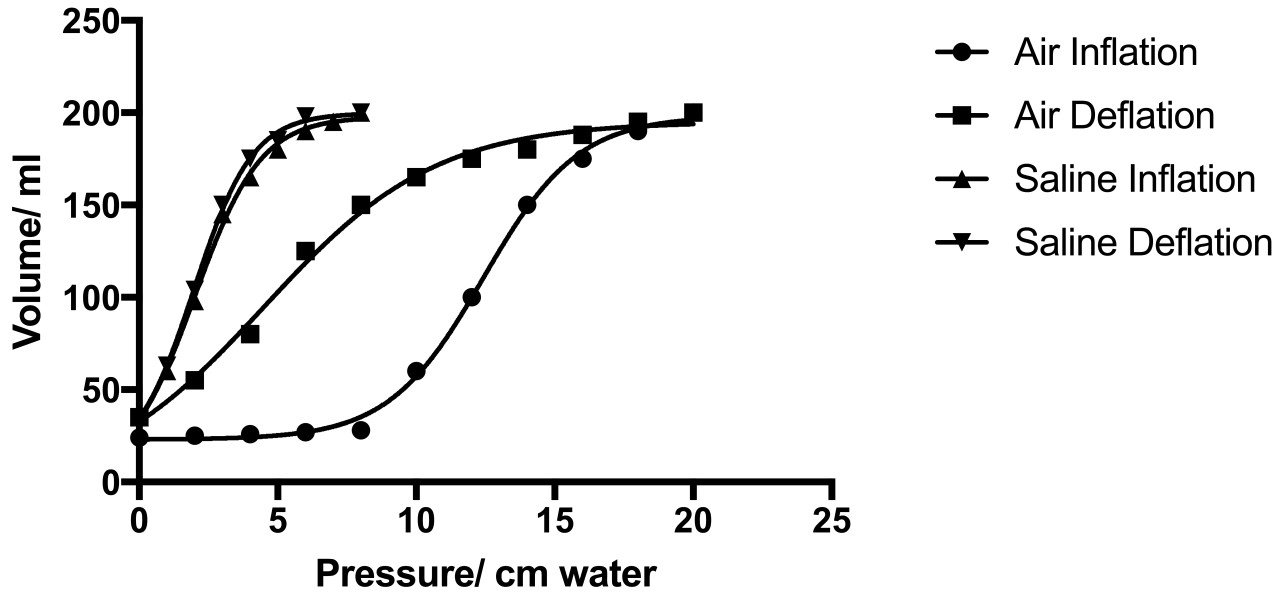

Figure 5. Compliance ($\Delta V/\Delta P$) curves of isolated cat lung inflated with air or saline. When the lungs are inflated with saline, the pressure applied results in greater expansion than with air, i.e. increased compliance. After West (26).

The first description of the physical properties of the alveolar lining layer was made by Pattle in 1955 (29). Pattle worked at the Chemical Defence Experimental Establishment at Porton Down, on countermeasures to chemical weapons. He became interested in lung oedema fluid and the properties of the resulting foam. Foam was obtained from lung lavage or by underwater expression from cut lung surface. It was found to be peculiarly stable; resistant to conventional anti-foaming agents and forming bubbles with a lining layer of about 5nm. By comparing lung bubbles with others including those formed by plasma he noticed that lung bubbles remained entirely stable for an hour when observed in air saturated water. Whilst the lung bubbles remained the same diameter, others disappeared within a few minutes. In order for this to happen, the surface tension must be zero. Were the lungs lined with an ordinary fluid with surface tension, he hypothesised, then a transudate would be drawn into the alveoli from the surrounding capillaries (29). In deaerated water the bubbles collapsed leaving a ghost, confirming that the lining film is, as expected, gas permeable (30). Thus at

large lung volumes, surface tension contributes to elastic recoil of the lungs and at low lung volumes reduced surface tension prevents collapse of alveoli; atelectasis.

Pattle and Von Neergaard both considered lung function in neonates but it was the work of Avery and Mead that showed how the surface tension of neonatal lung changes with birth weight and disease. Using a film balance they examined lung samples from neonates that died from Hyaline Membrane Disease (HMD now known as Neonatal Respiratory Distress Syndrome) compared to those from other causes. Neonatal Respiratory Distress Syndrome (NRDS) is characterised by collapsed alveoli, now known to be caused by a deficiency in surfactant. The lowest surface tensions were seen in neonates without HMD that were above about 1200g birth weight. Below 1200g and in all cases of HMD the surface tensions were much higher, from 20- 35 dynes cm^{-1}(31). They concluded that the disease could be explained by the lack of a surface active material and that this is formed as the fetus passes the 1200g point during gestation. They had marked the point previously alluded to by the German obstetrician Jörg in 1835, ‘die foetuslunge imgebornen kinde’; fetal lung in the baby who is born (28).

Without surfactant, alveoli would collapse which would cause a large increase in work of breathing in order to open them with every breath. Whilst open the, negative pressure would cause transudation of fluid from lung capillaries into the alveoli (32). Both of these effects are seen in NRDS.

1.2.2 Surfactant Composition

Pattle noticed that the films produced could be altered by trypsin and pancreatin (29) thereby showing that they are partly composed of proteinaceous material. Further analysis showed the presence of lipoprotein rich in phospholipid, cholesterol, triglycerides and fatty acids (30).

Surfactant is, in fact composed of about 90% lipid and 10% protein. The surfactant proteins are SP-A, SP-B, SP-C and SP-D. They have a range of

functions including stabilisation of the alveolar surface layer and in innate immunity (33).

Surfactant Protein A and D. SP-A and SP-D are the hydrophilic surfactant proteins. SP-A was the first to be described, much was hypothesised regarding its function as it was observed to form lattice frameworks, however SP-A -/- knockout mice showed no sign of respiratory disease. It is likely that SP-A performs an immune role along with SP-D; they both bind a wide range of pathogens, both being in the collectin family of lectins (33). SP-D -/- knockout mice demonstrate disordered surfactant homeostasis and alveolar macrophages preferentially internalise PG16:0/16:0 over PC16:0/16:0 when SP-A is absent, suggesting an additional regulatory role (33, 34).

Surfactant Protein B and C. SP-B and SP-C are the hydrophobic surfactant proteins. Mutations of SP-B are associated with some severe lung disorders including NRDS and it is probably key to modulating the surface tension at the alveolar surface layer (33). Like SP-A and SP-D, it too has an immunological function, in that it contains a sequence that binds lipopolysaccharides (LPS) present in the outer membrane of Gram- negative bacteria (35). SP-C is extremely hydrophobic and only 35 amino acids in length. It is highly conserved across species, reflecting mutant forms of SP-Cs' role in the development of a variety of interstitial lung diseases (36). The role of SP-C is in the stabilisation of the alveolar surface layer. Unlike the other Surfactant Proteins, SP-C is not found in any extrapulmonary tissues (33).

Glycerophospholipids make up the bulk of the lipid content of surfactant. They are made up of a glycerol backbone; phosphatidic acid (PA) with two fatty acid chains. The glycerol backbone has carbons numbered *sn-1* to *sn-3*. Fatty acids are present at *sn-1* and *sn-2* while the head group is at *sn-3*. Thus they are amphipathic with a polar 'head' and hydrophobic 'tails'. The 'head' groups are formed from PA and an alcohol (37):

Serine	+	PA	→	phosphatidylserine (PS)
Ethanolamine	+	PA	→	phosphatidylethanolamine (PE)
Choline	+	PA	→	phosphatidylcholine (PC)
Inositol	+	PA	→	phosphatidylinositol (PI)
Glycerol	+	PA	→	phosphatidylglycerol (PG)

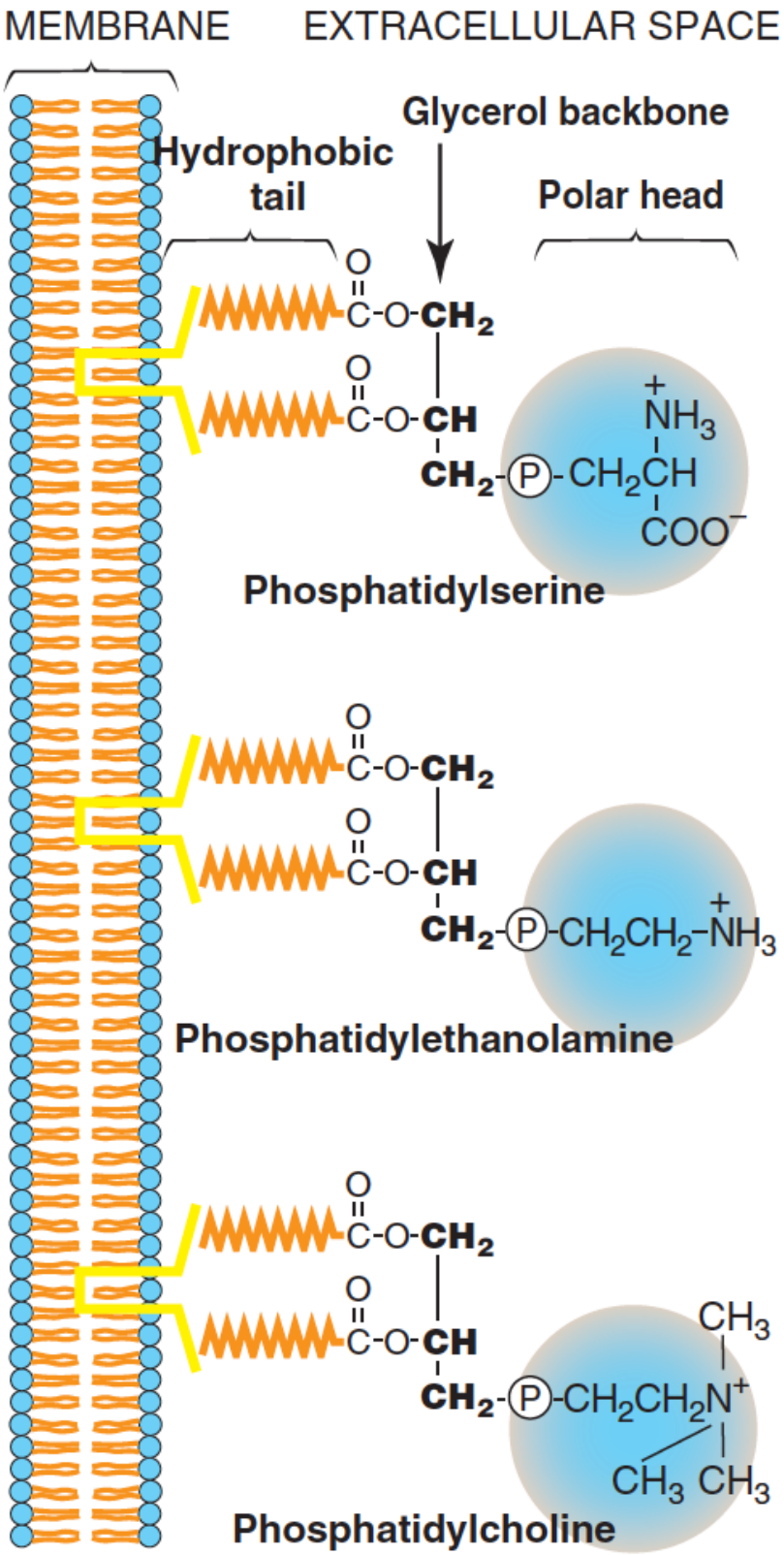
MEMBRANE
EXTRACELLULAR SPACE
Glycerol backbone
Hydrophobic tail
Polar head
Phosphatidylserine
Phosphatidylethanolamine
Phosphatidylcholine

Figure 6. Examples of some glycerophospholipids from Harvey and Ferrier (37). Note that Phosphatidylserine is present on the cell surface only in conditions of cell activation such as transformation or death (38).

A common means of describing phospholipids was first proposed in 2005 (39) and updated in 2009 (40). The 'head' groups are described as above and the side chains are numbered, for example PC 16:0/16:0 describes dipalmitoyl phosphatidylcholine (DPPC). Where double bonds occur they are *cis* (Z), numbered after the colon and bracketed numbers indicate where the double bonds are. Carbons are numbered from the glycerol backbone to the end methyl group, also known as omega. An example of PC with palmitoyl (16C) and stearyl (18C) side chains, with a single *cis* double bond in the stearyl chain at position 9 is PC 16:0/18:1(9Z) (39, 40).

Postle *et al*. examined the relative abundance of PC, PG and PI in surfactant across a small number of mammalian species but excluding pigs. The relative proportions of PC, PG and PI are shown in Figure 7. PC makes up approximately 80% of human surfactant glycerophosphates with PG accounting for approximately 17% and PI 3% (41). Previous work by Body in 1971 in analysing the phospholipid composition of pig lung surfactant (42) has been added to Postle's data and displayed in Figure 7. PC is overrepresented in porcine surfactant and PG is underrepresented, however the fatty acid compositions of PG and PC are similar, Figure 8 and Figure 9.

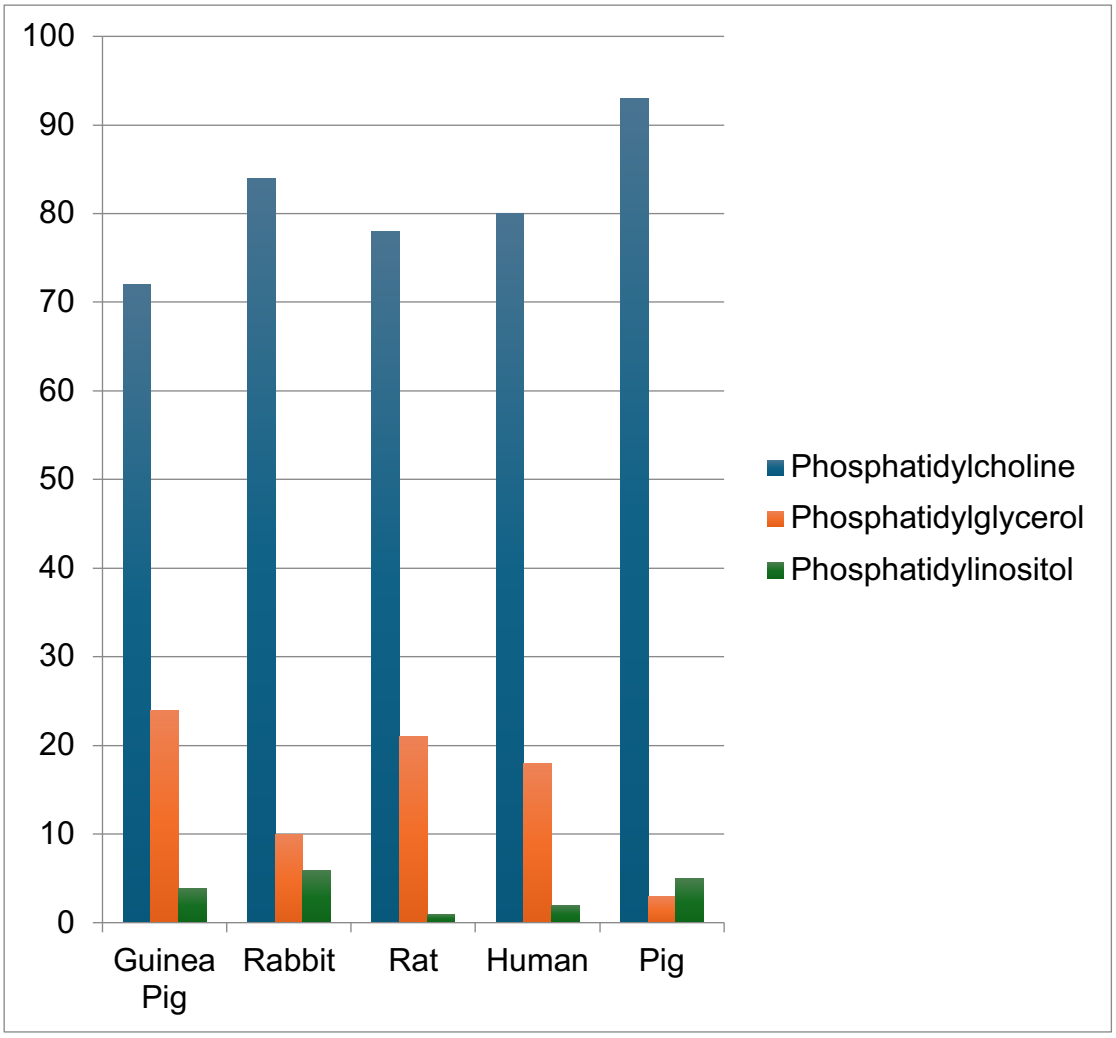

Figure 7. The relative proportions of PC, PG and PI in selected mammalian lung surfactant expressed as a percentage (41, 42).

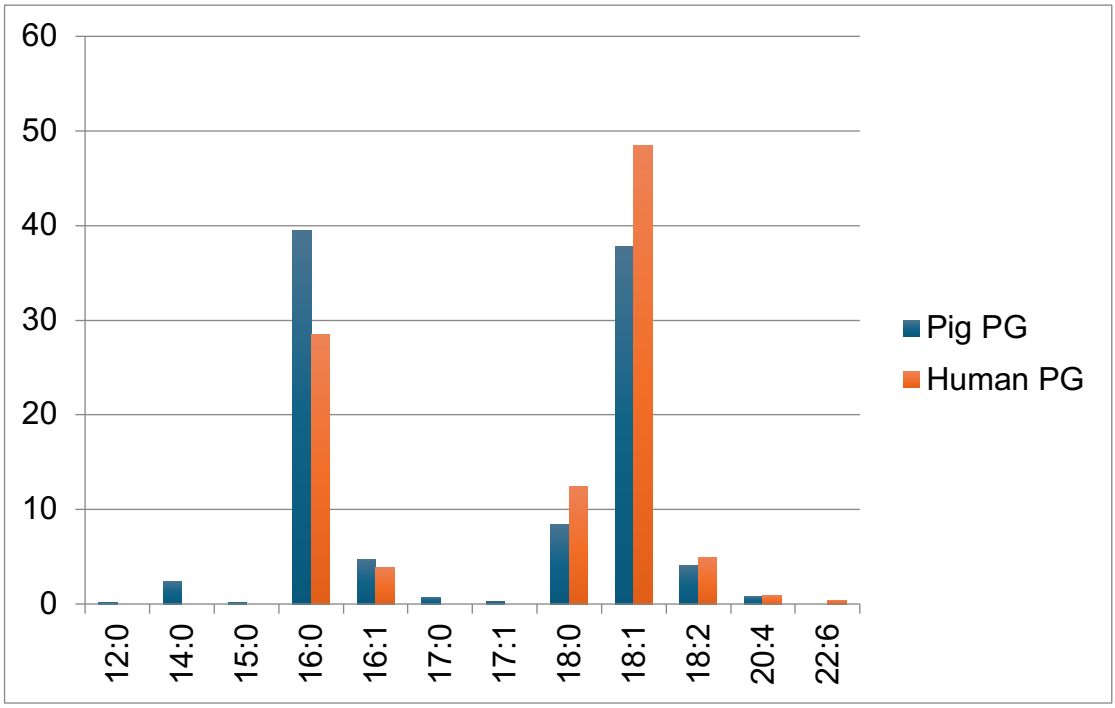

Figure 8. The relative proportions of porcine and human fatty acids of phosphatidylglycerol in lung surfactant.

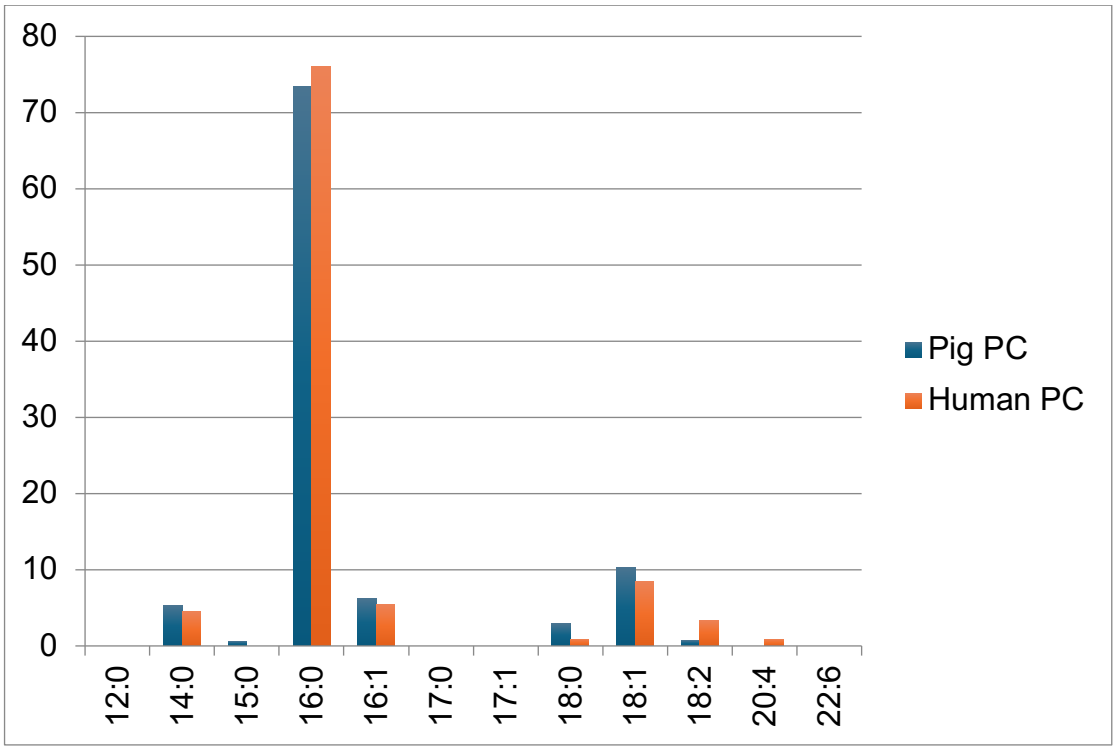

Figure 9. The relative proportions of porcine and human fatty acids of phosphatidylcholine in lung surfactant.

1.2.3 Glycerophospholipid Synthesis

Lung alveoli are mainly composed of type I pneumocytes whose main function is to act as a barrier between lung capillaries and air, however they are able to generate an inflammatory response. Type II pneumocytes have a role in maintaining the hypophase and repairing damaged alveoli; they are able to divide and differentiate to type I cells and have several immune functions (43, 44).

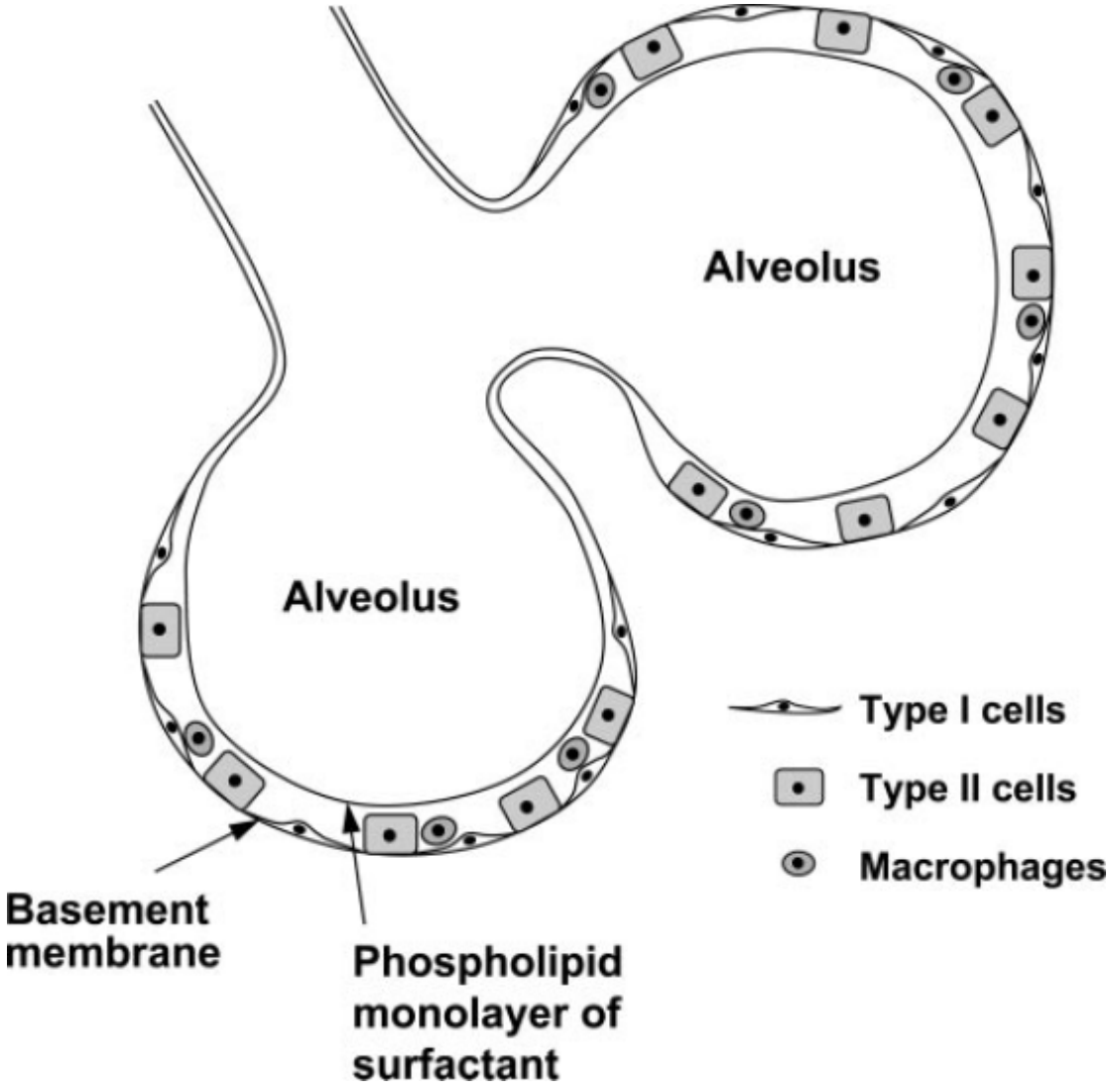

Figure 10. Arrangement of alveolar cells, the liquid lining the alveolus is termed the hypophase. Macrophages serve to phagocytose foreign and other unwanted particles (43).

Type II cells are the only cells to synthesise intact lung surfactant. At least three pathways for PC synthesis exist (45), the Kennedy pathway, Lands pathway and by methylation of PE by phosphatidylethanolamine methyl transferase (PEMT) in the liver. PEMT is localised to the endoplasmic reticulum in the liver only, and uses S-adenosyl methionine as a methyl donor (46). This pathway provides a means to produce choline in times of dietary deficiency (47).

Phosphatidic acid synthesis occurs on the outer membrane of mitochondria and the endoplasmic reticulum. It is an important intermediary in the synthesis of PI and PG (48) as shown in Figure 11. PC is synthesised on the endoplasmic reticulum via the Kennedy pathway. The Kennedy pathway refers to the de novo synthesis of phosphatidylethanolamine and phosphatidylcholine (49) and was first described by Kennedy and Weiss in 1956 working on rat liver (50). The alcohols ethanolamine or choline are first phosphorylated and then combined with cytidine triphosphate (CTP). This results in cytidine diphosphate (CDP)- choline or ethanolamine and pyrophosphate. Diacylglycerol then combines with the CDP- alcohol to form PE or PC and cytidine monophosphate (CMP) (49). The pathway is shown in Figure 12.

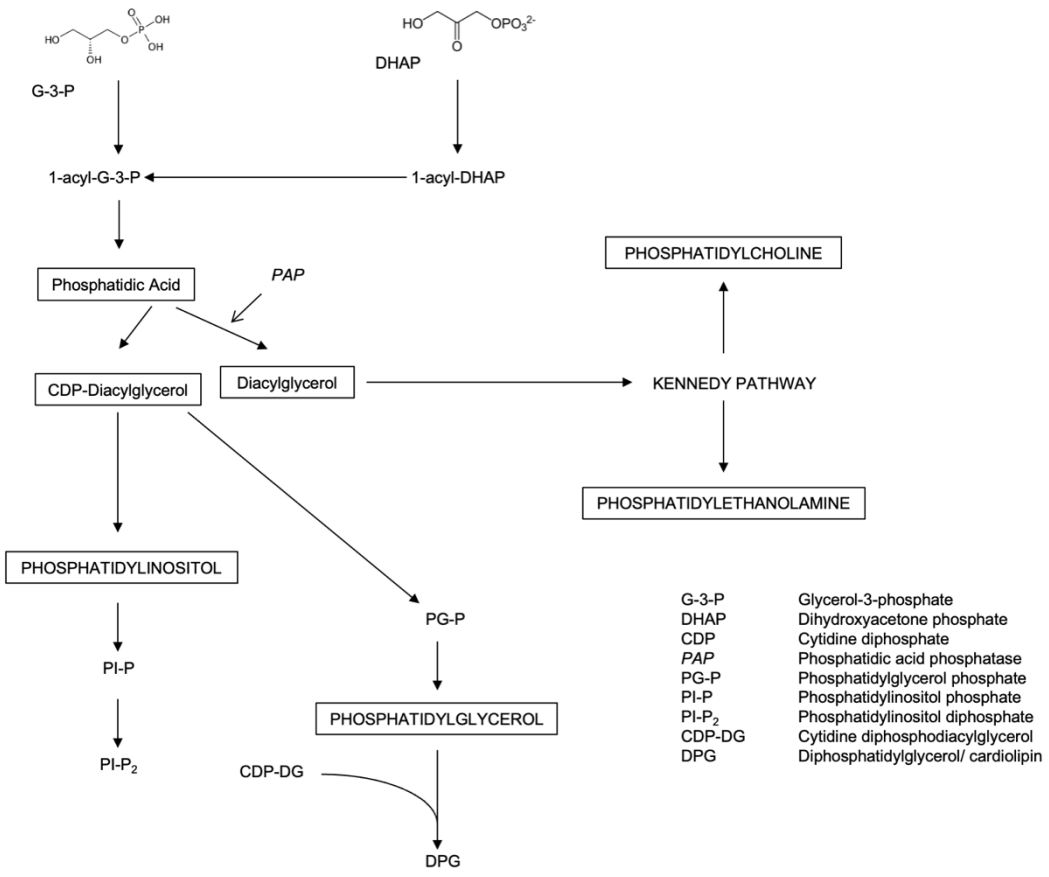

Figure 11. Glycerophospholipid synthesis (48). Phosphatidylcholine and phosphatidylethanolamine are both substrates for phosphatidylserine synthesis (not shown) (51).

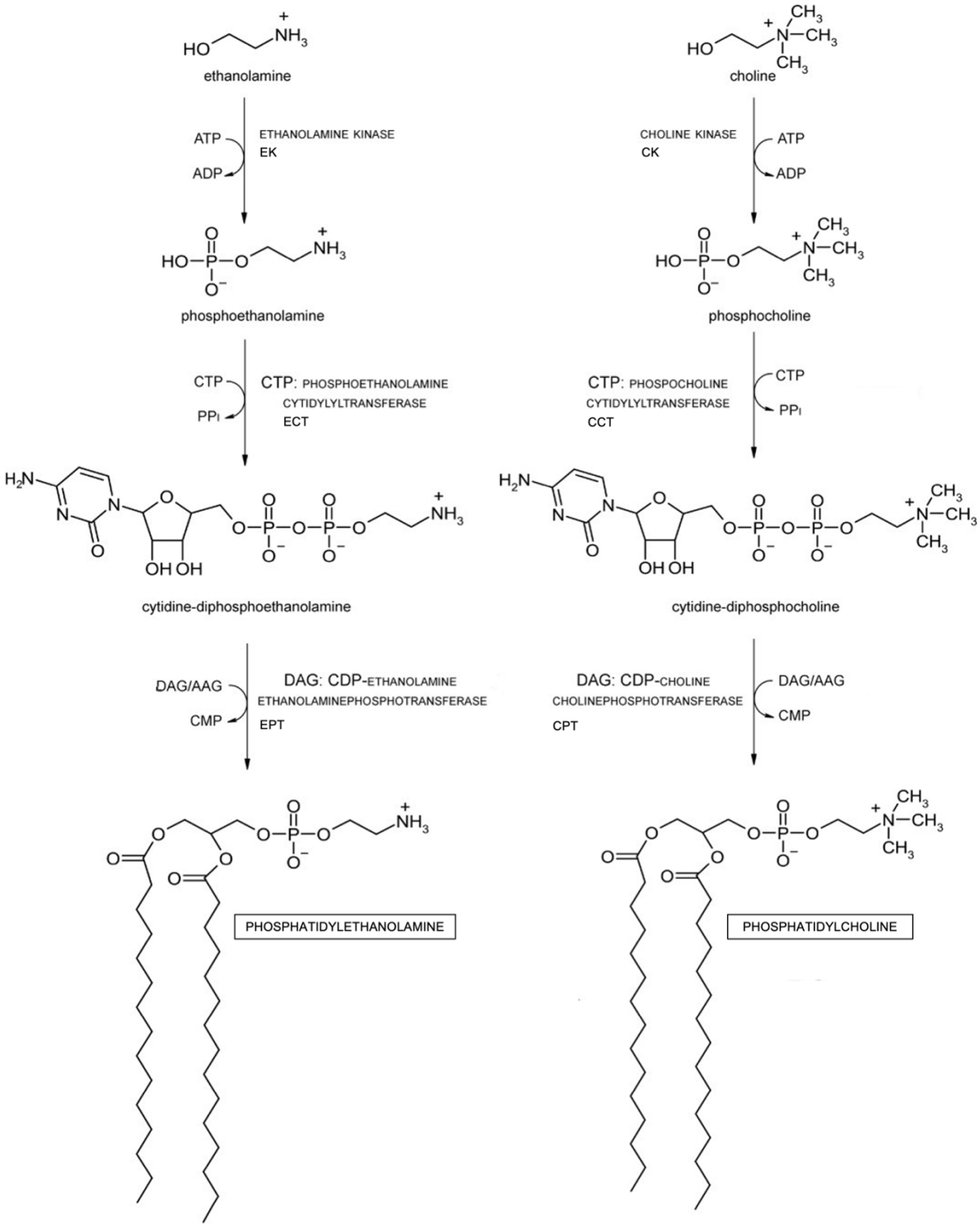

Figure 12. Formation of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) from ethanolamine and choline respectively in the Kennedy pathway.

In the Lands cycle, phosphatidylcholine (PC) is converted to lysophosphatidylcholine (LPC) and a fatty acid from the *sn-2* position, by phospholipase A_2s (PLA_2s). Fatty acids at the *sn-2* position are often polyunsaturated and can be converted to eicosanoids. LPC is either broken down further into lipid mediators or can be 'reacylated' by lysophosphatidylcholine acyltransferases (LPCATs) to reform PC. A generic outline of the Lands cycle is shown in Figure 13.

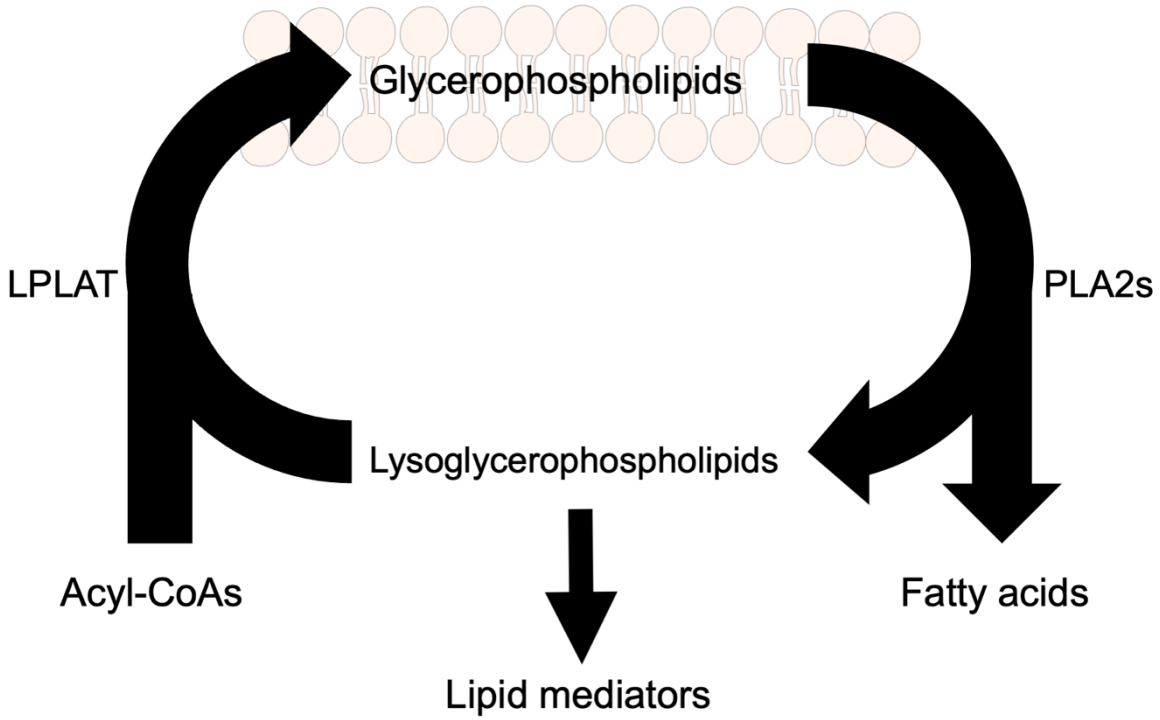

Figure 13. Lands cycle can exchange glycerophospholipid fatty acids at sn-2. PLA2s = phospholipase A2s. LPLAT= lysophospholipid acyltransferases. After Hishikawa *et al*. (52).

Lung surfactant is synthesised and secreted by type II alveolar pneumocytes. Using tritiated choline (choline-^{3}H) and electron microscopy Chevalier and Collet traced the incorporation of choline-^{3}H into phosphatidylcholine (PC)

and its secretion by type II pneumocytes (53). Their findings are outlined in Figure 14.

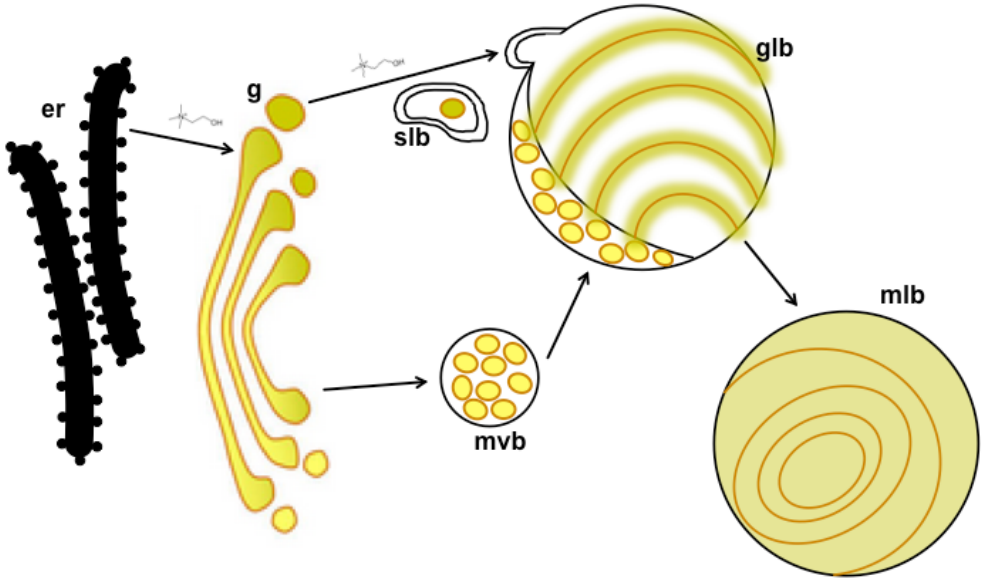

Figure 14. Choline-^{3}H is incorporated into the endoplasmic reticulum (er) whereupon it is moved to the Golgi apparatus (g) and possibly via the small lamellar bodies (slb), containing PC, to the growing lamellar bodies (glb). Fusion of multivesicular bodies (mvb), containing newly glycosylated proteins, with slb forms the glb. The glb then develops into the mature lamellar body (mlb), which is secreted into the alveolar cavity (53).

The pathway was further evaluated by Osanai *et al*. using Brefeldin A (BFA), a compound that leads to disassembly of the Golgi Apparatus (54, 55). It was first demonstrated that PC secretion is independent of a functioning Golgi apparatus, however it is still processed in lamellar bodies. Using a combination of immunolabelling and sucrose gradient centrifugation of organelles they showed that PC secretion was Golgi independent whilst Surfactant Protein- A (SP-A) and SP-B are Golgi dependent.

Adenosine triphosphate (ATP) Binding Cassette (ABC) transporters are a large family of membrane transport proteins of ancient origin, present in both prokaryotes and eukaryotes. They are typically formed from a series of transmembrane domains and two ATP- binding catalytic domains known as cassettes (56). Lipid translocation and secretion to form the surfactant monolayer is achieved using the ABC family and for surfactant lipids and the fat soluble SP-B and SP-C in particular, the ABCA subfamily (43). SP-B and SP-C are taken from the endoplasmic reticulum, through the Golgi apparatus and then formed with multivesicular bodies prior to secretion with surfactant lipid components. Indeed, SP-B is thought to be instrumental in the formation and evolution of these organelles (43). Absent or defective SP-B is lethal (57, 58). SP-C is a transmembrane protein involved with SP-B, a membrane bound protein, in the organisation of lipids in the alveolar subphase (43). There is a myriad of stimuli for surfactant secretion including the calcium mediated physical stimulus of alveolar stretch. Multiple other agonists effect secretion via three protein kinases, protein kinase C being the most potent (43).

1.2.4 Surfactant Clearance

A complex mix of biologically active molecules spread over a large area is highly susceptible to reaction with oxygen, inhaled toxins and pathogens. Surfactant can be divided into two subtypes by centrifugation, large and small aggregates. Large aggregates are far more surface active than small aggregates and contain more SP-B. Over time and in lung disease states, small aggregates increase in fraction prior to recycling by type II pneumocytes or degradation by alveolar macrophages, to a lesser degree (59-61). Plasma proteins can also accelerate this conversion (62), which has relevance in disease states involving lung oedema. Surfactant overall, is constantly reabsorbed and replaced in order to maintain the hypophase. It is cleared in four ways (34):

- Degradation by type II pneumocytes
- Recycling by type II pneumocytes
- Degradation by alveolar macrophages
- Mucociliary clearance

Endocytosis of surfactant lipids and surfactant proteins are closely linked, and this is engaged by type II pneumocytes and alveolar macrophages. Recycling of surfactant by type II pneumocytes is via clathrin coated vesicles and reinternalisation into lamellar bodies. This can be achieved rapidly with a turnover time for PC of 9- 10h (63). While type II pneumocytes will degrade and recycle surfactant lipids in roughly equal proportions, all that enters alveolar macrophages is degraded. Surfactant proteins are vital for trafficking lipids in type II pneumocytes, reinternalisation by type II pneumocytes is via a number of distinct pathways. These include a SP-A receptor mediated clathrin-dependent pathway and an actin-mediated pathway (64). Recycling is also modulated by SP-C and SP-D (62, 65). Granulocyte macrophage colony stimulating factor (GM-CSF) is implicated in alveolar macrophage catabolism of surfactant. (44, 66-68).

1.2.5 Glycerophospholipids in Lung Disease

Acute Respiratory Distress Syndrome (ARDS) was first described in 1967 as series of case reports of hypoxaemic lung disease, non- cardiogenic pulmonary oedema and reduced lung compliance in association with a variety of severe disease processes, not necessarily of pulmonary origin (69). Definitions or diagnostic criteria of this heterogenous condition have evolved over the years but can be summarised as (70):

- Occurring in temporal context of a significant illness, not necessarily of pulmonary origin
- Hypoxaemia of non-cardiac origin
- Bilateral opacities on lung imaging, not related to effusions or nodules

- P_aO_2/ F_IO_2 ratio defines severity
    - Mild ≤39.9kPa
    - Moderate ≤26.6kPa
    - Severe ≤13.3kPa

The stepwise increase of 13.3kPa is related to the same value in mmHg of 100. There is no pharmacological therapy for ARDS; treatment is directed at the cause but is otherwise supportive. Supportive ventilatory strategies were realised in the 1990s (71) and are the basis of ventilatory management, they are discussed further in Section 1.4.3.

Phosgene induced acute lung injury meets this definition and is therefore a form of ARDS. Established ARDS models provide essential context for interpreting phosgene induced acute lung injury, allowing mechanisms of alveolar- capillary disruption, surfactant disruption and inflammation to be identified and evaluated. While some aspects of the injury are shared, others are unique to chemical insult. Large animal models of chemical induced lung injury can thus be compared with ARDS models, to further evaluate the pathophysiology. Therapeutic strategies for chemical induced acute lung injury evaluated in this work, derive from ARDS management principles. Examination of the broader ARDS research base enables meaningful comparison, validation of experimental design and translational interpretation of findings.

Surfactant alterations in differing severe cases of respiratory failure have been described. A large study compared the phospholipid content of bronchoalveolar lavage (BAL) in 4 groups, ARDS without lung infection, severe pneumonia (subdivided into bronchial, alveolar and interstitial), ARDS and pneumonia and cardiogenic pulmonary oedema (72). BAL content is prone to recovery errors, making quantitative analysis difficult to report accurately. However, comparisons between classes of glycerophospholipids can be made. In this study, Günther *et al*. felt that overall concentrations of glycerophospholipids fell by a modest amount in the ARDS and pneumonic groups. BAL were taken at an unspecified time after diagnosis was made and

this could have been considerable, we cannot regard these findings as immediate in nature. Their more significant and accurate findings are shown in Figure 15 and Figure 16.

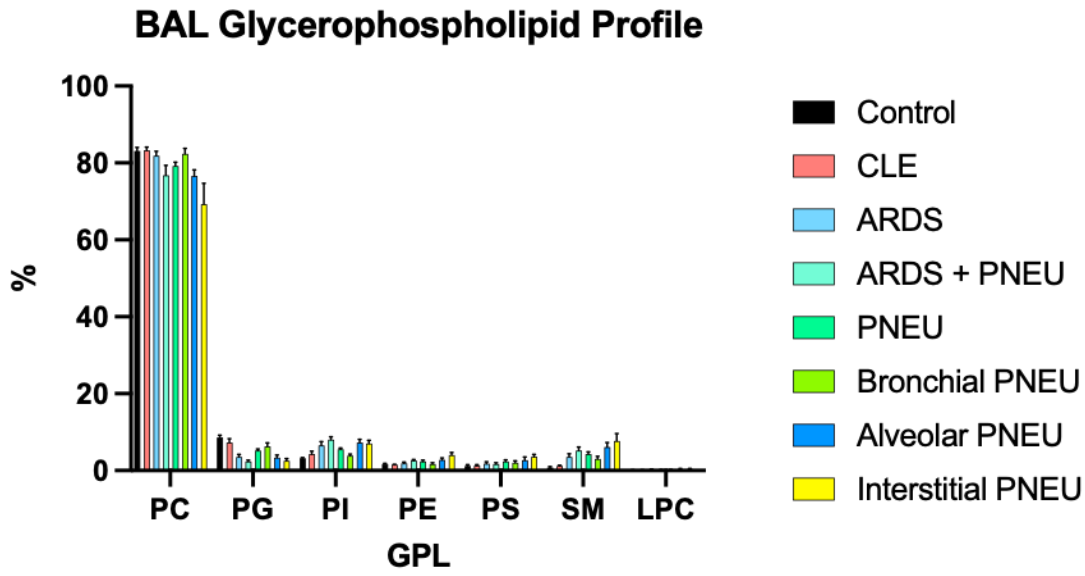

Figure 15. Fractional glycerophospholipid (GPL) profile of bronchoalveolar lavage (BAL) from Controls (N= 17), CLE (Cardiogenic Lung Oedema) (N= 10), ARDS (N= 15), ARDS + PNEU (ARDS + Pneumonia) (N= 28), PNEU (Pneumonia) (N= 64), Bronchial PNEU (N= 13), Alveolar Alveolar PNEU (N= 24) and Interstitial PNEU (N= 10). PC fraction is unchanged in CLE but decreases in all of the inflammatory conditions except Bronchial PNEU. Data are percentage ± SEM. PC= phosphatidylcholine. PG= phosphatidylglycerol. PI= phosphatidylinositol. PE= phosphatidylethanolamine. PS= phosphatidylserine. SM= sphingomyelin. LPC= lysophosphatidylcholine (72).

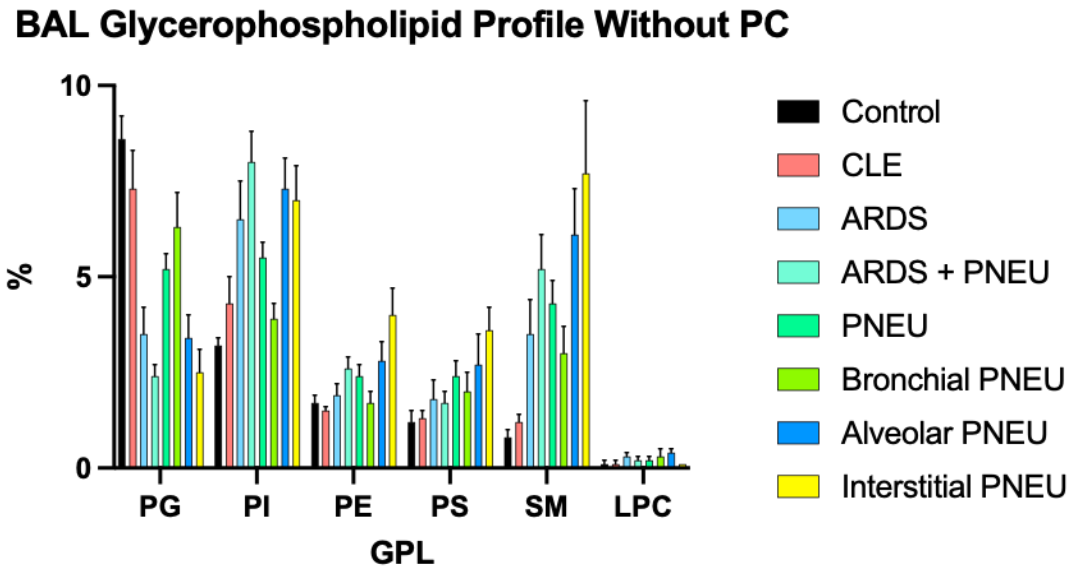

Figure 16. Fractional glycerophospholipid (GPL) profile of bronchoalveolar lavage (BAL) from Controls (N= 17), CLE (Cardiogenic Lung Oedema) (N= 10), ARDS (N= 15), ARDS + PNEU (ARDS + Pneumonia) (N= 28), PNEU (Pneumonia) (N= 64), Bronchial PNEU (N= 13), Alveolar PNEU (N= 24) and Interstitial PNEU (N= 10). PC fraction is excluded in order that changes in the other classes can more easily be interpreted. CLE fractions mirror Controls, and to some extent Bronchial PNEU. PG (as well as PC) is reduced in the inflammatory conditions shown but all other classes show a fractional increase. Data are percentage ± SEM. PC= phosphatidylcholine. PG= phosphatidylglycerol. PI= phosphatidylinositol. PE= phosphatidylethanolamine. PS= phosphatidylserine. SM= sphingomyelin. LPC= lysophosphatidylcholine (72).

The subgroups within the PNEU group were defined radiologically and it is noteworthy that bronchial infection/ inflammation experienced less change than the more distally involved conditions. In summary, PC and PG decreased in distal inflammatory airway disease and PI, PE, PS, SM and LPC increased. There is no explanation in this study for the reduction in PC and PG, the rise in LPC is not sufficient to explain conversion by the Lands

cycle. SM is largely intracellular (73, 74), and will increase in BAL as a result of lysis of dead cells.

In a small study of patients with pulmonary thromboembolism (PTE), BAL fluid was obtained and compared to 2 control groups. The control groups were patients without PTE or pneumonia but differed in their P_aO_2/ F_IO_2 ratios: Control A was >39.3kPa and Control B <39.3kPa (75). Markers of inflammation were massively increased in BAL fluid obtained from lung lobes affected by pulmonary embolism. Total protein was increased tenfold, platelet activating factor (PAF) was undetectable in controls but present at high concentrations in PTE and neutrophils were also raised. Total glycerophospholipid (GPL) concentration nearly doubles in the acute phase of PTE, returning to baseline by day 10. The changes in surfactant composition are displayed in Figure 17.

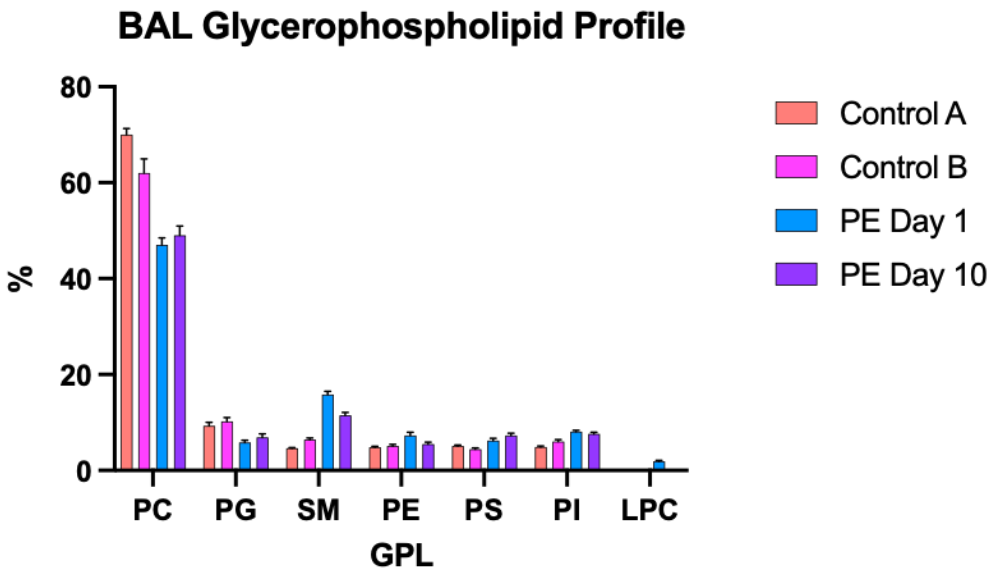

Figure 17. In pulmonary thromboembolism (PE Day 1 and PE Day 10), fractional composition of glycerophospholipids (GPL) changes are similar to other inflammatory lung conditions. PC and PG decrease, SM, PE, PS, PI and LPC increase. Control A have a higher P_aO_2/ F_IO_2 ratio and no group has microbiological evidence of pneumonia. Data are percentage ± SEM. PC= phosphatidylcholine. PG= phosphatidylglycerol. SM= sphingomyelin. PE= phosphatidylethanolamine. PS= phosphatidylserine. PI= phosphatidylinositol. LPC= lysophosphatidylcholine (75).

The marked increase in BAL protein content can be due to increased recovery or a real increase in pulmonary fluid protein content. This is feasible due to microemboli causing an increase in alveolar- capillary permeability as a result of lung tissue hypoperfusion with associated inflammatory response and/ or a rise in pulmonary capillary pressure. The changes in surfactant composition are likely due to contributions made by plasma as part of that increase in capillary permeability, contributions arising from the inflammatory process or a combination of both (76), as there is little to none sphingomyelinin in normal surfactant. It is likely that the total GPL concentration rises in the acute phase of pulmonary embolism despite the caveat of recovery errors. This can be due to combinations of increased

synthesis, increased secretion and decreased breakdown. The increase in secretion by type II alveolar cells can be triggered by several means, as diverse as hyperventilation (77, 78), catecholamines from associated sympathetic activation (43) or as part of the inflammatory response (79).

Alterations in species composition of phosphatidylcholine (PC) have been observed in patients with ARDS compared to controls (80). Enrolment of patients occurred up to 72h, then bronchoalveolar lavage (BAL) samples were taken from 10 patients with ARDS of mainly pneumonic aetiology and compared with 9 healthy non-smokers using electrospray ionisation mass spectrometry, a technique described in Section 2.2.3 Mass Spectrometry Overview. PC was very significantly decreased in ARDS affected lungs overall, mainly due to a reduction in PC16:0/16:0. Results are summarised in Table 1.

Increased	Decreased	Not significant
PC16:0/18:2	PC16:0/14:0	PC16:0/16:1
PC16:0/18:1	PC16:0/16:0	PC16:0/20:4
PC18:0/18:2		PC18:1/18:2
		PC18:0/18:1
		PC18:1/20:4
		PC18:0/20:4

Table 1. Differences in percentage phosphatidylcholine composition in the BAL of ARDS patients compared to controls at enrolment in the study. The proportion of saturated PCs is decreased in ARDS (80).

Overall, there was a reduction in saturated PC species which is in keeping with previous studies. However, this was the first study to look at individual PC species.

1.2.6 Summary

The peculiar changes in compliance of lungs noted by von Neergaard under different inflation conditions led to the search for a surface active film at the alveolar interface. It was as a direct result of research into pulmonary injury by chemical warfare agents that surfactant was first described by Pattle. Later analyses showed the film to be largely composed of lipids, in particular glycerophospholipids (GPL) of various species, the commonest being dipalmitoyl phosphatidylcholine (DPPC or PC 16:0/16:0). These molecules are amphipathic defined by a polar head bound to phosphatidic acid with two fatty acid tails. Surfactant proteins A- D have important stabilising and passive immune functions but proteins make up approximately 10% of surfactant. In this section we have seen that surfactant is synthesised in, secreted and recycled by type II pneumocytes via lamellar bodies, some of which are Golgi dependent.

The mechanisms of surfactant dysregulation in ARDS are likely to be complex and resulting from such processes as decreased synthesis or secretion, oxidative reactions or other breakdown promoting increased clearance, or functional inhibition by compounds exogenous to alveoli such as plasma proteins. Fractional composition of PC changes in bronchoalveolar lavage (BAL) and plasma in ARDS. Saturated PCs are decreased but mono and diunsaturates are increased in BAL. It is important to distinguish between total amounts and fractions as lipid recovery methods are variable. Total GPL falls in ARDS BAL and rises in acute PE. PC and PG fraction is reduced in ARDS and PE. SM rises, likely as a result of the release of intracellular contents. In non- inflammatory oedema, (cardiogenic) the lipid profile remains unchanged.

1.3 The Lipidome

1.3.1 Introduction

Surfactant lipids are discussed in Section 1.2. In this section I will present plasma lipids and their regulation, which is mainly by the liver. The mammalian cellular lipidome is more diverse than plasma. Cellular lipids are thought to number more than 1000 species (81), the plasma figure is more than 500 to 600 (82, 83) their presence in plasma is largely for transportation. The fraction of lipid classes present in human plasma has been characterised by Quehenberger *et al*. (82). These are shown in Figure 18. Sterol lipids form the largest molar fraction, and they number about 36 species, including cholesterol. There are approximately 160 species of glycerophospholipids in plasma. Glycerolipids are the acylglycerols and number 73 species. Sphingolipids include sphingomyelin and the ceramides, 204 species. Fatty acyls are fatty acids and eicosanoids, 107 species and there are 8 species of prenol lipids including coenzyme-Q.

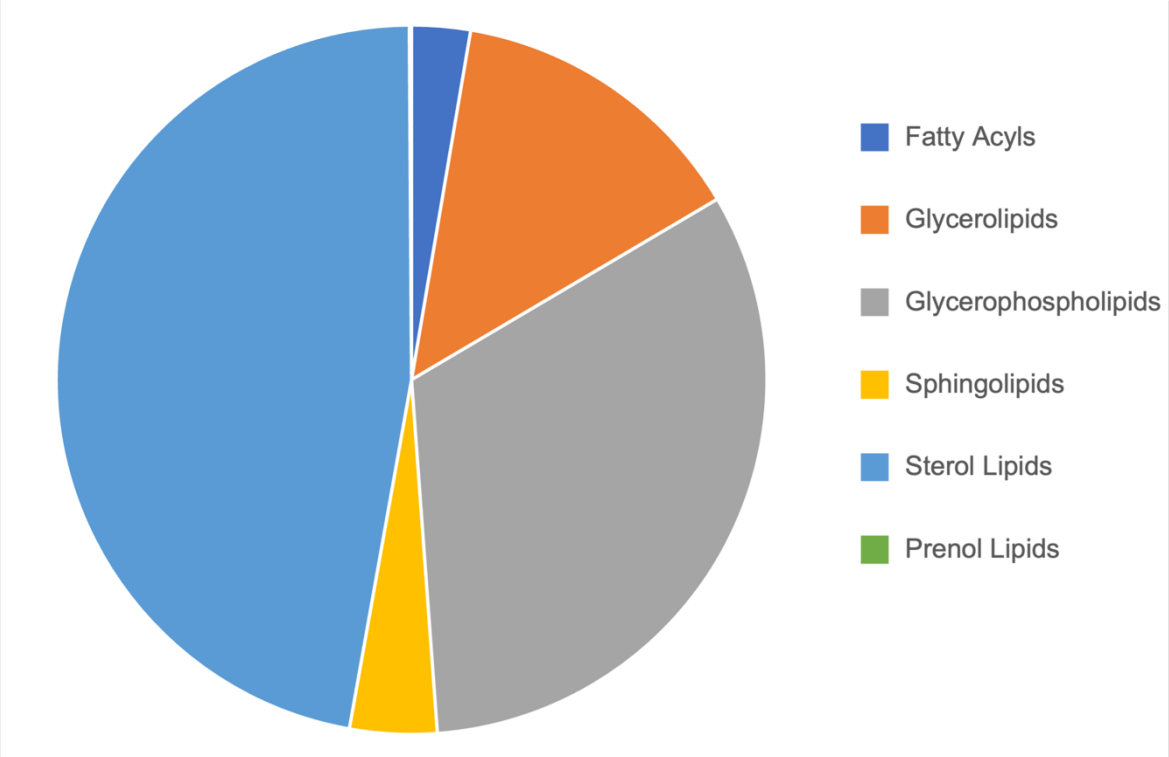

Figure 18. Fractional molar lipid composition of human plasma. Fatty acyls are present as fatty acids. After Quehenberger *et al*. (82).

1.3.2 Fatty Acids

Fatty acids make up a small fraction of total plasma lipids and they are produced by lipolysis, predominantly of adipose tissue according to metabolic demands. The majority is made up of 3 species, oleic acid (18:1), palmitic acid (16:0) and stearic acid (18:0) (82). The chief polyunsaturated fatty acids (PUFAs) are linoleic acid (18:2) and arachidonic acid (20:4).

Eicosanoids are derived from arachidonic acid (20:4) and related PUFAs by the actions of cyclooxygenases (COX-1 and COX-2), lipoxygenases (LOXs), cytochrome P450 epoxygenases and non-enzymatic pathways (84). This is illustrated in Figure 19. Eicosanoids have been studied extensively over the years due to their role in inflammatory conditions such as pain and asthma, and the drugs used to alter their balance.

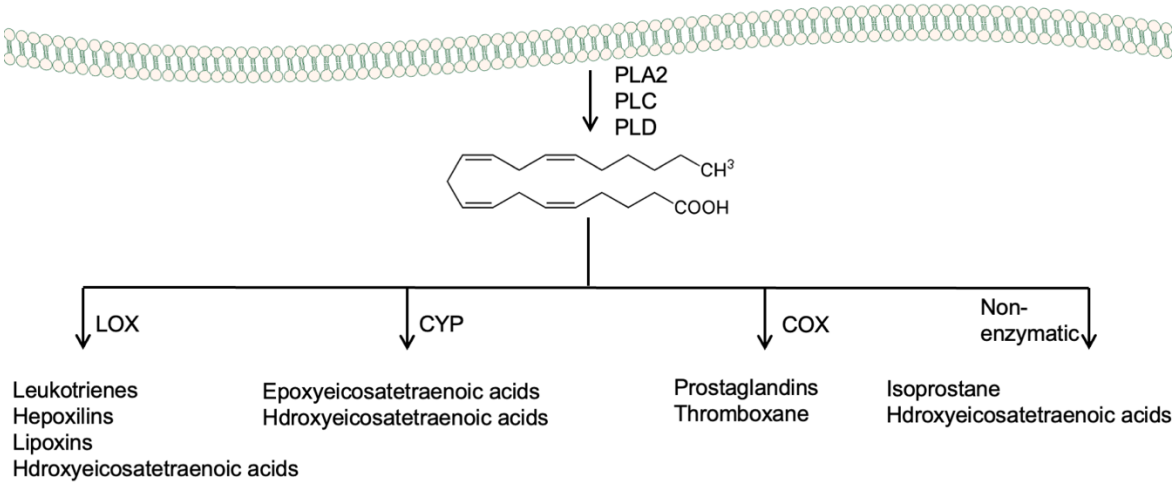

Figure 19. Arachidonic acid, shown here as its structural formula is formed by the action of three phospholipase enzymes on membrane bound phospholipids. PLA2= Phospholipase A2, PLC= Phospholipase C and PLD= Phospholipase D. Arachidonic acid can be converted to bioactive mediators by the actions of Lipoxygenases (LOX), Cytochrome P450 (CYP), Cyclooxygenases (COX (1-3)) or by peroxidation (non-enzymatic) (84, 85).

Lipoxygenases catalyse the insertion of molecular oxygen in arachidonic acid, to form compounds including leukotrienes. Leukotrienes are implicated in inflammatory conditions such as vascular diseases resulting from atherosclerosis. They also modulate inflammatory processes in the lung, acting as chemotactic agents for immune cells and stimulating cytokine pathways (85). Similarly, although by a different mechanism, CYP oxidises arachidonic acid to bioactive molecules that are thought to play a role in angiogenesis and other vascular functions (85).

The cyclooxygenases synthesise prostaglandins. COX-1 and COX-2 are the major enzymes, COX-3 is a variant of COX-1 (85). There is overlap in the function of COX-1 and COX-2, but with COX-1 more linked to thromboxane synthesis. Both catalyse the formation of a variety of prostaglandins which have effects on G-protein coupled receptors. Their effects are as diverse as the receptors and cells they act on. The effects of non-enzymatic derived metabolites are not known, however they can be used as markers of oxidative stress (84).

1.3.3 Glycerolipids

Plasma glycerolipids are the triacyl and diacylglycerols and are also known as tri and diglycerides. These are derived from diet and from synthesis by the liver, they are the major class of lipid present in adipose tissue. Normally dependent upon dietary intake, their presence in plasma is mainly as chylomicrons and very-low-density lipoprotein (VLDL) (82, 83).

1.3.4 Glycerophospholipids

Plasma glycerophospholipids (GPLs) make up a significant proportion of plasma total molar lipid fraction (Figure 18). The majority by molar fraction, are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species shown in Figure 20 (82).

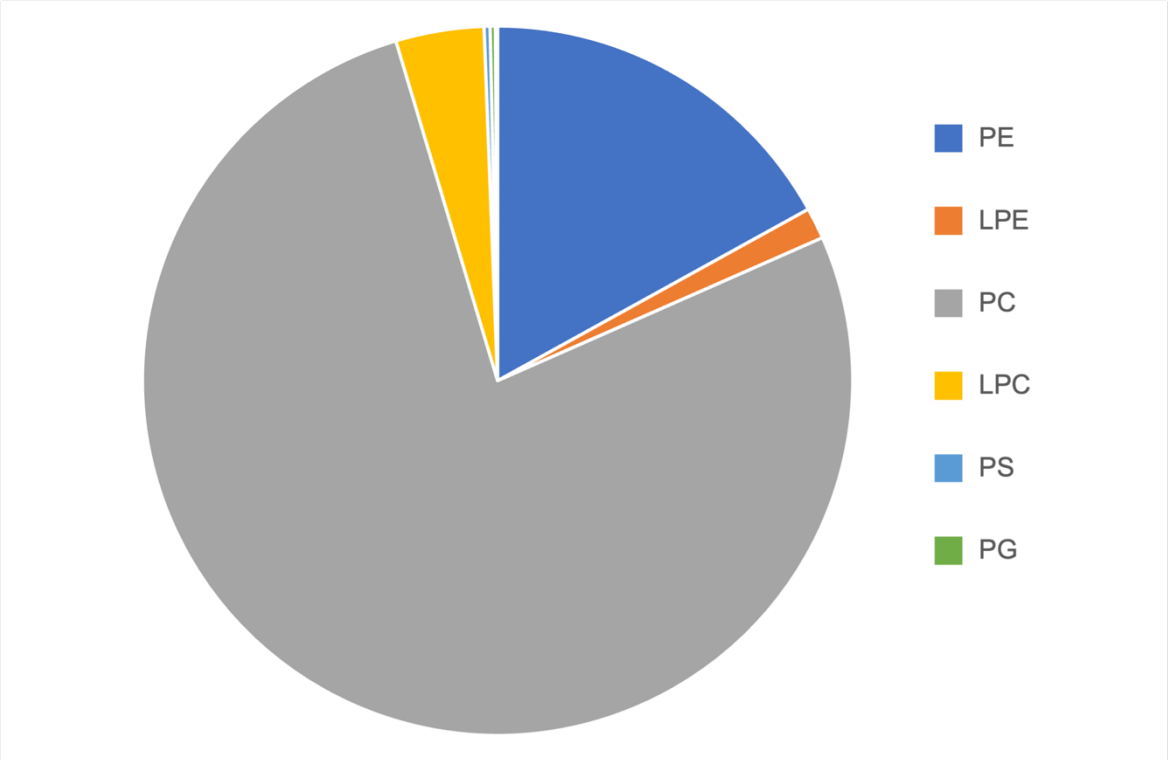

Figure 20. Fractional molar glycerophospholipid composition of human plasma. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are dominant. Removal of fatty acid from sn-2 results in Lyso PC and PE (LPC and LPE), less abundant fractions are phosphatidylserine (PS) and phosphatidylglycerol (PG). After Quehenberger *et al*. (82).

Plasma GPLs are secreted by the liver and exist as a component of lipoproteins (83, 86). Lipoproteins are used to transport water insoluble lipids in plasma and are composed of a shell of GPLs, proteins and unesterified cholesterol encasing a core of triacylglycerols (TAGs), cholesterol and cholesterol esters. The shell components are amphipathic, presenting polar moieties outwards to enable water solubility. Lipoproteins are classified chiefly by density, shown in Figure 21.

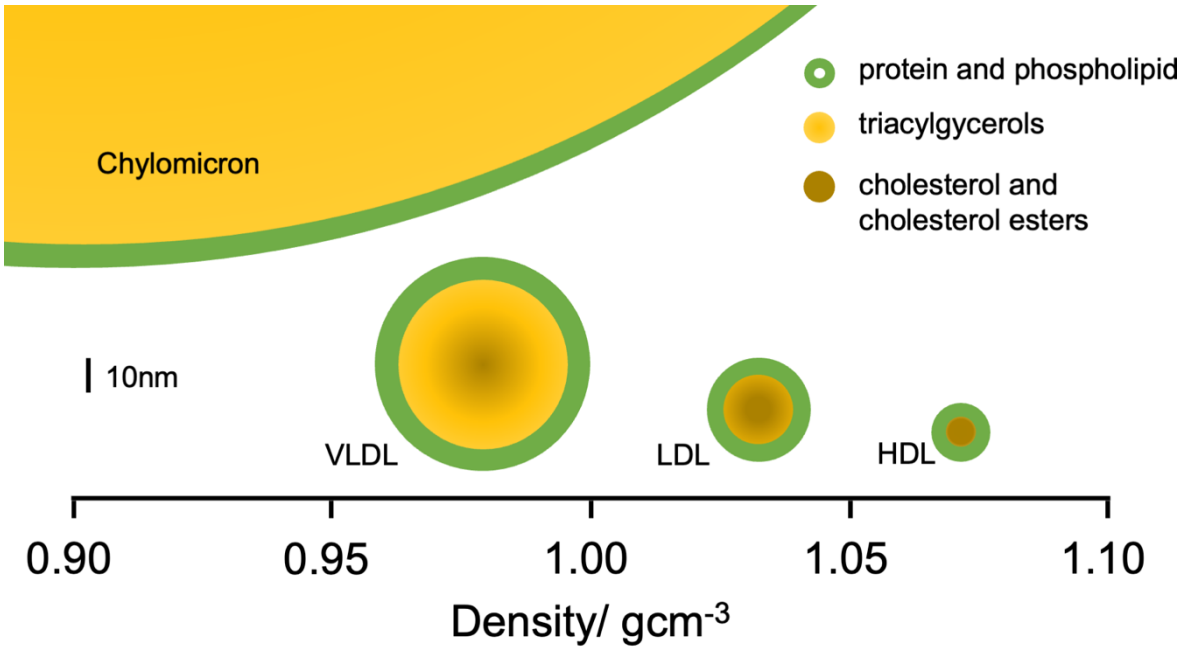

Figure 21. Size and density of plasma lipoproteins. VLDL= Very Low Density Lipoprotein. LDL= Low Density Lipoprotein. HDL= High Density Lipoprotein (37).

TAGs and cholesterol are absorbed from the gut or synthesised de novo. They are carried by chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) depending on the source. The proteins associated with GPLs in the spherical shell are known as apolipoproteins and divided into classes A, B, C, D and E. Apolipoproteins are thought to interact with cell surface receptors and enzymes as well as maintaining lipoprotein structure (37).

Chylomicrons are the largest lipoproteins and are formed in the intestinal mucosa from dietary TAGs, cholesterol, cholesterol esters and fat soluble compounds such as vitamins. Apolipoprotein B-48 is unique to chylomicrons (37). Chylomicrons are immediately altered in the circulation by degradation by lipoprotein lipase and addition of apolipoproteins such that they decrease in size. Lipoprotein lipase is prevalent on the endothelium of adipose tissue and muscle, where the breakdown products of fatty acids and glycerol are

stored or used as a source of energy. The degraded chylomicrons are known as remnants and are taken up by the liver (37, 83).

VLDL is synthesised and secreted into plasma by the liver. It is similarly broken down by lipoprotein lipase and undergoes modification of its apolipoprotein content. LDL provides cholesterol for tissues and the liver. Apo B-100/apo E (LDL) receptors present on the cell surface bind apolipoprotein B-100 in LDL and it is then endocytosed (37).

HDL is formed in plasma and acts as a reservoir for apolipoproteins, uptake and esterification of cholesterol and reverse cholesterol transport. Nascent, or discoidal HDL in plasma mainly contains GPLs, chiefly phosphatidylcholine (PC), and apolipoproteins. The high GPL content allows for the uptake of cholesterol which is then esterified by lecithin: cholesterol acyltransferase (LCAT). This process transfers a fatty acid from sn-2 of PC to cholesterol, which is then sequestered in the HDL core, the lyso PC remains in plasma. The uptake of peripheral cholesterol and transfer to liver is known as reverse cholesterol transport. Cholesterol is also transferred to steroidogenic cells for hormone synthesis, but in the liver, cholesterol is used for bile acid synthesis and excreted via bile (37, 83).

1.3.5 Sphingolipids

Sphingomyelin accounts for the largest fraction of human plasma sphingolipids. Sphingolipid presence in plasma has been implicated in some disease states but their physiological role is primarily in the cell membrane. Sphingolipid metabolites have a role in inflammatory processes including immune modulation (87).

1.3.6 Sterol Lipids

Section 1.3.4 Glycerophospholipids describes the existence of free and esterified forms of cholesterol in human plasma. 60- 70% of plasma cholesterol is present as LDL and 20- 30% as HDL. Cholesterol, including the

balance of LDL and HDL, is one of the foundations of cardiovascular risk stratification (83).

1.3.7 Plasma Lipids in Lung Disease

The relative contributions of the Kennedy pathway and phosphatidylethanolamine methyl transferase (PEMT) in the liver have been assessed using deuterated choline in ARDS patients (88). Electrospray ionisation mass spectrometry, described in Section 2.2.3 Mass Spectrometry Overview, was used to assess the incorporation of isotopically labelled choline via the Kennedy pathway. Isotopically labelled S-adenosyl methionine was not administered but PEMT activity could be indirectly measured. Deuterated *methyl*-D_9-choline is directly incorporated in the Kennedy pathway, it was infused into patients and controls with plasma samples taken at baseline and intervals following this. At baseline the ARDS patients had a slightly lower PC concentration than the controls. Changes in PC species are summarised in

Table **2**.

Decreased	Not significant
PC16:0/18:2	PC16:0/18:1
PC16:0/20:4	PC18:0/18:1
PC18:1/18:2	PC18:0/20:4
PC18:0/18:2	PC18:0/22:6
PC16:0/22:6	
PC18:1/20:4	

Table 2. Baseline fractional PC composition in the plasma of ARDS patients compared to healthy controls. Monounsaturates had a non-significant increase in proportion in ARDS.

When all species containing the non-essential fatty acid oleate (18:1) were compared, there was no significant difference between ARDS patients and controls. Those species containing the essential fatty acids linoleate (18:2) and arachidonate (20:4) were decreased in ARDS patients. Measurement of PEMT throughput was reliant on the oxidation of choline to the organic acid betaine, then acting as a methyl donor to homocysteine and ultimately forming S-adenosyl-*methyl*-D3-methionine. While isotopically labelled enrichment of PC was considerably varied between individuals, there was a significant increase in enrichment via the Kennedy pathway in ARDS patients. This was seen in all measured species of PC. PEMT activity was lower in ARDS patients when compared to controls.

Lysophosphatidylcholine (LPC) is formed by the hydrolysis of PC by phospholipase-A_2 (PLA_2) or secreted by the liver. Its fractional composition in ARDS patients differed in that LPC18:1 was increased and LPC18:2 was decreased. This could result from increased hepatic secretion of LPC18:1 or increased PLA_2 activity accounting for the decrease in plasma PC18:1/18:2 and PC18:1/20:4. Given that LPC18:2 decreased and LPC20:4 showed a non-significant increase it is likely that the changes are due to increased hepatic secretion of LPC18:1.

The profile of lung neutrophil arachidonate content is significantly altered in ARDS. A comparison of healthy controls' peripheral blood neutrophils and neutrophils in the BAL of ARDS patients was made (89). Neutrophils from the BAL of ARDS patients contained four times the amount of arachidonate as peripheral blood neutrophils in healthy controls. The majority of arachidonate was stored as phosphatidylethanolamine and triglycerides in the activated cells as illustrated in Figure 22.

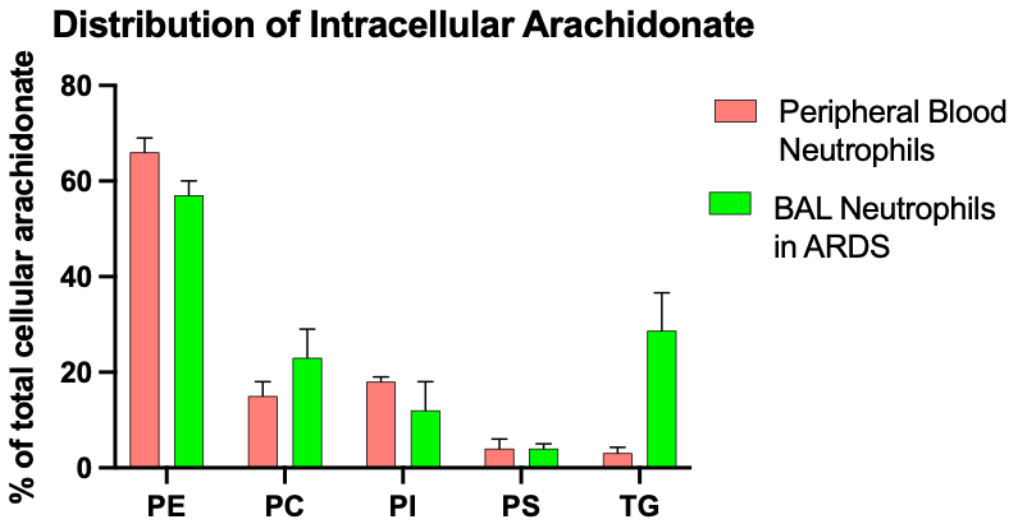

Figure 22. Arachidonate (C20:4) stores in activated neutrophils is increased in amount and differs considerably in which glycerolipid class it is held. The largest increase is seen in TG. Data are percentage ± SEM. PE= phosphatidylethanolamine. PC= phosphatidylcholine. PI= phosphatidylinositol. PS= phosphatidylserine. TG= triglycerides (89).

This arachidonate is a precursor for many lipid mediators of the inflammatory response. It is formed by the action of phospholipase A_2 on the sn-2 acyl bond, releasing arachidonic acid, as described in Figure 13, Lands cycle. This can then be converted to leukotrienes by 5-lipoxygenase or cyclic endoperoxides such as prostaglandins or thromboxane A_2 by cyclooxygenase (90). These eicosanoids have been observed in higher plasma concentrations in ARDS patients when compared to controls (91).

1.3.8 Summary

The predominant plasma lipids are sterols, glycerophospholipids (GPLs) and glycerolipids by fractional molar composition. Fatty acids are produced by lipolysis and have important roles as inflammatory mediators. GPLs are present, mainly in lipoproteins of varying density. Lipoproteins are used to transport triacylglycerols and sterols. The lowest density formation (chylomicrons) is secreted by the gut and undergoes refinement in plasma and by the liver to progressively higher density particles. Peripheral

cholesterol is taken up by High Density Lipoproteins (HDL), facilitated by the high GPL content, and transferred to the liver in a process known as reverse cholesterol transport.

There are 3 main routes for the synthesis of phosphatidylcholine (PC), with plasma measurements indicating that direct synthesis is increased in Acute Respiratory Disease Syndrome. Essential fatty acid species are decreased in plasma from ARDS patients. This may be from reduced gut absorption in illness or increased conversion to lipid mediators. Depletion of intracellular arachidonate-PC has been observed in neutrophils engaged in leukotriene synthesis after thimerosal stimulation (92). It is possible that upregulation of the Lands cycle and formation of leukotrienes from arachidonate is an important feature of acute inflammatory lung conditions and could lead to a reduction in arachidonate-PC.

1.4 Modern Evaluations of Phosgene Toxicity

1.4.1 Biochemistry

Prior to 1949 phosgene was thought to exert its toxicity by the formation of hydrochloric acid when in contact with water as described in Section 1.1.2:

$$\mathrm{COCl_2 + H_2O \longrightarrow CO_2 + 2HCl}$$

Potts *et al*. evaluated the contribution of diamide formation which occurs in two stages (93):

$$Cl- \overset{\overset{O}{\|}}{C} -Cl + R-NH_2 \longrightarrow Cl-\overset{\overset{O}{\|}}{C} -NH-R + HCl$$

$$Cl- \overset{\overset{O}{\|}}{C} -NH-R + R-NH_2 \longrightarrow R-NH- \overset{\overset{O}{\|}}{C} -NH-R + HCl$$

Their analysis concluded that the reaction of phosgene with tissue homogenates occurs in less than one second and that the total hydrolysis of a lethal dose of phosgene results in the formation of an insignificant amount of hydrochloric acid that is easily buffered by the circulation. Apart from tissue homogenates they reacted phosgene with a number of other biologically active chemicals, finding a significant reaction with sulphydryl groups. They hypothesised that an ingested non-toxic compound with amino or sulphydryl groups could act as pre-exposure prophylaxis, but would be in no way useful post exposure due to the rapid reaction of phosgene with tissues. These reactions will inevitably cause considerable disruption to biological systems.

Nash and Pattle built upon the work of Potts *et al*. by examining the absorption of phosgene in different solutions. They confirmed that the mechanism of toxicity was through molecular phosgene and not hydrochloric acid (94). For the first time an explanation of the effects of upper airway sparing, first noticed in World War 1 was provided. Phosgene is relatively insoluble in water and will react with solutes as described by Potts *et al*. The

aqueous mucus lining of the upper airways is sufficiently thick to prevent phosgene from reaching the cellular layers beneath. Additionally, its high reactivity means phosgene is likely to react with glycoproteins in the mucus. The resulting intense inflammatory response is further exacerbated by inflammatory cells such as neutrophils. Ghio *et al*. observed that phosgene induced lung injury, measured by protein content in bronchoalveolar lavage, was reduced in rats pre-treated with neutrophil-depleting drugs (95).

Nucleophilic groups or moieties are parts of molecules that can donate electrons to form covalent bonds. Nucleophilic groups in the biological context include primary and secondary amines, hydroxyl groups, sulphydryl (or thiol) groups and carboxylates. Phosgene is very reactive with these groups (96).

Pulmonary C fibres are parasympathetic nervous tissue that is involved with sensing noxious stimuli and mechanical strain. These fibres are transmitted by the vagus nerve and result in measures to protect the lungs such as coughing and transient apnoea (97). It has been proposed that C fibres contribute to pulmonary oedema. However, the data to support this is based upon assumed patterns of breathing thought to be mediated by C fibres and the pulmonary oedema observed occurs long after C fibre stimulation (98). In vagotomised dogs, there is no difference in phosgene induced acute lung injury, and in unilaterally vagotomised dogs, the lungs are indistinguishable after phosgene exposure (99).

As discussed in Section 1.2.1, surfactant prevents the ingress of oedema fluid from the expected surface tension within alveoli. After lung membrane disruption, alveolar fluid contains significant concentrations of proteins and other osmotically active molecules, tending to draw fluid from the now exposed high hydrostatic pressure circulation, into the lungs. In 1985, the effects of phosgene on the surfactant system were published by Frosolono and Currie (100). They hypothesised that an increase in surfactant components such as DPPC would have an antioedemogenic effect. Using rats exposed to sublethal doses of phosgene they found the changes in phospholipid components shown in Figure 23.

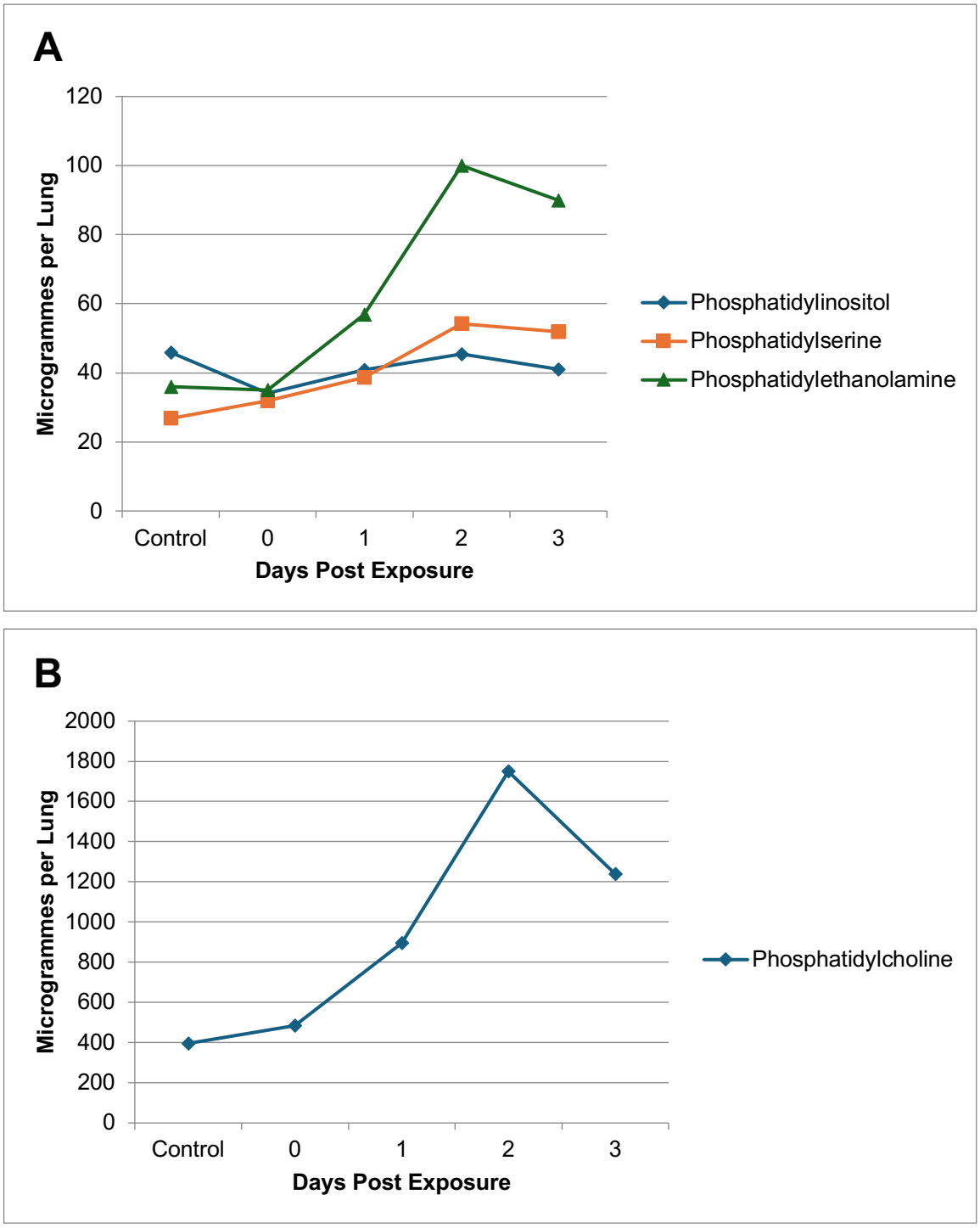

Figure 23. **A**. Response of glycerophospholipids, PI, PS and PE in rat lung to phosgene exposure. **B**. Response of PC in rat lung to phosgene exposure.

PI concentration declines and does not return to baseline. PE declines on day 0 post exposure but increases by day 1 and continues to rise. Both PS and PC increase from baseline from day 0. All of the post exposure peaks occur on day 2 of exposure with slight declines on day 3 before the animals were euthanised. Among the many signals promoting surfactant synthesis, water may be one that promotes the synthesis and secretion of PE, PS and PC. Frosolono and Currie suggested that some acyltransferases may have been initially compromised by phosgene, accounting for the delay in rise of PI and PE. It is likely that the alveolar damage will result in proliferation of type II pneumocytes regardless, in order to replace damaged type I cells.

Jugg *et al*. compared the change in surfactant composition by class in rat lungs exposed to perfluoroisobutene or phosgene by examining lavage fluid using high performance liquid chromatography (HPLC) (101). The sampling points are different to Frosolono and Currie and lend some insight to the earlier effects of phosgene on lung surfactant. PC, LPC, PG, PI, PE and sphingomyelin (SM) are examined in this study. The findings largely agree with the earlier rodent work, PC increases early and by a large amount, PE has increased by 24h. In contrast to the earlier work, PI increases from day 0 in this study. The results are displayed in ..

Aside from the changes in surfactant composition, there is a marked increase in protein content from 30min post phosgene exposure and changes in inflammatory cell populations. Macrophages decrease from 3h post exposure and the neutrophil count rises from about 4.5h in parallel with the oedema. There is a latent phase before changes in surfactant composition occurs, between 4.5- 6h. The animals were not distressed by phosgene exposure but later behaviour and physiological variables such as respiratory rate were not recorded, it is not known whether symptom onset mirrored this change.

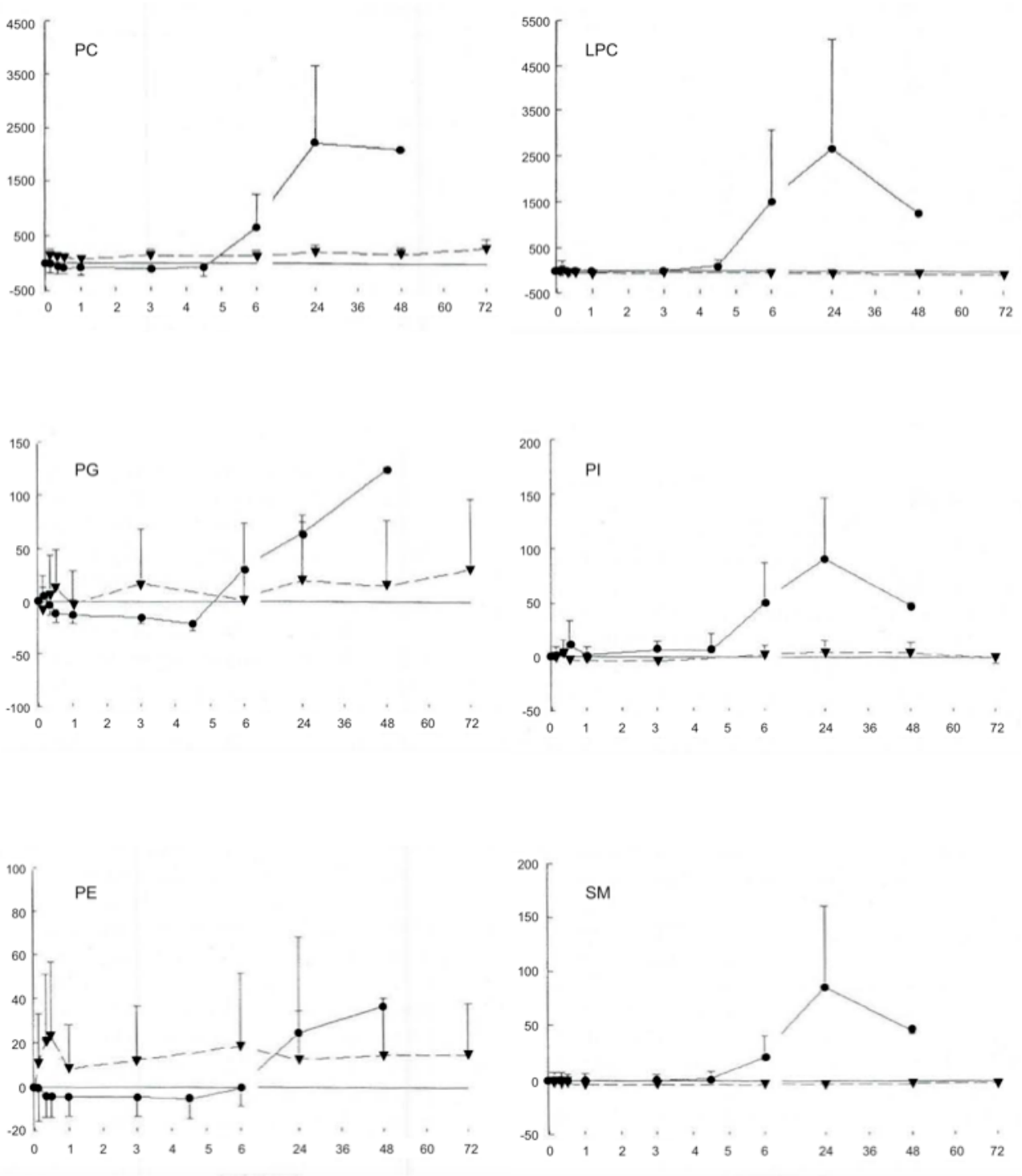

Figure 24. Changes observed expressed as a percentage of total amount of lung lavaged phospholipids of rat surfactant following exposure to air (▼) or phosgene (●) (101). Time on the x-axis is broken from 6-24h. PC= phosphatidylcholine, LPC= lysophosphatidylcholine, PG= phosphatidylglycerol, PI= phosphatidylinositol, PE= phosphatidylethanolamine, SM= sphingomyelin.

The other effects of the chemical reactions with nucleophilic moieties are an explosive activation of inflammatory pathways. Once phosgene has reacted with and exhausted surfactant, it will penetrate deeper and react with alveolar membranes and disrupt the pulmonary blood-gas barrier (102). The effects of this destruction include a massive inflammatory response with release of cytokines into the alveoli, further potentiated by oxidative stress and exhaustion of glutathione (103-105). There have been few studies in cell culture, the notable difference being that there is no observable latent phase. This is due to lack of the physiological processes and organ interactions necessary for its development. Aside from a reduction in IL-8, the inflammatory responses and depletion of glutathione are similar to those seen in vivo (106). The reduction in IL-8 may be due to a direct interaction with phosgene, this is less likely to occur in vivo due to shielding by other tissues.

1.4.2 Diagnosis of Phosgene Exposure

The variable latent period prior to symptoms is a large complicating factor in the diagnosis of phosgene induced acute lung injury. A large scale release would be extremely difficult to manage. The general approach to a phosgene release is doctrinally in keeping with other chemical releases and includes the difficulties in identifying the offending agent. Regrettably phosgene has the major complicating factors of how do you identify affected people? And how do you improve their oxygenation? During a mass release there may be large numbers of people awaiting triage, treatment and transport to hospital. Amongst them will be intoxicated patients who are asymptomatic yet will deteriorate at some point. It is crucial that these cases are not missed and it is the repetitive triage of patients that will identify them through an increase in respiratory rate or mild hypoxaemia on pulse oximetry.

It is unreasonable to expect people involved in a mass release of phosgene to accurately report exposure to a ‘new mown hay’ odour, particularly in a state of high anxiety. Equally it is unreasonable to expect smokers to report a

change in the taste of their cigarettes to rotten eggs (11, 12). Until we have a practical biomarker or means to test for acute lung injury in the prehospital population it is possible that a significant number of unintoxicated individuals will be treated. In fact, it is probably safe to wait for patients exhibit dyspnoea or mild hypoxaemia before treating (107).

A number of largely impractical methods for the early detection of phosgene induced acute lung injury have been proposed and include the wearing of indicator badges, measurement of serum lactate dehydrogenase (LDH), chest radiography, transthoracic impedance and diffusion capacity (108). Most of these are also unreliable. There are currently no markers of phosgene exposure (109). Patients with a history of potential phosgene exposure will require repeated physical examination including monitoring of pulse oximetry (SpO_2) until they show signs of deterioration (107).

1.4.3 Management of Phosgene Exposure

A number of pre- and post- exposure therapeutic interventions have been trialled in a number of different animal models. None of these has been shown to be of benefit at clinically relevant doses (104, 107) and salbutamol worsens physiological measures (110). Critical Care interventions that have made a positive impact were based upon modulation of fraction of inspired oxygen (F_IO_2), positive end expiratory pressure (PEEP) and tidal volume (V_T).

Titration of F_IO_2 to effect was an intervention used during World War 1 and the risks of oxygen toxicity were known (16, 17, 111). Despite a study confirming the harm of high fractions of inspired oxygen in phosgene poisoning in 1947 (112), these risks were taken less seriously as the century wore on and in 2000 some guidelines were produced that underpin our current management of invasively ventilated patients (71). These were built upon a large international study comparing tradition methods of ventilation with one of minimising F_IO_2 and V_T, generating an improvement in mortality. Regarding oxygen the guidelines support matching PEEP to F_IO_2 in order to

recruit collapsed alveoli, decrease extravascular lung water and minimise the risk of oxygen toxicity. Thus a patient in receipt of a high F_IO_2 should have a concomitant increase in PEEP to gain the above benefits.

Grainge *et al*. evaluated the effects of differing F_IO_2 in terminally anaesthetised phosgene exposed pigs (113). The animals were ventilated otherwise identically. Their primary end point was survival to 24h but they also measured a number of physiological and pathological variables. They exposed 30 pigs to a dose of phosgene likely to cause death, randomised to 5 groups:

Group 1 F_IO_2 0.30 throughout (n = 10)

Group 2 F_IO_2 0.80 immediately post exposure (n = 5).

Group 3 F_IO_2 0.30 from 30 min post exposure, increased to 0.80 at 6 h post exposure (n = 5).

Group 4 F_IO_2 0.30 from 30 min post exposure, increased to 0.40 at 6 h post exposure (n = 5).

Group 5 F_IO_2 0.30 from 30 min post exposure, increased to 0.40 at 12 h post exposure (n = 5).

The interventions were designed to replicate variable logistic arrangements for oxygen supply in a large scale release of phosgene with multiple casualties. The survival data is presented in Figure 25.

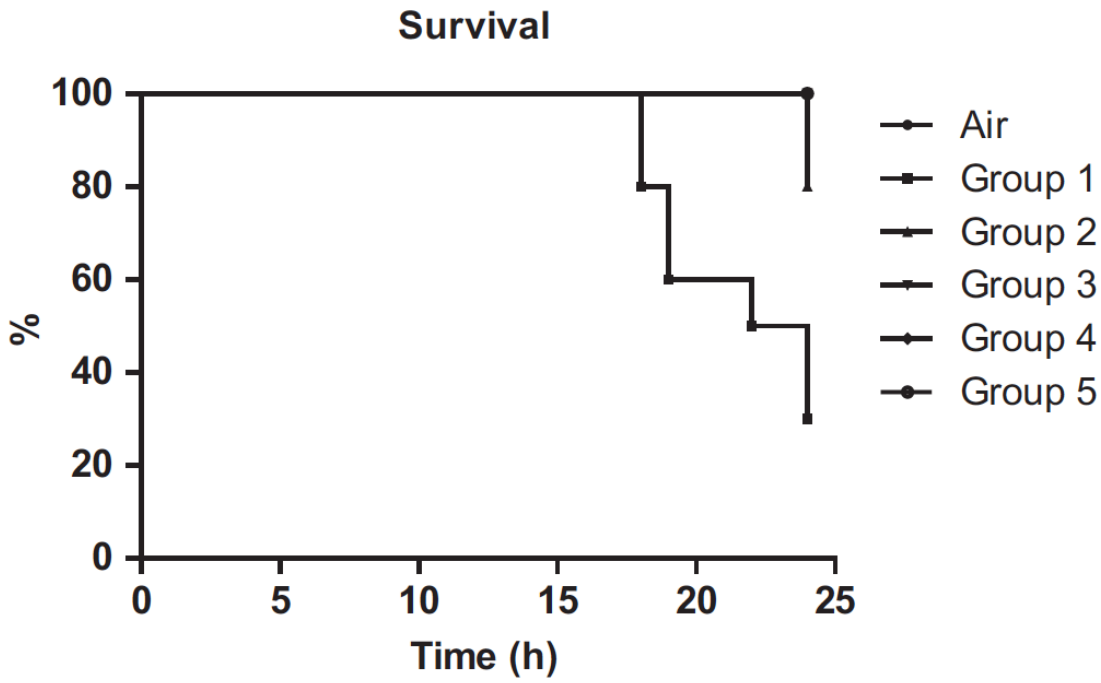

Figure 25. Kaplan-Meier plot showing that Groups 2- 5 (enriched oxygen) survival was better than Group 1 (F_IO_2 0.3) Group 2 (immediate F_IO_2 of 0.8) had a single death at 24h but all others in the delayed oxygen groups survived to 24h (upper line) (113).

Lung wet weight (g) to bodyweight (kg) ratio (LWW/BW) is a measure of extravascular lung water, representing pulmonary oedema as a result of alveolar permeability to plasma. In the pig model, air exposed controls have a ratio of 8.8± 0.4 (114, 115).

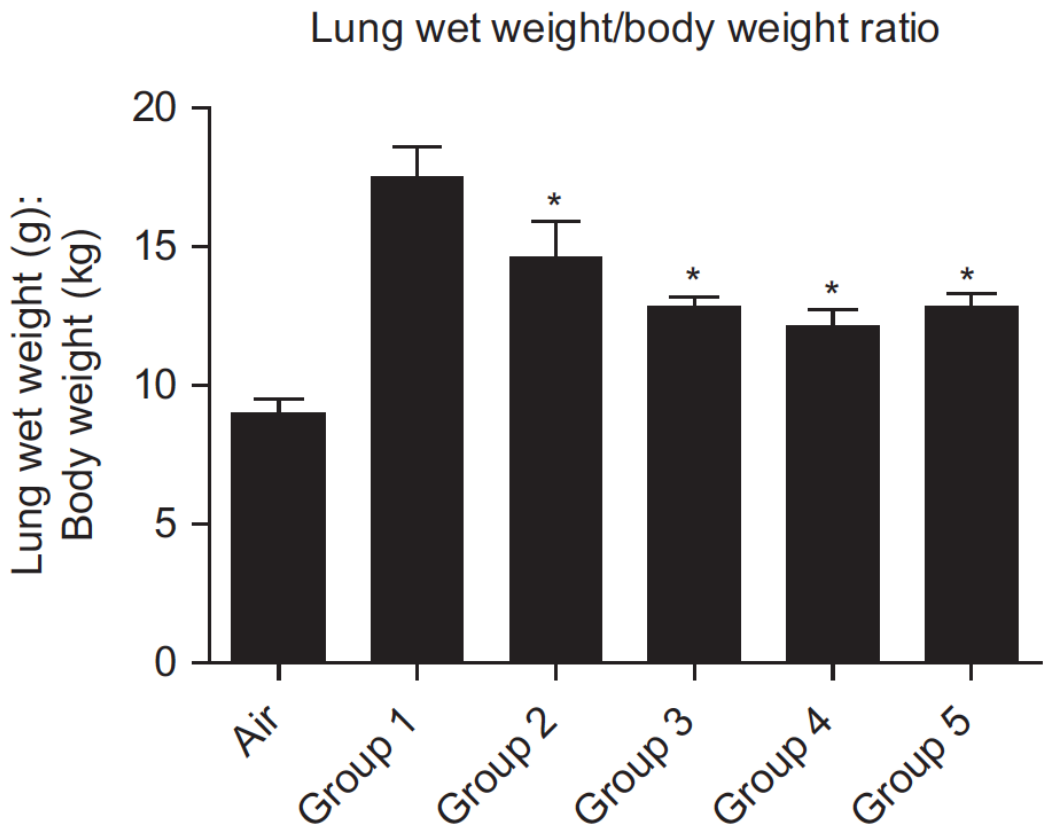

Figure 26. A comparison of air exposed controls with the oxygenation strategies of the 5 Groups. Group 1 (F_IO_2 0.30 throughout) show a significant increase in LWW/BW ratio compared to the groups receiving F_IO_2 of 0.8 or 0.4 at the delayed intervals. (*p= <0.05) (113)

Blood oxygen saturation was maintained in all groups except Group 1 (phosgene controls). What this study shows is that delayed oxygen therapy, even to 12h is safe and does not significantly alter the LWW/BW ratio. Additionally, it is sufficient to monitor pulse oximetry in phosgene exposed casualties.

Turning to protective ventilatory strategies in the intensive management of casualties Parkhouse *et al*. investigated V_T of 10mlkg^{-1}, 20 breaths min^{-1} and PEEP 3cmH_2O F_IO_2 0.24 for 6h then randomised to 3 groups (115):

CV continue 'conventional ventilation'

PV(A) V_T of 8mlkg^{-1} 20 breaths min^{-1} and PEEP 8cmH_20 F_IO_2 0.4

PV(B) V_T of 6mlkg^{-1} 25 breaths min^{-1} and PEEP 8cmH_20 F_IO_2 0.4

For a 50kg pig these equate to V_T of 400ml and 300ml and minute volumes of 8l and 7.5l respectively in the PV groups. The minute volume of the CV group would typically be 10l.

The results show a 100% survival in the PV groups and 30% survival in the CV group. LWW/BW ratios are shown in Figure 27. Oxygen saturation remained high in both PV groups, blood CO_2 climbed in the PV(B) group to approximately 12kPa from 8kPa at 6h and this is reflected in the observed reduction in pH. It is not clear what conditions the investigators were trying to replicate with the initial high V_T before intervention at 6h. It is unhelpful that in the PV arms, the F_IO_2 was increased but not in the CV arm. The previously discussed study showed a benefit of F_IO_2 at around >0.4 (113). However, I believe it is unlikely that the results can be fully explained by the F_IO_2 and there is likely to be an element of volutrauma induced by the high V_T in the CV Group. Protective ventilatory strategies apply to mandatory ventilated patients and these animals had their anaesthesia deepened in order to achieve this without the use of neuromuscular blocking agents. The minute volumes were such that the PV(B) group became profoundly hypercapnic to the point of causing a respiratory acidosis beyond a level that would be tolerated by attending clinicians.

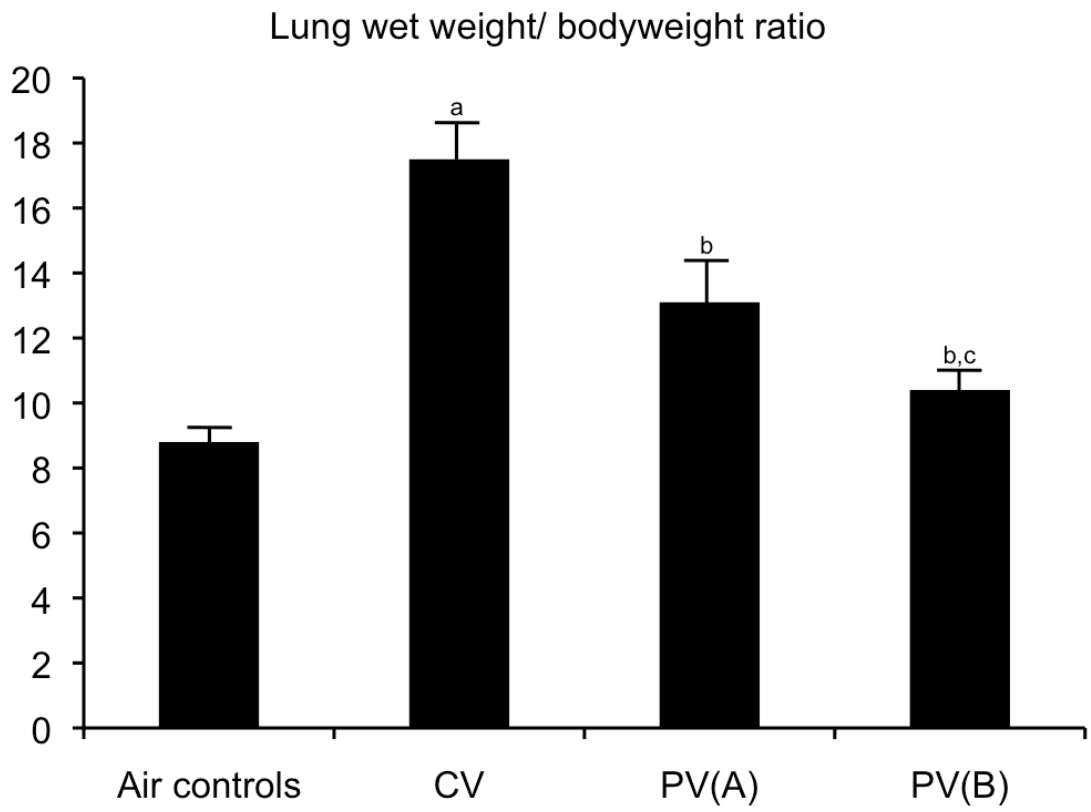

Figure 27. LWW/BW ratio is improved by protective ventilatory strategies. [a]p <0.005 compared to air exposed controls, [b]p <0.05 compared to air exposed controls, [c]p <0.05 compared to phosgene CV controls (115).

The management of multiple casualties requiring invasive ventilation is a very undesirable situation that would likely overwhelm medical logistics. The study changed too many variables and did not completely reflect clinical practice. A better design would have compared the three ventilatory strategies from the outset, using identical F_IO_2 and titrating the respiratory rate to maintain a near normal pH.

1.4.4 Summary

This section has built upon the earlier elegant experiments of the 1920s and the experience of World War 1 to explain the effects of phosgene at a molecular level, the effects on surfactant and the management of casualties.

Phosgene exerts its toxicity primarily through its reactivity with nucleophilic moieties on macromolecules, such as proteins and lipids. This results in acylation and structural modifications following inhalation exposure. The chemical disruption of these macromolecules impairs critical cellular functions, damages epithelial barriers, and triggers inflammation, leading to pulmonary oedema and respiratory failure. Mucous membranes are largely preserved due to the layer of mucus protecting the underlying tissues, phosgene primarily affects the lower airways and alveoli in particular. The effects on surfactant are a large increase in PC secretion occurring at 4.5h and variable effects on other glycerophospholipids including an initial reduction of secretion followed by an increase. This may represent poisoning of the cellular mechanics and subsequent repair. The mechanism for increased secretion is unclear and may be more than that expected from proliferation of type II pneumocytes differentiating to replace damaged type I pneumocytes. This increase in PC is not seen in ARDS. Total GPL falls in ARDS BAL with reductions seen in PC and PG and a rise in SM.

Diagnosis of phosgene toxicity is complicated by the lack of any tests or biomarker in a condition that can present late with serious consequences. Current guidance is to observe patients for signs of respiratory distress for 24h and, if they are asymptomatic after this time they can be discharged from hospital after a normal chest X-ray film (CXR) (116). Management is entirely supportive, and no therapeutic agents have been found to be effective. Inhaled therapies less likely to work in the presence of large volumes of pulmonary oedema fluid. If exposure has occurred within 6h then high dose methylprednisolone can be considered (107). The only effective interventions are titration of oxygen therapy to maintain oxygen saturation above 94% and protective ventilatory strategies should the patient require invasive ventilation.

1.5 Porcine Models of Lung Injury

Porcine models of phosgene induced acute lung injury have informed the management of phosgene exposure because porcine pulmonary anatomy and physiology approximate those of humans. The porcine lung anatomy is similar to human with 23 bronchial generations (117, 118). The lobar arrangement differs slightly to humans in that there are 4 lobes of the right lung; cranial (apical), middle, accessory and caudal. The left has 2 lobes, in common with humans; middle (apical) and caudal (119, 120). The right apical lobe is supplied by a tracheal bronchus, direct from the trachea, rather than the right main bronchus, a variant that is occasionally seen in humans; bronchus suis or pig bronchus (121, 122). Adult humans have collateral ventilation that is a survival adaption to enable oxygenation and ventilation bypassing lung or lower airway collapse in disease states (123). This may have significance in acute lung injury from pulmonary agents, however pig collateral ventilation is far less developed (124). A comparative summary of anatomical and physiological features of human and porcine lungs is provided in Table 3.

Feature	Human	Pig	Relevance to ARDS models
Lobar arrangement	R 3 Lobes L 2 Lobes	R 4 Lobes L 2 Lobes	Minor anatomical variation. Influences sampling.
Bronchial generations	23	23	Similar complexity. Supports translational studies.
Tracheal bronchus	Rare variant	Supplies R apical lobe	Minor technical differences in instrumentation.
Collateral ventilation	Well-developed	Poorly developed	May accentuate atelectasis.
Pulmonary circulation	Dual blood supply with extensive anastomoses	Similar	Comparable vascular responses to hypoxia and inflammation.
ARDS/ ALI susceptibility	Variable	Reproducible with triggers	Enables controlled modelling
Surfactant composition	PC ≈ 80% | PG ≈ 10% | minimal SM	Very similar proportions	Enables translational studies of surfactant changes

Table 3. Comparative features of human and porcine lungs relevant to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) models. R = right, L = left, PC = phosphatidylcholine, PG = phosphatidylglycerol, SM = sphingomyelin, TV = tidal volume, PEEP = positive end expiratory pressure.

Much is known of the pathophysiology of the porcine lung and it is of no surprise that research has focused on the most appropriate inducers of ALI/

ARDS rather than the animal model itself. Pig models have been established with ARDS induced with intravenous endotoxin, oleic acid or repeated bronchoalveolar lavage (BAL). Endotoxaemia induced ARDS in pigs has been shown to mimic the early stages of ARDS in sepsis related ARDS in humans (125). Indeed, a review compared the pathological changes observed in these 3 methods of inducing ARDS in pig, dog and sheep models. The comparisons with human ARDS are difficult only because of the timing of the transition from exudative to fibroproliferative phases which varies between species and sometimes even strains. The lavage model is less suitable for investigating the pathogenesis of ALI or ARDS, the oleic acid and endotoxin induced ARDS are the most suitable, endotoxin particularly in sepsis related ARDS, with oleic acid reproducing direct disruption of the epithelial- endothelial interface (126).

Other species have been used to study phosgene exposure, these include rat, mouse, Guinea pig, dog (Beagle), sheep and rabbit. Rodents are useful for mechanistic studies but not for evaluating ventilatory interventions; rodent lungs have major differences with human, dog and pig. Sheep are less susceptible to phosgene and studies in this field are rare. Rabbit susceptibility to phosgene is variable and there are few current models of lung injury. Dogs and pigs remain the most appropriate models of phosgene induced acute lung injury for translational pathophysiology (127). However, under current legislation, the use of dogs is subject to additional scrutiny. Interpretation of this legislation shows that non- human primates and companion species such as cats and dogs is permissible only when other species are unable to yield equivalent data (128).

Against this background, the value of porcine phosgene studies can be appreciated. Studies of phosgene in humans are not possible, however the effects observed in accidental exposure mimic those seen in the pig model. Nevertheless, there remain important gaps; no detailed surfactant compositional analysis in phosgene induced acute lung injury has been previously reported and no assessment of the systemic effects by analysis of plasma lipids has been done before. Finally, the assessment of ventilatory

countermeasures in prior phosgene studies have not included the simple addition of CPAP, which this work evaluates.

1.6 Thesis Aims

Environmental phosgene detectors appropriate for field are in existence (129), but no effective post exposure human biomarkers or immediate countermeasures exist. The management of affected patients remains entirely supportive.

1.6.1 Contemporary Relevance of Phosgene Exposure

The Geneva Protocol, signed in 1925 prohibited the use of poisonous gases including phosgene for use as weapons (130). However, this protocol did not preclude the manufacture and storage of phosgene. Nonetheless, phosgene was deployed by the Japanese in the Second Sino Japanese War (131) and by Iraq in the Iran Iraq War; Al Basrah, April 1987 (132). There have been many other claims of phosgene use as a chemical weapon since the 1925 protocol, but these are largely unsubstantiated. After many years of international negotiations, it was not until 1997 that the Chemical Weapons Convention came into force (133). The Convention lists phosgene in Schedule 3 which is the control framework for toxic chemicals that have legitimate large scale industrial uses. As a Schedule 3, or dual use chemical, phosgene is subject to verification measures, by way of on-site inspections, and the Convention's prohibition on development, production, stockpiling and use as a weapon. In the last few years there has been increased activity at a plant in North Korea that produces fertiliser. This could easily be repurposed to manufacture chemical weapons (134). Legacy munitions and abandoned stockpiles present an extreme hazard, particularly in unstable or collapsing regimes (135).

In the industrial setting, phosgene represents a significant public health risk in the absence of misuse. In Hamburg, 1928 a store of military grade phosgene for demobilisation ruptured and released 8m^3 of liquid phosgene that evaporated and poisoned 300 people up to 10km distant from the source, 10 of whom died (136). Exceptional measures to ensure the safe handling and use of phosgene have been put in place (137), yet despite this,

accidents still occur. In 2010, at DuPont's West Virginia plant, three separate incidents occurred within 36h of each other, one of which was a fatal phosgene release (138). 2012, in France an accidental phosgene release at a plant producing phosgene and chlorine for isocyanate production, resulted in the exposure of 4 workers (139). Unusually these workers were assessed and returned to work. In 2016, a fertiliser plant in Gujarat accidentally released phosgene, killing 4 workers and affecting 9 others (140). These are some examples of accidental phosgene release, but there are many more recorded by The Occupational Safety and Health Administration, US Department of Labor (141).

Non- state actors have used Toxic Industrial Chemicals (TICs) in the past due to their availability (142). However, there have been no substantiated cases of terrorists successfully deploying phosgene. The literature and assessments made by interested parties treat phosgene as part of the broader TIC threat (143, 144). A handbook, titled "Silent Death" by the pseudonymous, "Uncle Fester" is a sensationalist manual of biological and chemical terrorism (145). In it, Uncle Fester discusses methods of manufacturing phosgene described in Section 1.1.2 and then dismisses them in favour of devices that combine oleum with tetrachloromethane. The reaction produces phosgene, $COCl_2$ which is supposed to bubble out of the mixture, leaving sulphurochloridic acid as a liquid mixed with residual sulphuric acid:

$$H_2SO_4 + SO_3 \rightleftharpoons H_2S_2O_7 + CCl_4 \rightarrow COCl_2 + 2HSO_3Cl$$

This reaction is likely to be highly exothermic and result in co-release of sulphur trioxide which is also extremely toxic by inhalation, forming sulphuric acid when combined with water, causing a rapid chemical pneumonitis and a burning sensation in the upper airways (146). The toxicity of oleum and tetrachloromethane to individuals and the environment are such that their availability in UK is highly restricted (147, 148). Without applying for a valid license, the potential terrorist would need to illegally source reagents from abroad or steal from legitimate institutions such as universities, chemical plants or other industrial users.

Nowadays phosgene is manufactured by the combination of chlorine with carbon monoxide, in an excess of carbon monoxide to minimise the formation of undesirable chlorine based side reactions (137). Storage and transport are limited to the industrial site of use, and in the minimum volumes required. Multiple layers of safety are in place to reduce the risk of leaks, in the form of equipment and handling processes. Its main uses are in the production of isocyanates, polycarbonates, pesticides and pharmaceuticals (137). The estimated world production of phosgene for 2013 was $10x10^6$ tonnes from more than 200 plants, and this is set to increase (137).

1.6.2 Operational Medical Response and Rationale for CPAP

In a large scale release of phosgene involving multiple people, there will be considerable medical and logistical problems. The chief difficulty is the latent phase. The need for triage, treatment and transport of casualties could easily overwhelm local resources. Management of the 'worried well' could needlessly consume healthcare resources, as there is no way of excluding the diagnosis of phosgene induced acute lung injury (ALI) in this group. Oxygen therapy can safely be delayed in all patients until the oxygen saturation begins to fall, and this can also be used to diagnose phosgene induced ALI. Where multiple casualties exist, the triage, treatment and transport phases of the incident could easily become delayed by sheer numbers of casualties.

These difficulties necessitate early scalable interventions that could be applied prehospitally. Can the use of Continuous Positive Airway Pressure (CPAP) be used to delay the onset of signs of phosgene induced ALI? CPAP machines are a commercial off the shelf (COTS) device consisting of a tight fitting facemask and an electrically powered air pump that maintains air at a set pressure during both inspiration and expiration. It is therefore not a means of ventilation and will only improve oxygenation by reducing the closing volume of the lungs and redistributing extravascular lung water. Their use could be a substitute for oxygen therapy. In a large scale release, CPAP

masks could be used to extend the time window for the safe triage, treatment and transport of affected persons. They may also ameliorate the effects of phosgene induced ALI.

What are the precise changes in surfactant composition in phosgene induced ALI? The major groups of glycerophospholipids have been investigated but not down to individual molecular species. The investigation may reveal a surfactant metabolite only found in phosgene induced ALI that may act as a marker of clinically relevant exposure. Future work could develop a point of care test that could be used in the field to diagnose exposure.

Hypotheses:

- CPAP can delay the onset and decrease the adverse effects of phosgene induced ALI.
- There is a difference in lipid composition of phosgene exposed individuals compared to unexposed.

2 - METHODOLOGY

2.1 The Large Animal Model

2.1.1 Ethical Approval

All this work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 (149), and the work was issued a Project Licence by the Home Office. Large White female juvenile pigs (47- 55kg, n= 22) were obtained from a commercial source approved by the Animal and Welfare Ethical Review body at Defence Science and Technology Laboratory (DSTL) Porton Down. Animals were housed in pairs with access to standard pig diet and water *ad libitum* until the night before the study when food was removed. The standard diet was cereal based; approximately 80%, supplemented with protein from Oilseed Rape and Soya, and a small proportion of added fats. This was representative of UK commercial maintenance feed for porcine research. Neuromuscular blocking agents were not used at any point during the study.

2.1.2 Surgical Procedures

The placement of lines and tubes in the animals is previously described (114, 150). These were placed by Technicians and overseen by a Veterinary Surgeon credited in Acknowledgements. The materials and methods included premedication with intramuscular midazolam 0.5mg kg^{-1} (Roche Products Limited, Welwyn Garden City UK) prior to gaseous induction of anaesthesia with 5% isoflurane (IsoFlo, Abbott Laboratories, Kent, UK) in oxygen and nitrous oxide (30%). All of the study animals underwent tracheal intubation, once anaesthetised, using a size 8.0mm cuffed endotracheal tube (Portex, Smiths Medical Int. Ltd, UK). The endotracheal tubes remained in place, in all animals until the post mortem examination; none of the animals underwent tracheal extubation during the monitoring period. Anaesthesia was maintained with 1-2% isoflurane and nitrous oxide 30- 50% in oxygen. One

carotid artery and both internal jugular veins were surgically exposed and cannulated using an 8-French gauge catheter (Dog catheter 50cm, Portex, Smiths Medical Int Ltd, UK) and connected to a pressure transducer and the blood pressure monitored. Electrocardiogram (ECG), pulse oximetry, end-tidal carbon dioxide, and central venous pressure were measured using a Propaq 246 monitor (Protocol systems Inc., Beaverton, USA). A 'Pulse Contour Continuous Cardiac Output' (PiCCO) catheter was inserted into the femoral artery using the Seldinger technique and attached to a PiCCO monitor (PV8115, Pulsion Medical Systems, Munich, Germany), allowing continuous measurement of cardiac output, systemic artery pressure, stroke volume, and core temperature. Periodic thermal dilution analysis allows estimation of extravascular lung water (EVLW). This was performed by myself and DSTL Scientists credited in Acknowledgements. A Bonanno bladder catheter was introduced into the bladder via an open cystotomy, urine output was measure using a urine bag (Kendall PRECISION™ 400 Urine Meter, Covidien, Massachusetts, USA). To replace insensible fluid losses, animals were maintained on an infusion of Vetivex 11 (Hartmann's solution, 2ml kg^{-1} h^{-1}). Following surgery and for the duration of the experiment, anaesthesia was maintained intravenously with propofol 2% (~10–12mg kg^{-1} h^{-1}) (Astra Zeneca, UK; 20 mg ml^{-1}) and alfentanil hydrochloride (~0.5–2.5µg kg^{-1} h^{-1}) (Janssen Pharmaceuticals Ltd, Ireland; 500µg ml^{-1}). Maintenance of anaesthesia was titrated by myself and DSTL Scientists, and overseen by a Veterinary Surgeon credited in Acknowledgements. Neuromuscular blocking agents were not used at any point, not even for laryngoscopy.

2.1.3 Study Protocol

Baseline physiological measures were recorded and blood sampling done at various time points relating to exposure to phosgene/ air. Sampling was done by myself and DSTL Scientists credited in Acknowledgements. Arterial and venous blood gases were sampled at -1h, -40min, -20min, +0.5h, +1h and then hourly until the experiment ended. Arterial samples were taken as

follows for haematological and biochemical analysis at the following time points:

-40min, +0.5h, +1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18h, and then hourly until the experiment ended.

Measured and derived PiCCO values were obtained by cold injectate analysis using 10 ml chilled saline administered via the PiCCO catheter every 4 h post exposure. Urine was collected at -20 min and then hourly post exposure and analysed using a urine dipstick (Siemens Multistix 10 SG urinalysis multistrip, Siemens, Surrey, UK). 4.5 ml was decanted and stored at -80 ^{o}C for further analysis.

The animals were randomly assigned to the following groups:

• Air-exposed controls (n = 4)

• Phosgene-exposed controls (n = 10)

• Phosgene + CPAP (n = 8)

Group sizes were set in advance, in line with the Home Office 3Rs (Replacement, Reduction, Refinement) and the cost and logistical constraints inherent to large animal critical care studies. The Resource Equation can be used to estimate the minimum and maximum numbers of animals required. When the standard deviation and effect sizes cannot be assumed, and in a study that anticipates multiple endpoints requiring an analysis of variance, the Resource Equation can be used as a design check. (151, 152). The Resource Equation gives a value, E that should lie between 10 and 20. $E = N - k$, where N is total number of animals and k the total number of groups. For this study, $E = 19$ which was satisfactory.

The air-exposed control animals remained breathing air, whilst the other groups were exposed to phosgene (BDH, UK) diluted with nitrogen for approximately 10 min to accurately deliver a target inhaled dose. The target dose was selected to match established DSTL porcine phosgene models and to provide an intervention responsive lung injury that remains reproducible.

An exposure of $C_t \approx 2.3x10^3 mg.min^{-1}m^{-3}$ for 10min had been used previously (110, 113-115). Using the measured inspired phosgene concentrations and minute volume in these studies, this corresponds to an inhaled dose of approximately 0.23- 0.27$mg.kg^{-1}$. In an evaluation of inhaled furosemide, an inhaled phosgene dose of 0.256- 0.266$mg.kg^{-1}$ was reported and led to the deaths of 3/8 of the controls before 24h (153). A target dose of 0.24 $mg.kg^{-1}$ was selected for this study as it was expected to produce a relevant clinical picture of severe acute lung injury, yet still rescuable by plausible interventions. The dose balanced scientific value with animal welfare; it produced a meaningful injury enabling the study of an intervention, rather than simply observing inevitable death. The exposure apparatus has been described previously (114) and is shown in Figure 28.

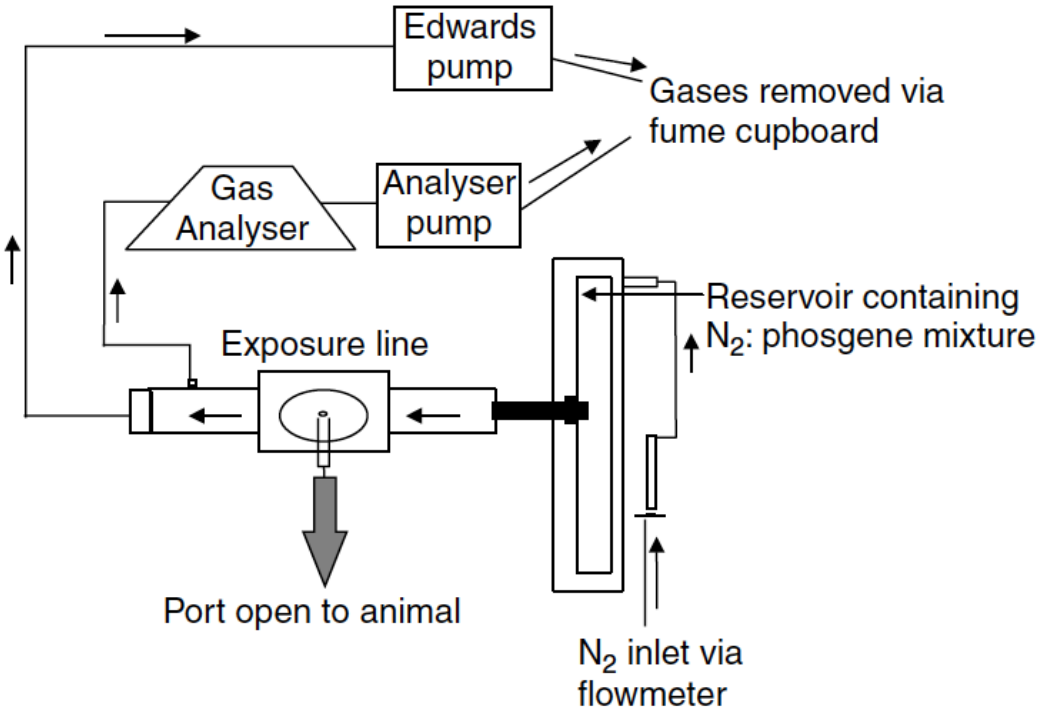

Figure 28. Exposure apparatus consisted of a 15/22mm connection to the animal's endotracheal tube. Ambient air was drawn through the apparatus at a rate of 78- 85l min^{-1} to which phosgene could be added from a reservoir.

The apparatus consisted of a delivery system, an exposure line, effluent and analyser. Ambient air flowed through the exposure line, to which the animal was connected via the endotracheal tube as described. 10min was allowed for acclimatisation to the apparatus prior to exposure to air or phosgene, which then occurred at time= 0h. The delivery system consisted of a 10l Tedlar® reservoir bag mounted within a rigid Perspex box and filled with phosgene (BDH, UK) diluted with nitrogen. For phosgene exposures, nitrogen flowing into the Perspex box was used to compress the Tedlar® bag, delivering a regulated flow of phosgene into the exposure line at approximately 250ml min^{-1}, mixing with the ambient air flow. Phosgene concentration within the exposure tube was monitored continuously using a Miran infrared gas analyser (Foxboro Instruments, UK). This allowed for adjustment of nitrogen gas flow into the Perspex box and highly accurate dosing. Phosgene concentration was recorded every 15s and integrated over time to calculate the dosage (concentration (mg m^{-3}) x time (min); C_t) delivered to the animal. The Inhaled dose was determined from the mean phosgene concentration (mg m^{-3}) x total inhaled volume (m^3, l/1000) divided by body weight (kg). Following exposure the animal remained attached to the exposure apparatus for 5min which allowed for any residual phosgene to clear which was confirmed by the analyser (114, 150).

In selected animals (3, 5, 6, 7, 8, 9 and 10), time-expired D-9 labelled choline was administered intravenously over 1h after exposure to air or phosgene, to explore phosphatidylcholine synthesis and turnover. Doses ranged from 1.2-3.6mg kg^{-1}, according to limited supply and with the intention to assess changes in plasma PC. Plasma samples from these animals were not released by DSTL, or not received in Southampton. At the doses used, and with BAL sampled post mortem, after 7- 24h, labelled PC did not reach detectable levels in BAL. The procedure is noted here for completeness.

2.1.4 CPAP

Continuous Positive Airway Pressure (CPAP) is a description used for non-invasive elevation in airway pressure. The animals had undergone tracheal intubation and by convention, a continuous elevation in airway pressure applied invasively is termed Positive End Expiratory Pressure (PEEP). Animals in the CPAP arms of the study were connected to a ventilator (Evita XL, Dräger Medical) with PEEP and pressure support both set at 0cmH_2O. Fraction of inspired oxygen (F_IO_2) was set at 0.21 using medical air by myself and DSTL Scientists credited in Acknowledgements. The animals were spontaneously breathing with anaesthesia maintained with propofol and alfentanil infusions adjusted as described above. At 1h the CPAP arms had PEEP increased to 5cmH_2O and then incremental increases by 1cmH_2O every 5min until reaching the target of 10cmH_2O. PEEP was applied at 1h to balance the anticipated delay in access to specialist medical intervention and the associated toxicological progression, against the need to preserve the utility of subsequent observations during the remaining 24h period. The PEEP was then not adjusted for the duration of each experiment. In porcine models of acute lung injury, rapid increases in PEEP or peak inspiratory airway pressure result in significant cardiovascular embarrassment (154). This is associated with a large decrease in cardiac output, manifest as profound bradycardia and associated apnoea. For these reasons PEEP was cautiously increased incrementally to 10cmH_2O. This is a cardiovascular complication, not typically seen in humans, and does not undermine the relevance of the porcine lung model. Air and phosgene exposed controls were not attached to the ventilators and remained spontaneously breathing ambient air via the endotracheal tube. This uptitration of PEEP was done by myself or DSTL Scientists credited in Acknowledgements.

2.1.5 Naming of Experimental Groups

The North Atlantic Treaty Organisation (NATO) designation for phosgene is CG (155 p.402). This designation is used to describe the phosgene exposed

groups. The experimental arms described in Section 2.1.3 were named as follows:

- Unexposed controls = **Air Controls** (n = 4)
- Phosgene exposed, no PEEP applied = **CG Controls** (n = 10)
- Phosgene exposed, PEEP applied = **CG CPAP** (n = 8)

2.1.6 Physiological Measurements

Measurements were recorded at 20min intervals during the 1h baseline period followed by 30min intervals up to 24h or death. Two animals at a time were run almost in parallel, with one team caring for each. As with Intensive Care, each subject required several staff, and large teams were used in shifts to manage the experiments. As a Home Office Licence holder for this project, I maintained anaesthesia, drew samples and performed cold injectate analysis. Arterial and venous blood gas samples were taken hourly and immediately analysed (GEM Premier 3500 blood gas and co-oximetry analyser, Instrumentation Laboratories UK Ltd) and adjusted for temperature. Blood was also taken for haematological analysis using an Advia120 Haematology instrument (Siemens UK) and for analysis of plasma lipids. These samples were drawn and analysed by myself and Scientists credited in Acknowledgements. Cardiac Output, Shunt fraction (Qs:Qt) and Extravascular Lung Water were calculated by and from data measured by a PiCCO monitor (PV8115, Pulsion Medical Systems, Munich, Germany) and estimated using standard formulae (156).

Cardiac output is derived by the PiCCO monitor, from the temperature change of cold saline injected via the central venous catheter and measured again at the femoral arterial line. It is related to the area under the curve of temperature change (ΔT) measured at the femoral artery over time by the Stewart Hamilton equation:

$$CO = \frac{KV_i(T_b - T_i)}{\int_{t_0}^{t_1} \Delta T(t)\ dt}$$

where K is a constant relating to densities and specific heat capacities of injectate and blood; V_i is injectate volume; T_b is baseline blood temperature; T_i is injectate temperature; $t_0 \rightarrow t_1$ defines the first pass curve before recirculation.

Extravascular lung water (EVLW) is calculated by the PiCCO monitor by subtraction of the intrathoracic blood volume (ITBV) from the intrathoracic thermal volume (ITTV):

$$EVLW = ITTV - ITBV$$

ITTV is calculated by:

$$ITTV = CO \times MTt$$

where MTt is the time taken for half the cold injectate to pass, or mean transit time

ITBV is calculated by:

$$ITBV = GEDV \times 1.25$$

where GEDV (Global End Diastolic Volume) is:

$$GEDV = CO \times (MTt - DSt)$$

and DSt is the time taken for the natural log- transformed thermodilution curve to decrease exponentially, or downslope time.

Other variables are derived from these calculations by the PiCCO monitor:

- Stroke volume (SV) is CO divided by heart rate
- Contractility (dP/dt) derived by the PiCCO algorithm
- SV Variation (SVV) is the relative change in SV during the respiratory cycle and is an indicator of intravenous fluid responsiveness
- Systemic vascular resistance (SVR) is Mean Arterial Pressure (MAP)-Central Venous Pressure (CVP) divided by CO

Shunt Fraction is calculated by:

$$\frac{Q_s}{Q_t} = \frac{(C_{cO_2} - C_{aO_2})}{(C_{cO_2} - C_{vO_2})}$$

where Q_s is shunt blood flow per minute; $Q_t = CO$ is total cardiac output per minute; C_{cO_2} is pulmonary end- capillary oxygen content (≈ alveolar O_2 content); C_{aO_2} is arterial O_2 content; C_{vO_2} is mixed venous O_2 content (O_2 content of blood in the pulmonary artery). The pulmonary artery was not accessed in this study, central venous blood oxygen saturation (S_{cvO_2}) was used as a surrogate for mixed venous blood O_2 content (C_{vO_2}); $C_{vO_2} = ([Hb] \times 1.34 \times S_vO_2) + (0.003 \times P_vO_2)$. $S_{cvO_2}$is approximately 5% higher than mixed venous O_2 saturation (S_vO_2) and will slightly but consistently underestimate shunt (157, 158).

The animals were physiologically too unstable for antemortem bronchoscopy.

2.1.7 Post Mortem Examination

The experiments were run up to 24h. Animals were killed with an intravenous overdose of sodium pentobarbitone 150mg kg^{-1} ("Euthatal" 200mg ml^{-1} Rhone Merieux Ltd., UK). An animal was killed before 24h if it became moribund, defined as periods of asystole or a central venous oxygenation ($S_{cv}O_2$) <15%. The thoracic and abdominal contents were examined with the trachea clamped distal to the endotracheal tube as quickly as possible. This was to prevent fluid loss from the lungs or contamination. The thorax was

then opened fully and the heart and lungs removed intact. The right median lobe was accessed bronchoscopically (Olympus BF-4B2, KeyMed Ltd, Essex, UK). Bronchoalveolar lavage (BAL) was performed via the flexible bronchoscope using sterile saline. 4 aliquots of 40ml were instilled into the lobe, the fluid was aspirated between each instillation and placed on ice. Lavage fluid was analysed for total WBC count using an Advia120 Haematology instrument (Siemens UK) and for differential cell count. Protein content of BAL supernatant was determined using the Coomassie blue method (159). Remaining supernatant was stored at -80°C for subsequent lipid analysis. Following lavage, the lungs were weighed with the weight of the remaining lavage saline taken into account for lung wet weight to body weight (LWW:BW) ratio determinations. Measurements of lung wet weight to dry weight (LWW:DW) ratios were made for each lung lobe. Tissue block sections were made of each lung lobe, except the right median lavaged lobe, which were weighed and dried in a ventilated oven at approximately 60°C until weight was constant. BAL fluid was recovered and analysed by myself and DSTL Scientists credited in Acknowledgements.

2.1.8 Summary of my Role and Contributions to the Large Animal Model

The experiments conducted to generate the clinical data were a collaborative effort involving multiple team members. My specific responsibilities during these experiments included the following:

- Anaesthetic Support:
  I provided anaesthetic management for some of the animal subjects during the experimental procedures.
- Sample Collection:
  I participated in collecting physiological data, BAL and blood samples during the study. This included real-time monitoring and documentation of physiological variables and collection of

samples for laboratory analysis at point of care and for use with other instruments.

- Data Handling:
  While some of the physiological data were initially provided in pre-analysed graphical formats by collaborators, I performed a comprehensive reanalysis. This involved selecting appropriate statistical tests, applying them to the raw data, and critically interpreting the results.

- Experimental Planning and Protocol Adjustment:
  I contributed to the refinement of experimental protocols, including responses to challenges during the study.

The following tasks were undertaken by collaborators and are acknowledged in this chapter and Acknowledgements:

- Surgical procedures.
- Elements of the post-mortem examinations and some additional sample processing.
- Storage of physiological data.

This part of the work represents a significant team effort that cannot be achieved by a single person due to the time taken and multiple parallel tasks. My contributions focused on the experimental design, data collection, and analysis.

2.2 Lipid Extraction and Analysis

2.2.1 Lipid Extraction Technique Overview

Plasma and BAL samples were prepared with 10µl of 2% butylated hydroxytoluene (BHT) (Sigma- Aldrich), to limit lipid oxidation, prior to storage at -80°C. Lipid extraction was carried out using a modification of the technique developed by Bligh and Dyer (160). They described a rapid method of separating lipids in methanol and chloroform (trichloromethane) with non-lipids remaining in water and tissue residue. In this method we used dichloromethane (DCM) in place of chloroform.

2.2.2 Lipid Extraction Method

Samples were first thawed. For plasma 100µl and for BAL 800µl were removed and placed in a borosilicate glass tube. A final volume of 800µl was achieved with the addition of 700µl of 0.9% saline (Fluka/ milliQ) before adding 2ml of methanol (Fisher Scientific) and 1ml of dichloromethane (DCM, Fisher Scientific). 100µl of Internal Standard was added to plasma and 10µl to BAL samples. Internal Standard composition is listed in Table 4.

10µmol	DMPC	14:0/14:0 PC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
4µmol	DMPE	14:0/14:0 PE	Dimyristoyl phosphatidylethanolamine
2µmol	DMPG	14:0/14:0 PG	1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol
2µmol	DMPS	14:0/14:0 PS	1,2-dimyristoyl-sn-glycero-3-phospho-L-serine
1µmol	LPC	17:0 LPC	1-Heptadecanoyl-sn-glycero-3-phosphocholine
1µmol	06:0 SM	d18:1/6:0	N-hexanoyl-D-erythro-sphingosylphosphorylcholine

Table 4. Internal Standard for all mass spectrometric analyses. Contents were made up to 100ml with dichloromethane (DCM).

Internal Standard is used to quantify Lipids, compensating for variability in sample preparation, instrument response and other analytical conditions. Constituents were chosen for structural similarity and distinguishability from species of interest. Lids were placed on the tubes containing samples and extraction solvents and they were shaken vigorously by hand. Following this the tubes were centrifuged at 2200rpm for 10min at 20ºC with the brake on. The supernatant was then taken from each tube and placed in a fresh one. To the supernatant in fresh tubes were added 1ml of DCM and 1ml of High Performance Liquid Chromatography (HPLC) grade water. These were then sealed and shaken vigorously by hand, the phospholipids settling in the lower DCM phase. To sharpen the interface the samples were centrifuged at 2200rpm for 10min at 20ºC with the brake off. After centrifugation the lower phase was aspirated using a Pasteur pipette. Extreme care was taken to avoid contamination by the upper aqueous phase, the lower phases were placed in smaller borosilicate tubes and loaded into a dry block at 37ºC. Nitrogen gas was then delivered by needles above the samples to dry them. Protein and lipid residue would be left in the glass tubes once dry. After drying 500µl of DCM were added to the tubes without mixing and placed in a new borosilicate tube. This was repeated with the second 500µl added to the first to make a total of 1ml of DCM containing the extracted lipid, leaving the majority of protein residue in the first drying tubes. Once dry the tubes were

sealed and stored at -80ºC ready for mass spectrometric analysis. At all stages meticulous labelling of sample tubes prevented the mix up of samples.

2.2.3 Mass Spectrometry Overview

Mass spectrometry is a technique used to separate ions by charge and mass and present the results as a mass spectrum. Relative abundance, which is proportional to the detected signal intensity is presented on the y-axis, and mass, or mass/ charge ratio on the x- axis.

Electrospray ionisation (ESI) is a means of producing ions from macromolecules by applying a high voltage to a liquid and creating an aerosol. The advantage of this technique is that macromolecules can then be analysed in a mass spectrometer with minimal fragmentation occurring during the ionisation process.

Tandem mass spectrometry (MS/MS) is a two stage analysis of a mixture of ions including a selective examination of ion fragments. For this study a triple quadrupole mass spectrometer (TQMS) was employed to allow greater sensitivity, selectivity, accuracy and reproducibility. The arrangement is of two mass analysing quadrupoles (Q1 and Q3) and one collision cell (q2) depicted in Figure 29.

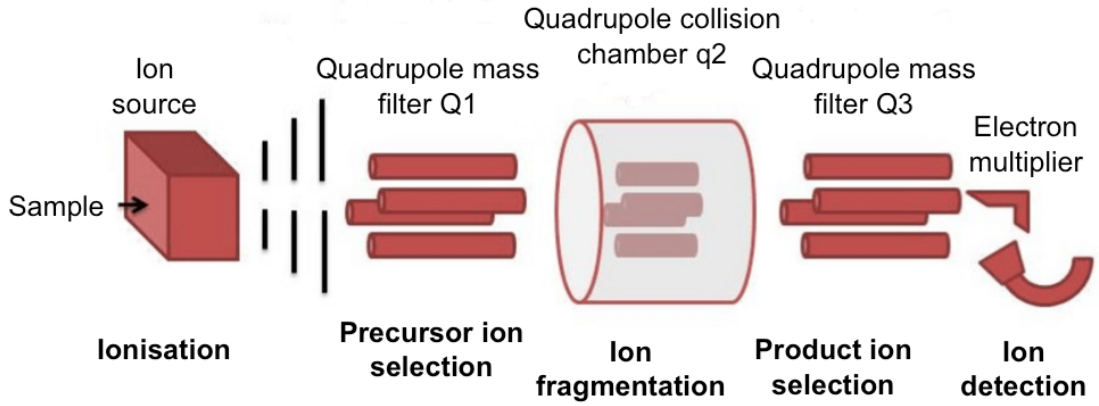

Figure 29. Arrangement of a triple quadrupole mass spectrometer. In this study ions were produced using electrospray ionisation (ESI) and the collision chamber, q2 filled with nitrogen. The three scan modes are described below. (*Image adapted from Wikimedia Commons*.)

Radio frequency and direct current potentials allow for mass selection in Q1 and Q3. A fixed radio frequency voltage is used in q2, used as a collision chamber (161). The arrangement allows three scan modes, product ion scan, precursor ion scan and neutral loss scan.

Product ion scan. (Q1 fixed/ Q3 scan) Ions of a given mass/ charge ratio are selected using Q1 and passed into q2. There they are induced to collide and fragments are analysed in Q3. A product or daughter ion spectrum is observed and the fragmentation data can be used to identify molecules in the sample (161).

Precursor ion scan. (Q1 scan/ Q3 fixed) Q3 is set to allow only ions of a certain mass/ charge ratio to pass. Q1 is set to scan over a certain mass range. Ions passing through Q1 will only be detected if, after fragmentation, the preselected product is produced and passes through Q3. This is also known as a parent ion scan (161).

Neutral loss scan. (Q1 scan/ Q3 scan) Both Q1 and Q3 are scanning together but with a constant mass/ charge ratio offset between them. Results can be used to identify ions that have lost a neutral fragment in q2 (161).

2.2.4 Mass Spectrometry Method

The prepared sample tubes from Section 2.2.2 were brought to room temperature. Lipids were then suspended in 300µl of mass spectrometry solvent, composition listed in Table 5.

Methanol	66% v/v
Dichloromethane	30% v/v
HPLC grade Water	3% v/v
Concentrated Ammonia solution	1% v/v

Table 5. Mass spectrometry solvent.

Using a syringe driver connected to capillary tubing the samples were introduced to an electrospray ionisation (ESI) chamber and then to a triple quadrupole mass spectrometer (TQMS) (Waters, USA) at a flow rate of 8µlmin^{-1}. Spectra were acquired in both ES+ and ES- polarities across a mass range of 2- 1300. Other conditions set were capillary 2.75kV, cone 30V, source temperature 150°C and MS/MS collision energy 19- 42eV. Collision induced fragmentation, or dissociation, and neutral loss scans were used to identify glycerophospholipid species. Collision induced fragmentation was used to identify glycerophospholipid species using the following scans:

- m/z184^{+}:
  - PC
  - LPC
  - SM
- m/z241^{-}:
  - PI
- m/z153^{-}:
  - PG

Neutral loss scans were used to identify PE and PS:

- m/z141^{+}:
  - PE
- m/z87^{-}:
  - PS

The instrument was managed and data collected using MassLynx^{TM} software (Waters, USA). Raw spectra collected from this process were then analysed using an analytical spreadsheet, analyserV4V5V5ab.xlsm (Koster, G), in Microsoft® Excel for Mac Version 16.96.1 (Microsoft Corporation, Redmond, WA, USA). All glycerophospholipids were selected and the major classes of PC, LPC, PG, PI and SM were further selected. Values for species of interest contributing >1% of the total signal were analysed. The internal standard enabled quantitative analysis by the analytical spreadsheet. Processed values were exported into respective Excel files for further handling and statistical analysis.

Lipid species are reported at the sum composition level; total acyl carbons: total double bonds, e.g. PC34:1, rather than assuming the commonest explicit acyl identities, e.g. PC16:0/18:1. The direct infusion MS/MS approach used here does not resolve isomeric acyl combinations, sn- positions or double bond locations. For lyso- species, the single acyl chain is given, e.g. LPC16:0. Figures therefore use sum composition labels, common species may be noted in the text, e.g. PC32:0, commonly PC16:0/16:0 or DPPC.

2.3 Statistical Analysis

2.3.1 Data Processing

Data were analysed using Microsoft Excel for Mac Version 16.96.1 and GraphPad Prism (Version 10.4.0 (527) for macOS, GraphPad Software, San Diego, CA, USA).

2.3.2 Physiological Data

Measurements of the following variables were assessed for normality using the Shapiro-Wilk test in GraphPad Prism:

- Respiratory Rate
- Oxygenation: P_aO_2
- Haemoglobin Saturation: S_aO_2
- Ventilation: P_aCO_2
- Lung Wet Weight: Body Weight Ratio
- Lung Wet Weight: Dry Weight Ratio
- BAL Protein Concentration
- BAL White Blood Cell Counts
- Arterial pH
- Arterial Bicarbonate
- Arterial Haematocrit
- Arterial Neutrophil Cell Count
- Central Venous Pressure
- Arterial Lactate Concentration

For each variable, normality was tested independently at each time point within each experimental group described in Section 2.1.5. At most time points the data were consistent with a normal distribution ($p > 0.05$), however some deviations from normality were observed. This was mainly in the Air Control group (n = 4), on account of the small group size, and most evident in

BAL Protein, Arterial Lactate and CVP measurements. Normality was more frequently observed in the CG CPAP group (n = 8).

Due to the variable sample size and the presence of non-normality at multiple time points, a non-parametric test was applied consistently to all groups to ensure methodological uniformity and statistical robustness. The Kruskal-Wallis test, a non-parametric equivalent of one-way ANOVA was used in GraphPad Prism to compare groups across the monitoring period. This test ranks all values and compares the distribution of ranks between groups. The test statistic (H) approximates a chi squared (χ^2) distribution with degrees of freedom (df) equal to the number of groups minus one, allowing calculation of a p value. The higher the H statistic, further apart the average ranks of groups are. Where significant differences were detected ($p < 0.05$), a post hoc Dunn's multiple comparison test identified specific group differences. However, GraphPad Prism does not support Kruskal- Wallis testing across multiple groups at individual time points in repeated measures datasets. Therefore, analyses are presented as overall differences across the monitoring period with Dunn's test used to identify specific group differences.

Where raw data were available, results are presented as mean ± Standard Error of the Mean (SEM), calculated as $SEM = \frac{SD}{\sqrt{\{N\}}}$ where N represents the number of animals per group, reflecting the precision of the mean estimate. In figures where raw data were not available and analysis was performed by collaborators credited in ACKNOWLEDGEMENTS, results are shown as mean ± Standard Deviation (SD), as originally provided, (Figure 45- Figure 49). This accounts for the difference in error bar representation between figures.

Derived measurement data was not made available to me in raw format, i.e. Shunt Fraction, Cardiac Output and Extravascular Lung Water. From the 24 h monitoring period, these data were analysed by collaborators, credited in ACKNOWLEDGEMENTS, using a linear mixed-effects model (http://CRAN.R-project.org/package=lme4) that describes the relationship between a response variable and one or more covariates recorded with it

(treatment, time and animal). The arms were compared against each other at all time points throughout the study. The fixed effects are compared using Tukey's multiple comparisons test within the lsmeans package within R (https://cran.r-project.org/web/packages/lsmeans/index.html) and the results were determined based on a 95% confidence level so found to be significant at $p < 0.05$. Results from these analyses are shown as mean ± SD, since this was the form in which the data were provided, and reanalysis was not possible without raw values. Arterial white blood cell count (WBC) data in Figure 45 were analysed by collaborators credited in ACKNOWLEDGEMENTS. A preliminary statistical screen to ensure acceptable fit for a one-way analysis of variance (ANOVA) was completed. Where data was shown to be non-normally distributed, a non-parametric ANOVA (Kruskal Wallis) was applied. Where the nonparametric ANOVA (Kruskal Wallis) was applied, and the H value found to be significant ($p < 0.05$) a Dunn's Test was used to examine planned comparisons of treatment medians (150). Results from the arterial WBC analyses in Figure 45 are shown as mean ± SD, since this was the form in which the data were provided, and reanalysis was not possible without raw values.

2.3.3 BAL Lipid Findings

For all bronchoalveolar lavage (BAL) lipid analyses, replicate measurements for each sample were averaged to produce a single data point per animal to avoid artificial inflation of sample size. Data are presented as mean ± SEM, calculated as $SEM = \frac{SD}{\sqrt{\{N\}}}$ where N represents the number of animals per group, using Excel and Prism.

Normality of distribution was assessed using the Shapiro- Wilk test. The vast majority (>92%) of datasets were consistent with a normal distribution, therefore a parametric method; two- way ANOVA, was applied throughout.

Two-way ANOVA was used to assess the effects of group on lipid amounts. Where ANOVA revealed significant effects, Tukey's multiple comparison test

was used to assess pairwise differences between exposure groups for lipids of interest. Statistical significance was defined as $p < 0.05$. Graphical illustration was generated by GraphPad Prism 10.

Linear regression was used to evaluate relationships between lipid species and between measured physiological variables and lipid species. The slope and goodness-of-fit (R^2) were reported, and the significance of the slope was assessed using an F-test. Group-specific analyses were conducted alongside pooled analyses to evaluate overall trends.

2.3.4 Plasma Lipid Findings

Normality of plasma lipid data was assessed using the Shapiro-Wilk test in GraphPad Prism. Several datasets, particularly fractional lipid species values and glycerophospholipid GPL classes, deviated from normality ($p < 0.05$) while others were consistent with a normal distribution. To ensure methodological uniformity and statistical robustness, all comparisons between CG Controls and CG CPAP were performed using the Mann-Whitney U test (two-tailed). This test ranks all values from both groups together and calculates the U statistic which describes the degree of overlap between rank distributions. A smaller U indicates a greater difference between groups.

This non-parametric test was selected because of deviations from normality in the data and there being only two independent groups. Kruskal-Wallis test is mathematically equivalent, however it cannot be implemented in GraphPad Prism when only two groups are present. Statistical significance was defined as $p < 0.05$. Graphical illustration was generated by GraphPad Prism 10.

3 - CLINICAL RESULTS

3.1 Introduction

The baseline characteristics of all groups were broadly similar and presented in Table 6, all Large White female pigs with a weight of 47- 55kg. Individual weights were not made available. The model showed that survival to 24h was significantly improved by the application of Positive End Expiratory Pressure (PEEP) as a surrogate for Continuous Positive Airway Pressure (CPAP). All CPAP treated phosgene exposed animals survived to 24h and all the air-exposed controls survived to 24h. Inhaled phosgene dose was extremely accurately delivered with a mean of 0.241 ± 0.008mgkg^{-1}. This resulted in death before 24h in half the untreated phosgene exposed animals, validating the dose as ideal, i.e. significant but not immediately lethal to the point of preventing data capture over a reasonable time. The mean *Ct* required to provide this inhaled dose was 1846 ± 432mgmin^{-1}m^{-3} (*C* = 185.4 ± 38.5 mgm^{-3} (~ 45 ppm); *t* = 9.94 ± 0.53 min). Survival data are shown in Figure 30. *p* values are represented as $p \leq 0.05$ = ★, $p \leq 0.01$ = ★★, $p \leq 0.001$ = ★★★ and $p \leq 0.0001$ = ★★★★.

Variable	**Air Controls** n = 4	**CG Controls** n = 10	**CG CPAP** n = 8	***p***
pH	7.38 ± 0.05	7.4 ± 0.04	7.43 ± 0.04	0.298
P_aO_2 (kPa)	8.05 ± 2.32	8.70 ± 1.91	8.99 ± 1.40	0.823
P_aCO_2 (kPa)	8.10 ± 1.24	7.63 ± 0.90	7.14 ± 0.64	0.248
HCO_3^- ($mmol.l^{-1}$)	31.65 ± 0.44	31.57 ± 1.73	32.19 ± 0.99	0.454
HCT (%)	31.3 ± 3.3	31.6 ± 2.5	30.4 ± 1.9	0.991
SpO_2 (%)	87.5 ± 10.1	90.5 ± 10.8	94.0 ± 5.2	0.516
CVP (mmHg)	1.3 ± 1.5	-2.3 ± 1.7	-2.3 ± 2.3	0.020 ✶
Resp Rate (bpm)	24 ± 6	29 ± 7	36 ± 11	0.121
Lactate ($mmol.l^{-1}$)	1.25 ± 0.60	1.32 ± 1.14	0.73 ± 0.32	0.268
Neutrophils ($x10^9.l^{-1}$)	4.94 ± 1.80	3.50 ± 1.75	4.48 ± 1.46	0.215

Table 6. Comparisons between groups' baseline characteristics were performed using Kruskal- Wallis test with Dunn's multiple comparison post test. Central Venous Pressure (CVP) differed significantly across groups ($p = 0.020$) with Air Controls showing higher values than CG Controls (Dunn's $p = 0.031$). No other baseline differences were significant (all $p > 0.05$). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.2 Respiratory Rate

There was a significant increase in respiratory rate in the phosgene control animals, exhibiting rapid shallow breathing, shown in Figure 31. Rates were

significantly higher than in both air exposed controls and CPAP treated animals, while Air Controls and CG CPAP did not differ. The change in respiratory rate is most easily explained by the alveolar air equation for the reasons outlined in Section 1.1.4. A significant drive to increase minute volume will result from detection of hypoxaemia by peripheral chemoreceptors (26). The factors resulting in hyperventilation in phosgene exposure were abolished in the CPAP arm.

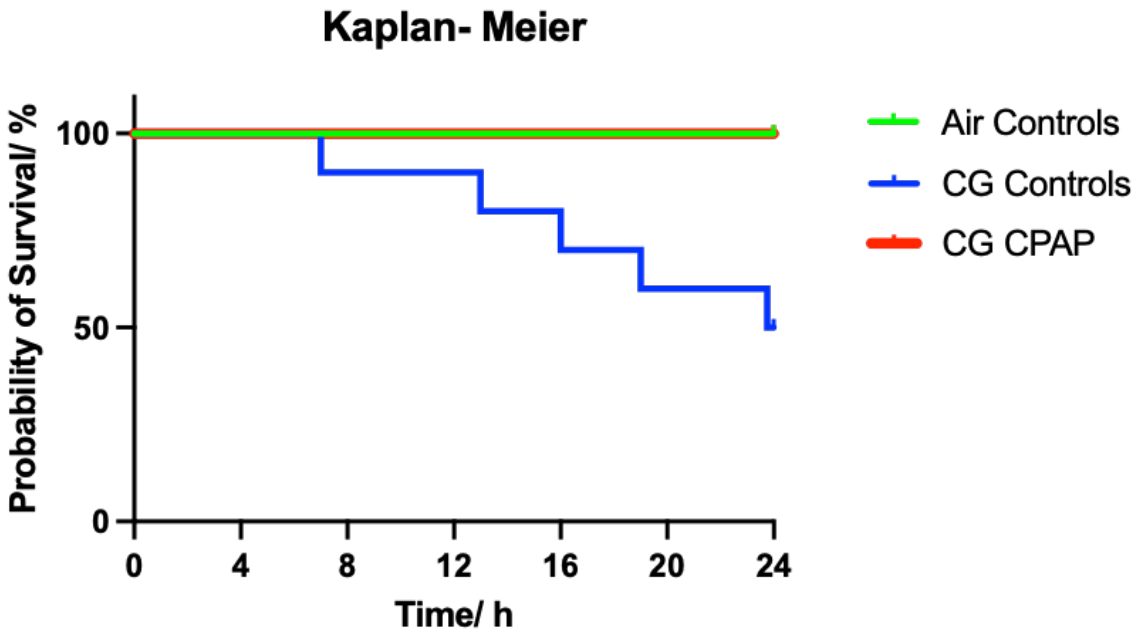

Figure 30. Kaplan-Meier survival graph for the three experimental groups. There were no deaths before 24 in the Air Controls and the CG CPAP treated animals. Half the CG Controls died before 24h, the majority after 12h. Survival differed significantly between groups (log-rank (Mantel-Cox) $\chi^2\,(2) = 7.65,\ p = 0.022$. Initial sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

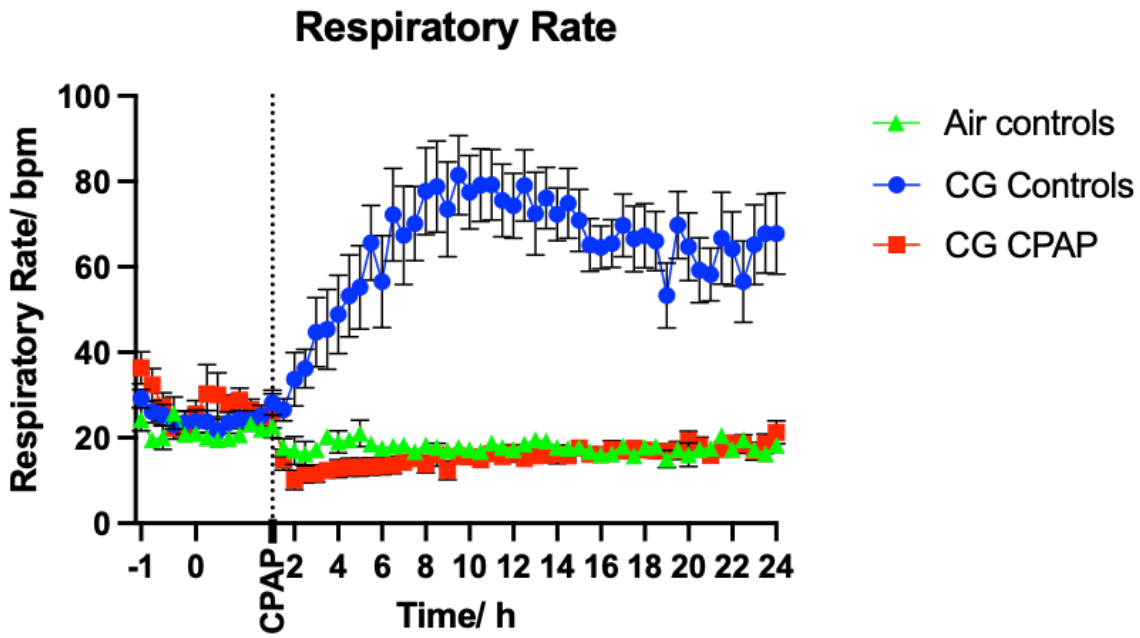

Figure 31. Changes in respiratory rate during the monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP 1h post exposure. CG Controls exhibited profound tachypnoea. Kruskal- Wallis test showed a significant overall difference among groups ($H = 110.3, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that respiratory rate was significantly higher in CG Controls compared with both Air Controls ($p < 0.0001$) and CG CPAP ($p < 0.0001$), whereas Air Controls and CG CPAP did not differ ($p = 0.193$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.3 Gas Exchange

3.3.1 Oxygenation

Phosgene exposure resulted in a significant reduction in partial pressure of oxygen in arterial blood (P_aO_2). Across the monitoring period, P_aO_2 was significantly lower in phosgene exposed animals compared with air exposed controls. While the initiation of CPAP attenuated this reduction, P_aO_2 did not return to the levels observed in the air control group, shown in Figure 32.

Oxygen saturation of arterial haemoglobin (S_aO_2) was significantly reduced in CG Controls and CG CPAP compared to Air Controls, Figure 33. Impairment to gas exchange resulted from the development of pulmonary oedema. Spatially heterogeneous pulmonary oedema will result in regions of lung attempting to minimise shunt by hypoxic pulmonary vasoconstriction. Additionally, the formation of microemboli contribute to ventilation/ perfusion mismatch.

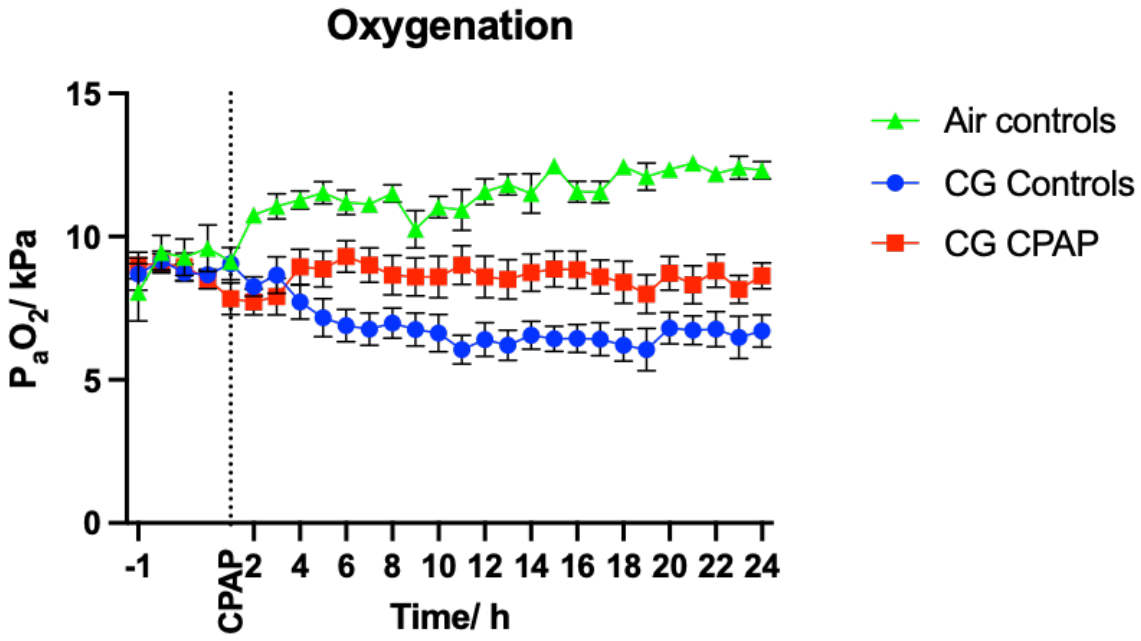

Figure 32. Changes in partial pressure of arterial oxygen (P_aO_2/ kPa) during the monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant overall difference among groups ($H = 60.13, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that P_aO_2 differed significantly between all groups: Air Controls v CG Controls ($p < 0.0001$), Air Controls v CG CPAP ($p < 0.0001$) and CG Controls v CG CPAP ($p = 0.0062$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

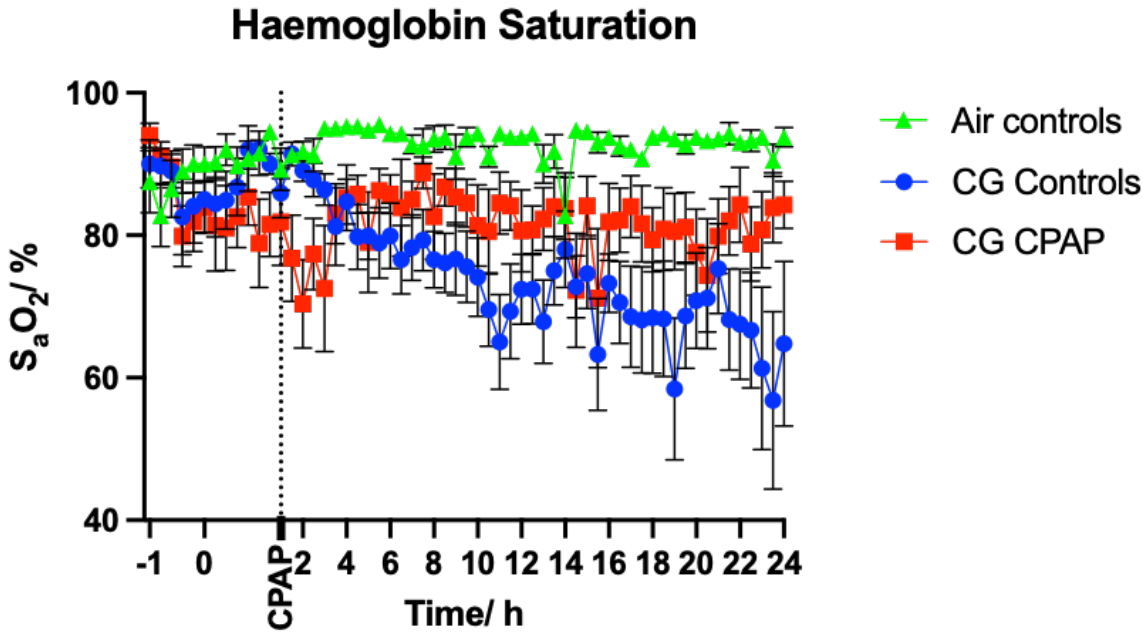

Figure 33. Changes in proportion of arterial haemoglobin saturated with oxygen (S_aO_2/ %) during the monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant overall difference in haemoglobin saturation among groups across the monitoring period ($H = 107.8, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that S_aO_2 was significantly lower in both phosgene exposed groups compared with Air Controls ($p < 0.0001$ for both), whereas CG Controls and CG CPAP did not differ significantly ($p = 0.0615$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.3.2 Ventilation

Figure 34 illustrates that the arterial partial pressure of carbon dioxide (P_aCO_2) was significantly higher in the phosgene with CPAP group compared to the air and phosgene control groups after CPAP initiation. No significant differences in P_aCO_2 were observed between the air and phosgene control groups. A fall in P_aCO_2 is expected in untreated animals as a result of hyperventilation to support oxygenation as explained in Section 1.1.4. In the

CPAP group, however P_aCO_2 rose after the application of PEEP. The increase in dead space by the physical attachment of a ventilator circuit to apply PEEP in the CPAP arm may have accounted for some of the increase in P_aCO_2. This was partially compensated by an increase in bicarbonate, helping to buffer pH. Cardiac output and temperature, other determinants of P_aCO_2 were not significantly different between the groups.

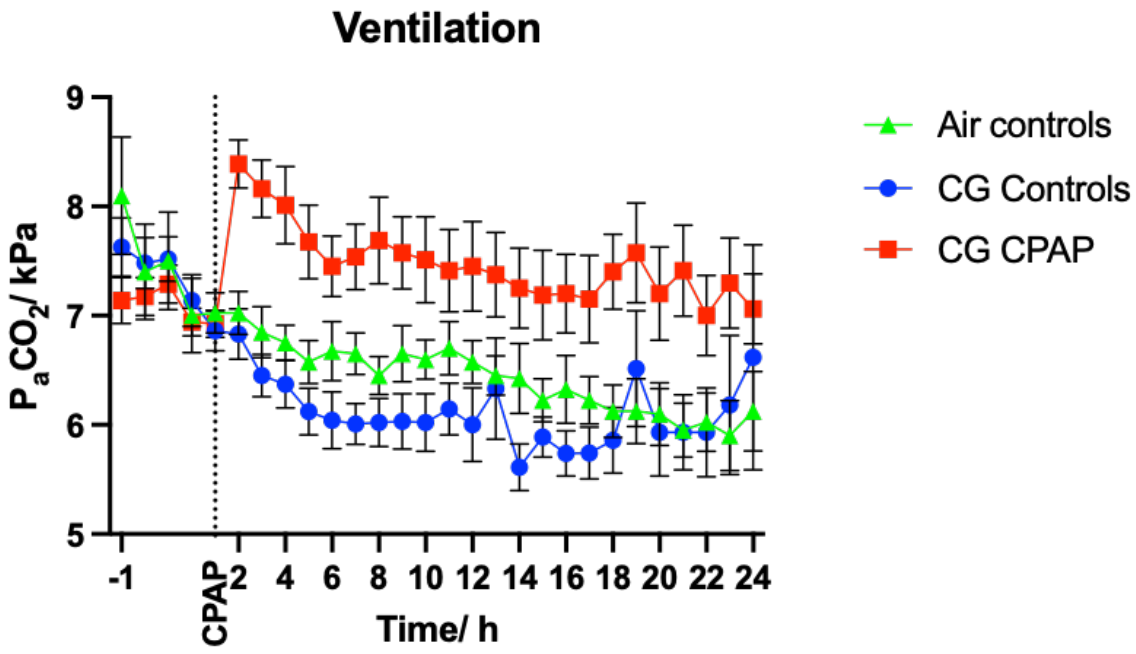

Figure 34. Changes in partial pressure of arterial carbon dioxide (P_aCO_2/ kPa) during the monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant overall difference between groups ($H = 40.58, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that PaCO2 was significantly higher for CG CPAP versus both Air Controls and CG Controls ($p < 0.0001$ for both), whereas Air Controls and CG Controls did not differ significantly ($p = 0.311$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.4 Measures of Lung Oedema

3.4.1 Lung weights

Lung weights reflect the degree of pulmonary oedema, or extravascular lung water (EVLW) resulting from increased alveolar permeability. Weights were measured post mortem and expressed as pooled variables irrespective of the time of death of the animals in the phosgene control group. Lung wet weight to bodyweight (LWW:BW) ratio data is displayed in Figure 35 and shows a significant increase in LWW:BW in the phosgene control group as compared with the air control group and no significant difference between the phosgene with CPAP group and the other groups. Lung wet weight to dry weight ratios (LWW:DW) were elevated significantly in both phosgene exposed controls and CPAP treated animals, in left and right apical lobes, compared with air exposed controls. Figure 36 illustrates the apical lung lobe findings. The right medial lobes were lavaged and could not be assessed for LWW:DW. A reanalysis of whole LWW:DW ratio revealed significantly higher lung water in CG Controls compared with Air Controls. The CG CPAP group exhibited intermediate values but did not differ significantly from either group. This supports the interpretation that PEEP applied one hour post exposure attenuates phosgene induced pulmonary oedema; CG CPAP resembled Air Controls more closely than CG Controls. PEEP is known to improve oxygenation through stabilisation of atelectatic alveoli and maximising volume of lung where alveolar pressure exceeds vascular pressure. Reduction in extravascular lung water (EVLW), measured indirectly or at post mortem has not been a consistent observation (162) and increases in extravascular lung water observed under some circumstances may be due to other effects of PEEP or CPAP impairing pulmonary lymphatic drainage (163). However, these effects may not be relevant to porcine models (164). The findings are consistent with previous porcine studies of phosgene in as much as lung oedema is greater in phosgene exposed animals but there is no statistically significant reduction in oedema in CPAP treated animals compared with phosgene controls.

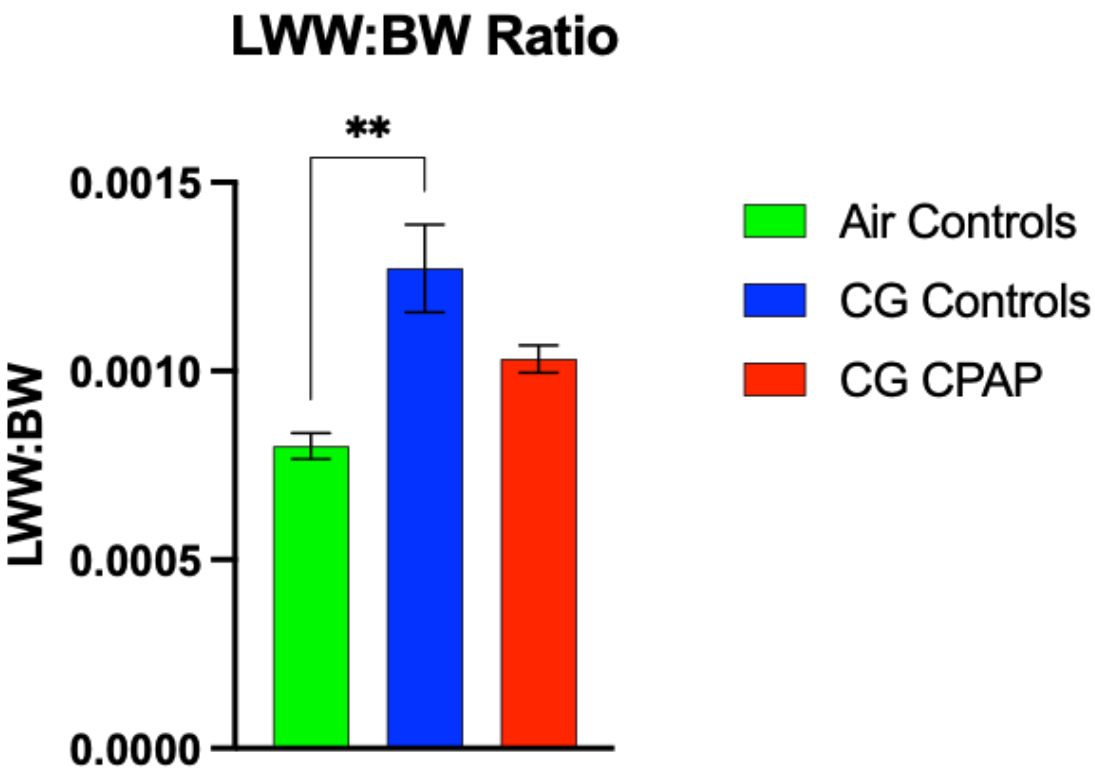

Figure 35. Lung wet weight to bodyweight (LWW:BW) ratio. Data expressed as mean ± SEM. Kruskal- Wallis test showed a significant difference between groups ($H = 9.47, df = 2, p = 0.0038$). Dunn's multiple comparisons confirmed that the LWW:BW ratio was significantly increased in CG Controls when compared to Air Controls ($p = 0.0067$), ★★. No significant differences were detected between the CG CPAP group and the two control groups ($p = 0.0552$ v Air Controls), ($p > 0.9999$ v CG Controls). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

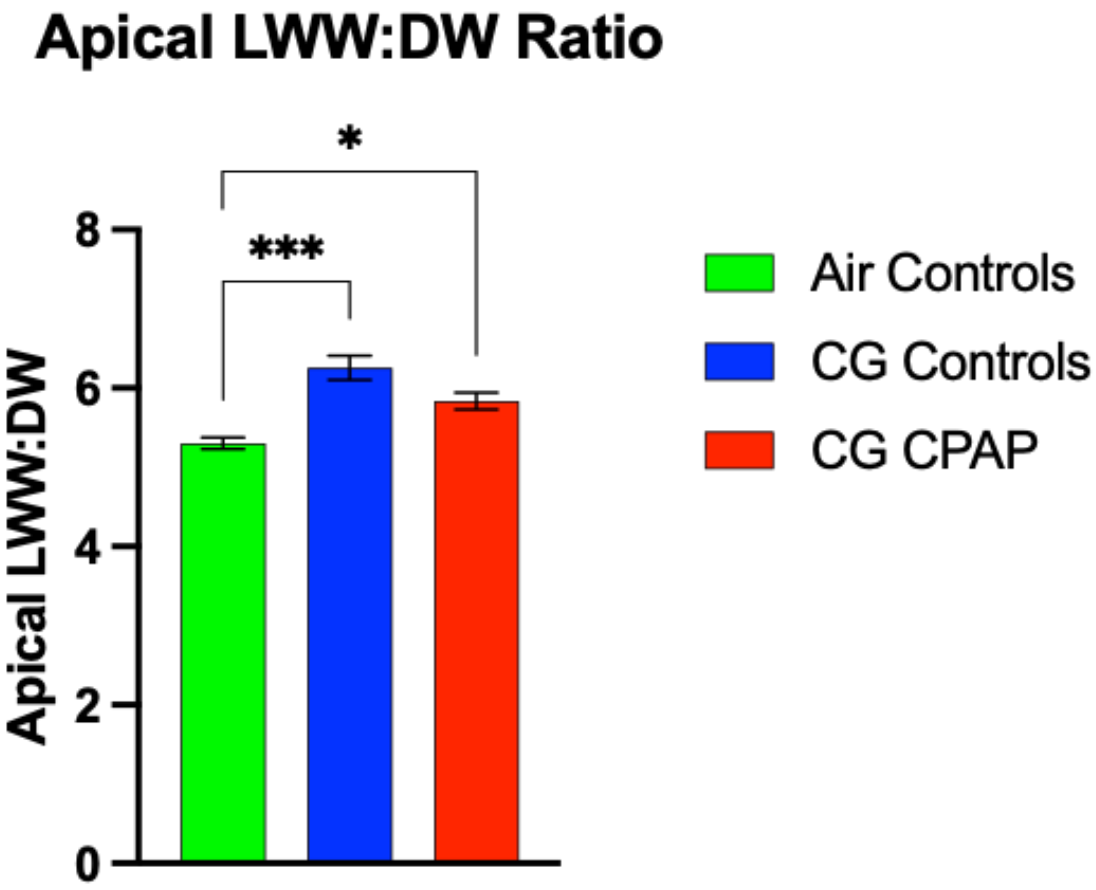

Figure 36. Lung wet weight to dry weight (LWW:DW) ratio in apical lobes. Data expressed as mean ± SEM. Kruskal- Wallis test showed a significant difference between groups ($H = 17.23, df = 2, p = 0.0002$). Dunn's multiple comparisons confirmed that LWW:DW was significantly higher in both phosgene exposed groups compared with Air Controls (Air Controls v CG Controls, $p = 0.0001$, ★★★ and Air Controls v CG CPAP $p = 0.0196$, ★), whereas there was no significant difference between CG Controls and CG CPAP ($p = 0.291$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

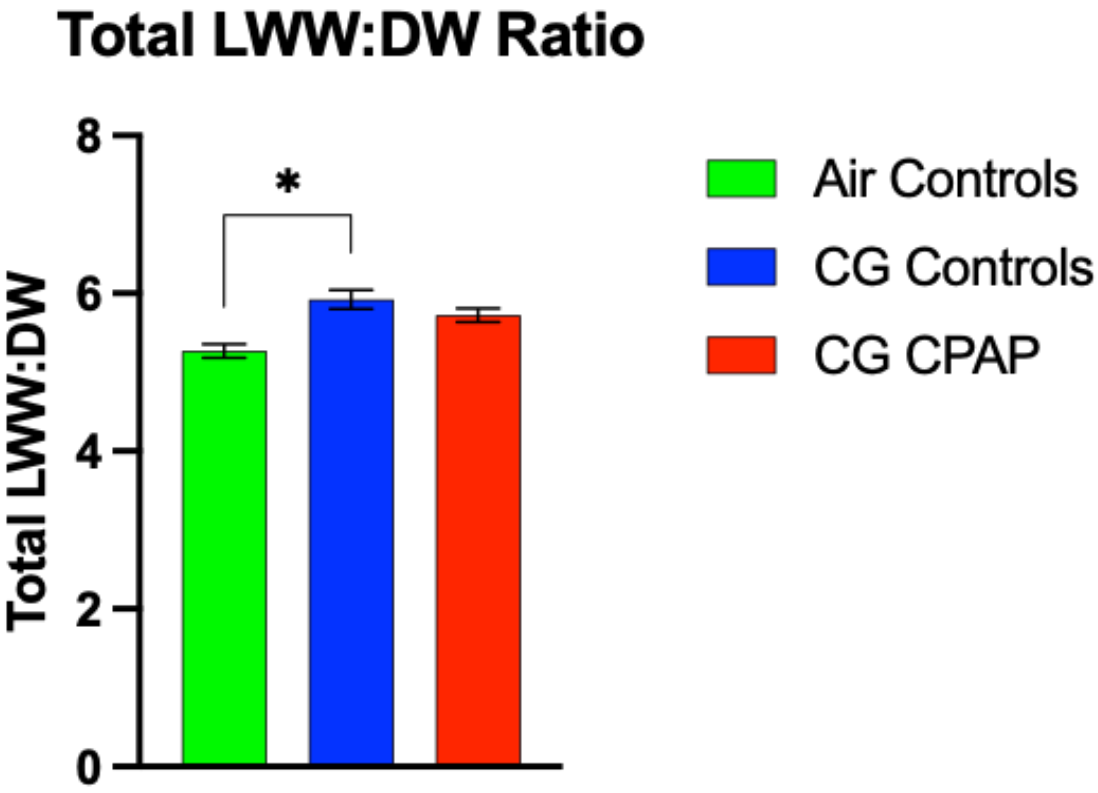

Figure 37. Total Lung Wet Weight: Dry Weight (LWW:DW) ratio. Data expressed as mean ± SEM. Kruskal- Wallis test showed a significant difference between groups ($H = 8.562, df = 2, p = 0.0077$). Dunn's multiple comparisons confirmed that LWW:DW was significantly higher in CG Controls compared Air Controls ($p = 0.0106$, ✶) whereas there was no significant difference between CG CPAP and either Air Controls ($p = 0.0866$) or CG Controls ($p > 0.9999$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.4.2 Bronchoalveolar Lavage Protein

Phosgene caused a significant increase in the protein concentration of terminal lavage fluid from the right medial lobes when compared to the air controls as measured using Coomassie blue (159) (Figure 38). Protein concentration in phosgene with CPAP animals was intermediate and not

significantly different from either control group suggesting attenuation of lung injury by PEEP. This implies that the mechanisms described previously are partially modified by PEEP.

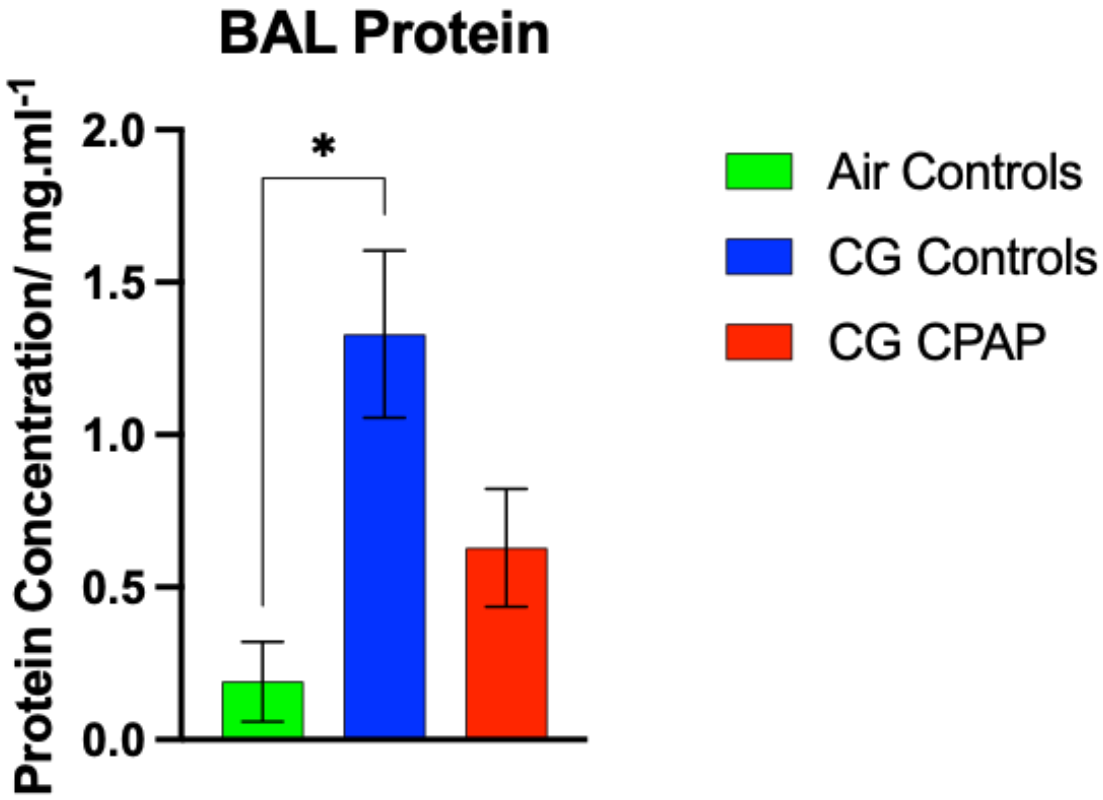

Figure 38. Terminal bronchoalveolar lavage protein concentration expressed as mean ± SEM. Kruskal- Wallis test showed a significant overall difference among groups ($H = 8.26, df = 2, p = 0.0096$). Dunn's multiple comparisons confirmed that protein concentration was significantly higher in CG Controls compared Air Controls ($p = 0.0153$, ✶) whereas there was no significant difference between CG CPAP and either Air Controls ($p = 0.431$) or CG Controls ($p > 0.326$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.4.3 Bronchoalveolar Lavage White Blood Cell Counts

The absolute white blood cell (WBC) counts of terminal BAL fluid showed that the most abundant cells were alveolar macrophages. Neutrophil numbers increased markedly in the phosgene exposed animals compared with air controls. No significant differences were found in lymphocyte and alveolar macrophage counts, as shown in Figure 39. Increased neutrophils in BAL were expected because of the inflammatory nature of phosgene induced acute lung injury and this has been observed in previous studies of ALI/ ARDS, including phosgene (70, 72, 101, 165). Neutrophil influx into alveoli is a defining characteristic of ARDS and is mediated by a number of chemokines (166, 167). Neutrophil content of BAL fluid is positively correlated with inflammation severity and ultimately risk of death and the findings here are consistent with previous reports (168-170).

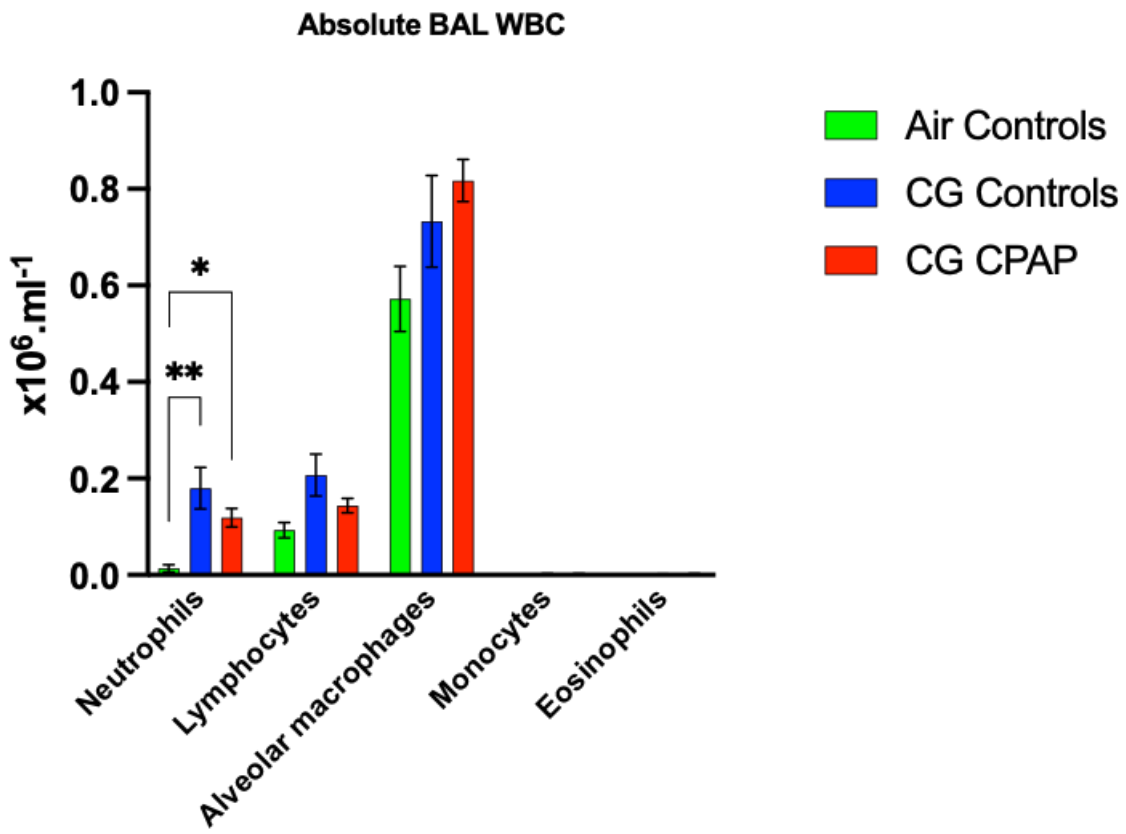

Figure 39. Absolute white blood cell (WBC) counts of terminal bronchoalveolar lavage (BAL) fluid. Data expressed as mean ± SEM. Differences between groups were not significant for lymphocytes or alveolar macrophages. However, Kruskal- Wallis test showed a significant difference in neutrophils among groups ($H = 10.13, df = 2, p = 0.0022$). Dunn's multiple comparisons confirmed that neutrophil counts were significantly higher in both phosgene exposed groups compared with Air Controls. Air Controls v CG Controls ($p = 0.0046$, ★★), Air Controls v CG CPAP ($p = 0.0480$, ★), whereas CG Controls and CG CPAP did not differ significantly ($p > 0.9999$). Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

The increase in absolute neutrophil count in the terminal BAL fluid resulted in a concomitant decrease in fraction of alveolar macrophages in the phosgene exposed controls. The increase in neutrophil fraction in phosgene exposed

controls was significant when compared with air exposed controls. PEEP treated animals showed intermediate values that did not differ significantly from either group. The corresponding reduction in alveolar macrophage fraction did not reach statistical significance and lymphocyte fractions were less affected. The differential WBC counts are shown in Figure 40.

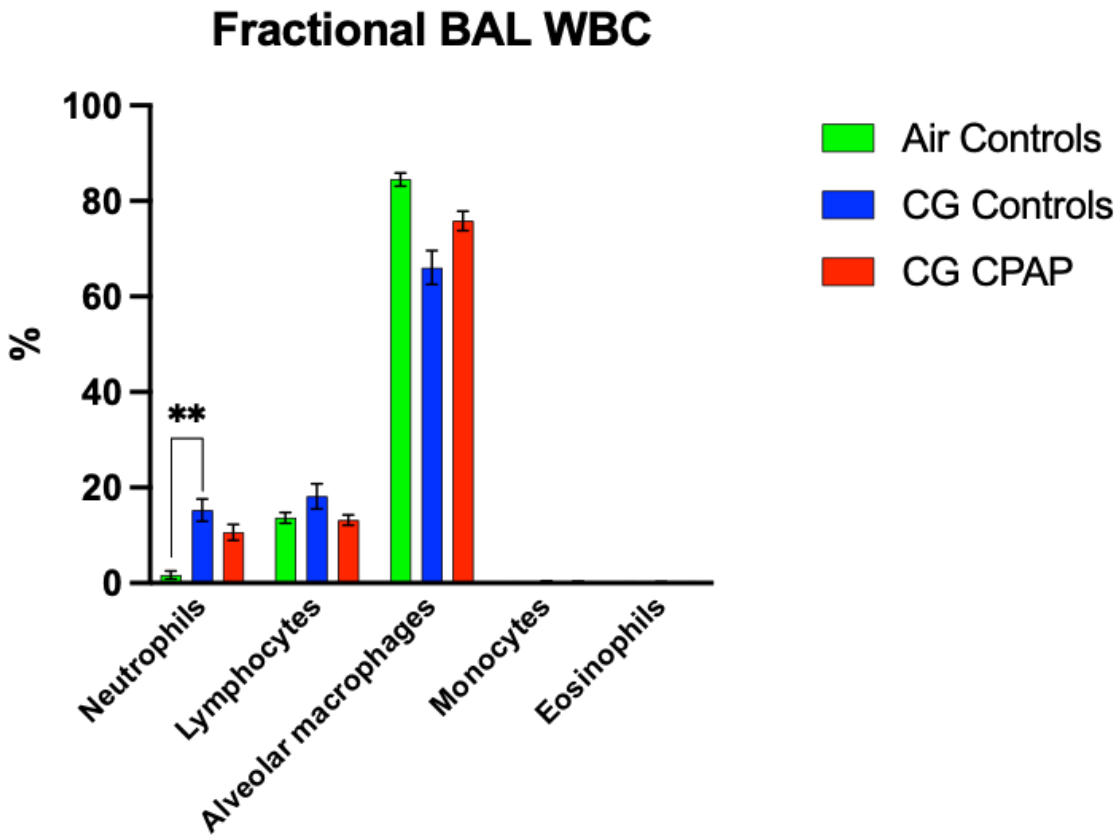

Figure 40. Fractional white blood cell (WBC) counts in terminal bronchoalveolar lavage (BAL) fluid. Data expressed as mean ± SEM. Kruskal- Wallis test showed a significant difference in the neutrophil fraction among groups ($H = 11.22, df = 2, p = 0.0009$). Dunn's multiple comparisons confirmed that the neutrophil fraction was significantly higher in CG Controls compared with Air Controls ($p = 0.0025$, ★★), whereas CG CPAP did not differ significantly from either group. (Air Controls v CG CPAP, $p = 0.0866$; CG Controls v CG CPAP, $p = 0.5294$). No significant differences were detected between groups for lymphocytes or alveolar macrophage fractions. Sample sizes: Air Controls (n = 4), CG Controls (n = 10, decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.5 Acid Base

3.5.1 pH

Arterial pH was significantly lower in the phosgene with CPAP animals compared with both air and phosgene controls (Figure 41). This decrease in pH followed the application of PEEP and mirrored the rise in P_aCO_2 observed in the same group, as described in Section 3.3.2. Air and phosgene control groups did not differ significantly in arterial pH. These findings suggest that the hypercapnia, induced by the PEEP apparatus was not fully compensated for by renal bicarbonate retention until later in the experiment. This is consistent with the known time course of renal compensation for respiratory acidosis (171).

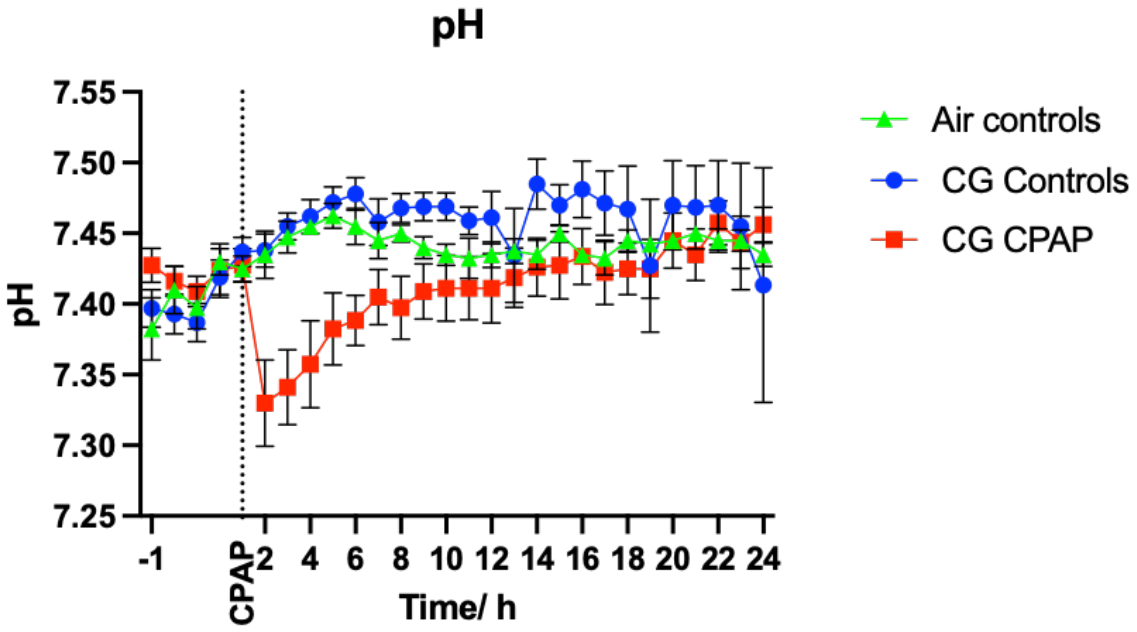

Figure 41. Changes in arterial pH over the course of the monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 27.08, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that pH was significantly lower in CG CPAP compared with both Air Controls ($p = 0.0072$) and CG Controls ($p < 0.0001$). No significant differences were observed between Air Controls and CG Controls ($p = 0.0969$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.5.2 Bicarbonate

There were no significant differences in arterial bicarbonate concentration between the air and phosgene control groups. Arterial bicarbonate was significantly increased in the phosgene with CPAP group compared to the phosgene control group. Air exposed control animals showed intermediate values that were not significantly different from either group (Figure 42). This increase observed in the CPAP group is consistent with renal compensation for the respiratory acidosis induced by the increase in dead space of the ventilator circuit used to apply PEEP (171).

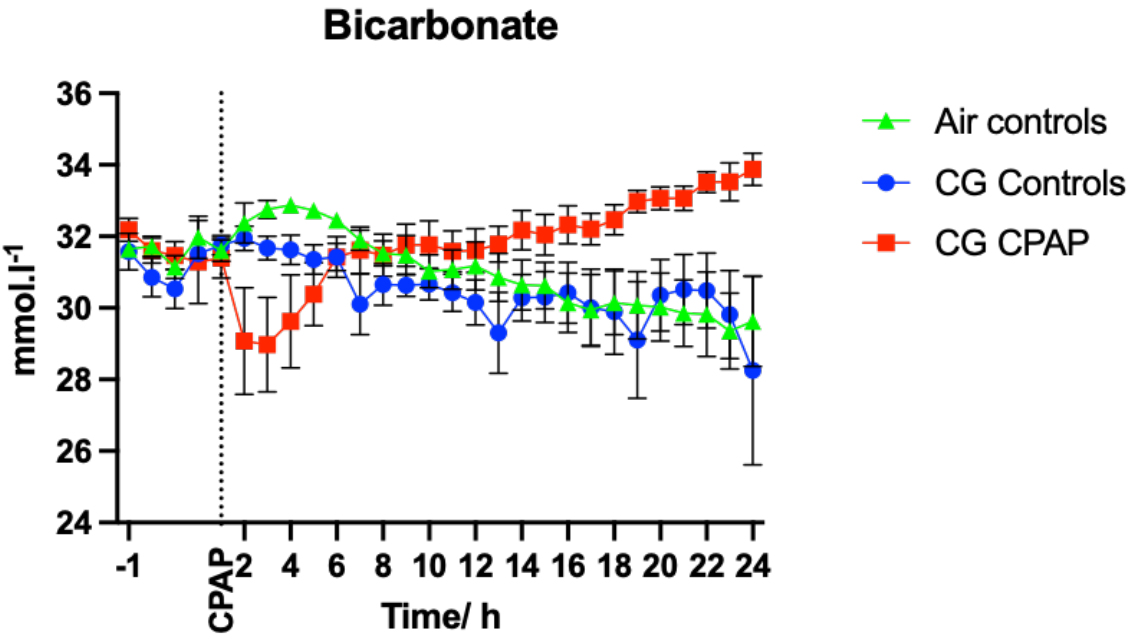

Figure 42. Changes in bicarbonate observed over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 16.23, df = 2, p = 0.0003$). Dunn's multiple comparisons confirmed that bicarbonate was significantly higher in the CG CPAP group compared with CG Controls ($p < 0.0002$), whereas Air Controls did not differ significantly from either group (Air Controls v CG Controls $p = 0.2991$, Air Controls v CG CPAP $p = 0.0546$) Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.6 Haematology

3.6.1 Haematocrit

Haematocrit differed significantly between groups. A significant increase in haematocrit was observed in the phosgene control animals when compared to the air exposed controls. Phosgene exposed CPAP animals experienced an intermediate but significant increase in haematocrit. This pattern is consistent with haemoconcentration from intravascular fluid loss in phosgene

exposed animals, partially attenuated by PEEP. This is illustrated in Figure 43. As was noted in casualties in World War 1, significant fluid losses occur in phosgene poisoning. These losses are so massive that they result in intravascular depletion so great that haematocrit is increased. The change in haematocrit observed in the phosgene controls is from 0.3 to 0.35 and this can be used to *estimate* fluid losses:

Assumptions:

- Weight = 50kg; blood volume (**BV**) = 70ml kg^{-1}
- Haematocrit (**HCT**) increased from 0.3 to 0.35 in CG Controls
- **HCT** is unchanged in the Air Controls
- Red Cell Volume (**RCV**) is constant
- Intravenous maintenance Vetivex 11 (Hartmann's solution) = 2ml kg^{-1} h^{-1} (100ml h^{-1}) as described in Section 2.1.2.
- ~25% of isotonic crystalloid remains intravascular at steady state (172)
- Urine output and temperature are the same in all groups

*Plasma volume (**PV**) changes by **HCT***:

- Initial **BV**, $\mathbf{BV_0} = 70 \times 50 = 3500ml$
- $\mathbf{RCV} = \mathbf{BV_0} \times \mathbf{HCT_0} = 3500ml \times 0.3 = 1050ml$
- Final **BV**, $\mathbf{BV_1} = \mathbf{RCV/HCT_1} = \mathbf{1050/0.35 = 3000ml}$
- Initial **PV**, $\mathbf{PV_0} = \mathbf{BV_0} - \mathbf{RCV} = 2450ml$
- Final **PV**, $\mathbf{PV_1} = \mathbf{BV_1} - \mathbf{RCV} = 1950ml$
- Observed fall in **PV**, $\Delta\mathbf{PV} = \mathbf{PV_0} - \mathbf{PV_1} = 2450 - 1950 = 500ml$

Intravascular contribution of maintenance fluid:

- Infused $100ml h^{-1} \times 24 = 2400ml$
- Intravascular share $0.25 \times 2400 = 600ml$

Total intravascular loss: $\Delta\mathbf{PV} + 600 = 1100ml$

Thus, the haematocrit rise from 0.3 to 0.35 implies an estimated 1100ml intravascular fluid loss, consistent with a substantial capillary leak that was clearly manifest as copious airway fluid losses in the phosgene exposed controls.

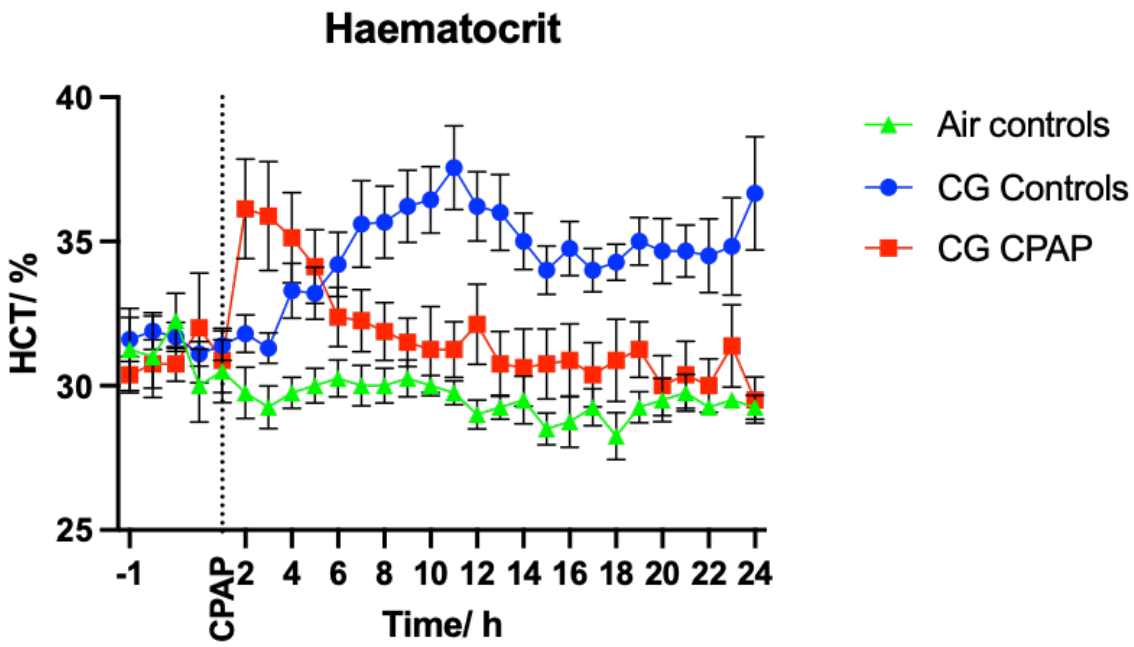

Figure 43. Changes in haematocrit observed over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 55.28, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that haematocrit was significantly lower in Air Controls compared with both CG Controls ($p < 0.0001$) and CG CPAP ($p = 0.0002$), and significantly higher in CG Controls compared with CG CPAP ($p = 0.002$. Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.6.2 Arterial White Blood Cells

The air exposed controls maintained a white blood cell profile within normal ranges for the duration of the study. After phosgene exposure, the blood neutrophil count increased, most markedly in the CPAP treated group, Figure 44. When phosgene exposed controls were compared to air exposed controls, the neutrophil count did not differ appreciably. CPAP treated animals showed higher counts, in keeping with the inflammatory recruitment occasionally observed in ARDS patients (165). The corresponding differential white blood cell counts are shown in Figure 45.

Although circulating neutrophils increased most noticeably in CPAP treated animals, BAL results suggested that CPAP may have limited neutrophil migration into the alveolar space. Both of the phosgene exposed groups showed elevated neutrophils in BAL compared with air exposed controls, but values in the CPAP group were intermediate. This pattern is compatible with CPAP as a stabiliser of alveolar- capillary integrity and reducing neutrophil trafficking into the lungs (173), even in the face of systemic neutrophil mobilisation. There is a paucity of data to support such a difference in blood and BAL compartments in response to PEEP. The observed effect supports CPAP's potential to protect alveoli in an inflammatory environment.

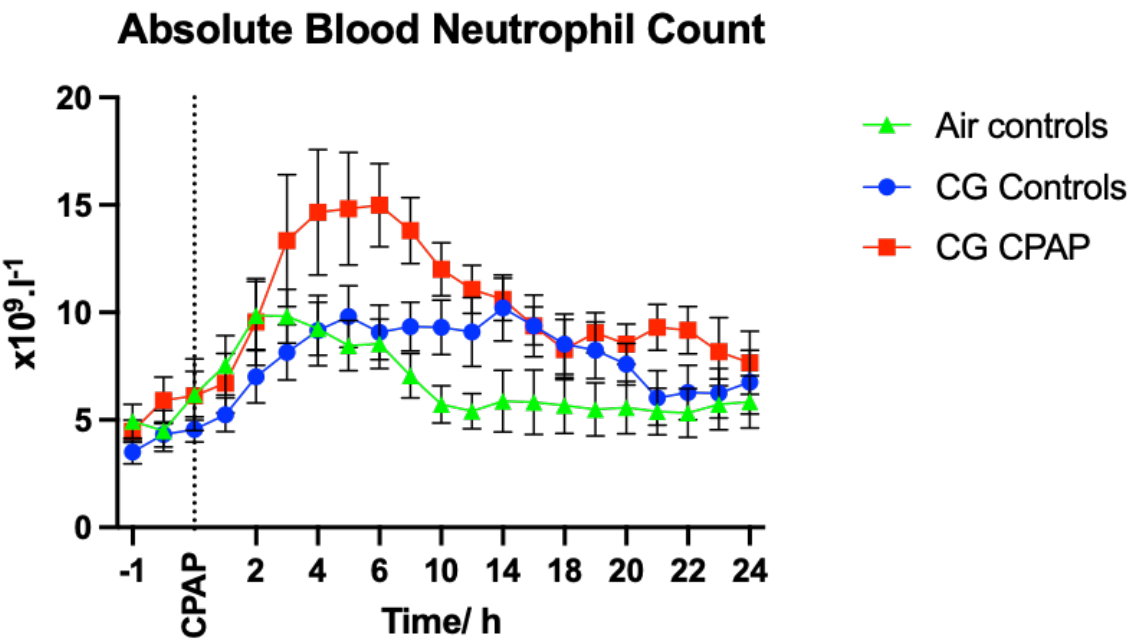

Figure 44. Changes in blood neutrophil count observed over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant overall difference among groups ($H = 14.7, df = 2, p = 0.0006$). Dunn's multiple comparisons confirmed that neutrophil counts were significantly higher in CG CPAP compared with Air Controls ($p = 0.0004$), whereas differences between Air Controls and CG Controls ($p = 0.352$) and between CG Controls and CG CPAP ($p = 0.352$) did not reach significance. Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

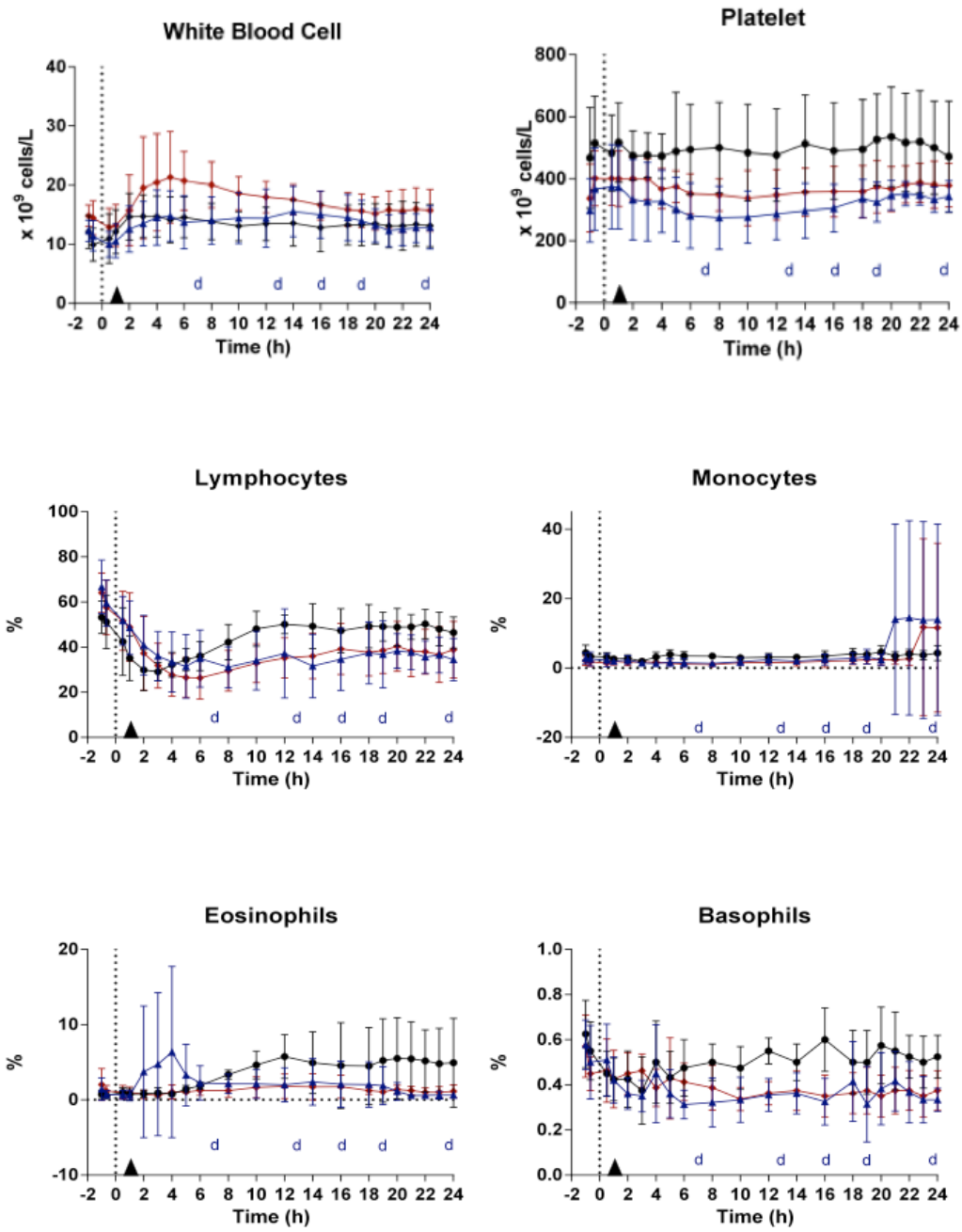

Figure 45. Changes in blood total white blood cell count (WBC), platelets and differential white cell count observed over the course of the 24h monitoring period expressed as mean ± SD, as provided by collaborators credited in ACKNOWLEDGEMENTS and explained in Section 2.3.2 (raw data not available for reanalysis). Air Controls (● n=4). CG Controls (▲ n= 10 decreasing to 5). CG CPAP (◆ n=8). ▲ denotes initiation of CPAP at 1h. d represents the death of an animal from the phosgene control group. CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

In recent years Neutrophil to Lymphocyte Ratio (NLR) has emerged as a marker of prognosis in a diverse range of diseases. These include sepsis, pneumonia and COVID-19 (174). NLR increased after phosgene exposure, both phosgene exposed groups showed significantly higher NLR values than air controls, whereas PEEP treated animals did not differ significantly from phosgene controls, Figure 46. This suggests that phosgene exposure triggered a systemic inflammatory response that was not substantially modified by the application of PEEP. The increase in NLR indicated a systemic inflammatory response and could serve as a non-specific indicator of exposure. However, NLR did not correlate with survival, or treatment effect, therefore it is not appropriate for prognostication in this context.

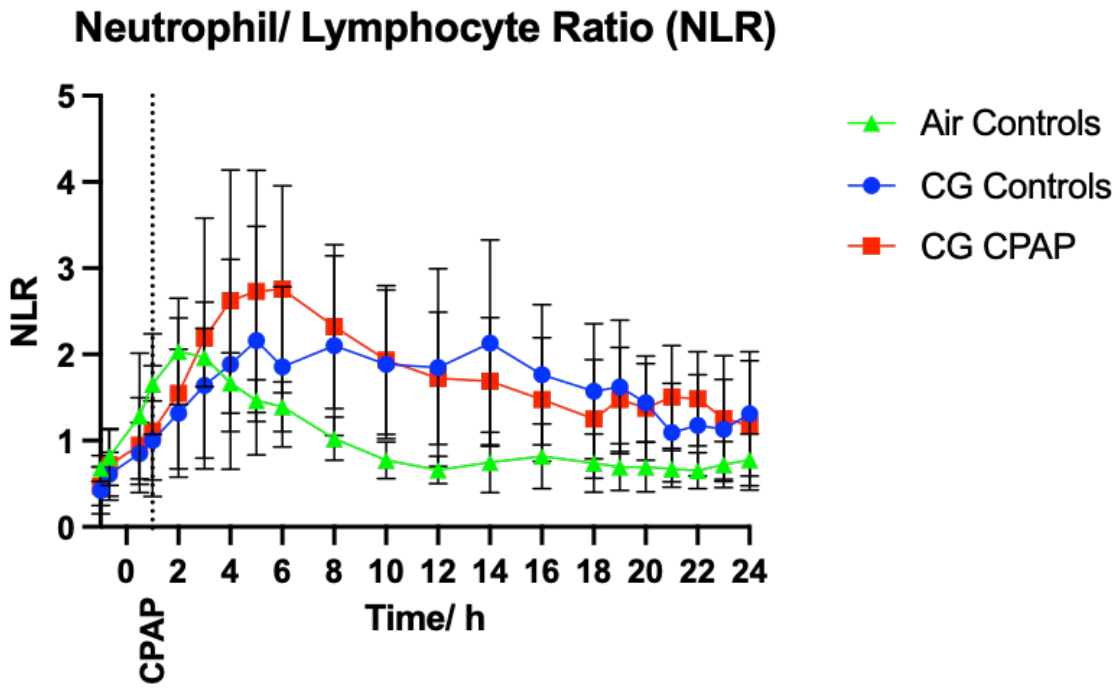

Figure 46. Neutrophil/ lymphocyte ratio (NLR) over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 10.38, df = 2, p = 0.0056$). Dunn's multiple comparisons confirmed that NLR was significantly higher in both phosgene exposed groups compared with Air Controls; Air Controls v CG Controls, $p = 0.0323$; Air Controls v CG CPAP, $p = 0.0086$. CG Controls and CG CPAP did not differ significantly ($p > 0.9999$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.7 Derived Measurements

Derived measurements were calculated by a PiCCO monitor (PV8115, Pulsion Medical Systems, Munich, Germany) and estimated using standard formulae as described in Section 2.1.6.

3.7.1 Shunt Fraction

Shunt fraction is the proportion of blood passing through areas of unaerated lung. In the collaborator provided analysis, there was a significant increase in shunt fraction in the phosgene control animals compared to air exposed controls from 5h and it remained high for the duration of the monitoring period, Figure 47. This is most likely due to volumes of unaerated lung in excess of compensatory mechanisms such as hypoxic pulmonary vasoconstriction. The shunt fraction of the phosgene with CPAP group was intermediate and not significantly different to either of the control groups.

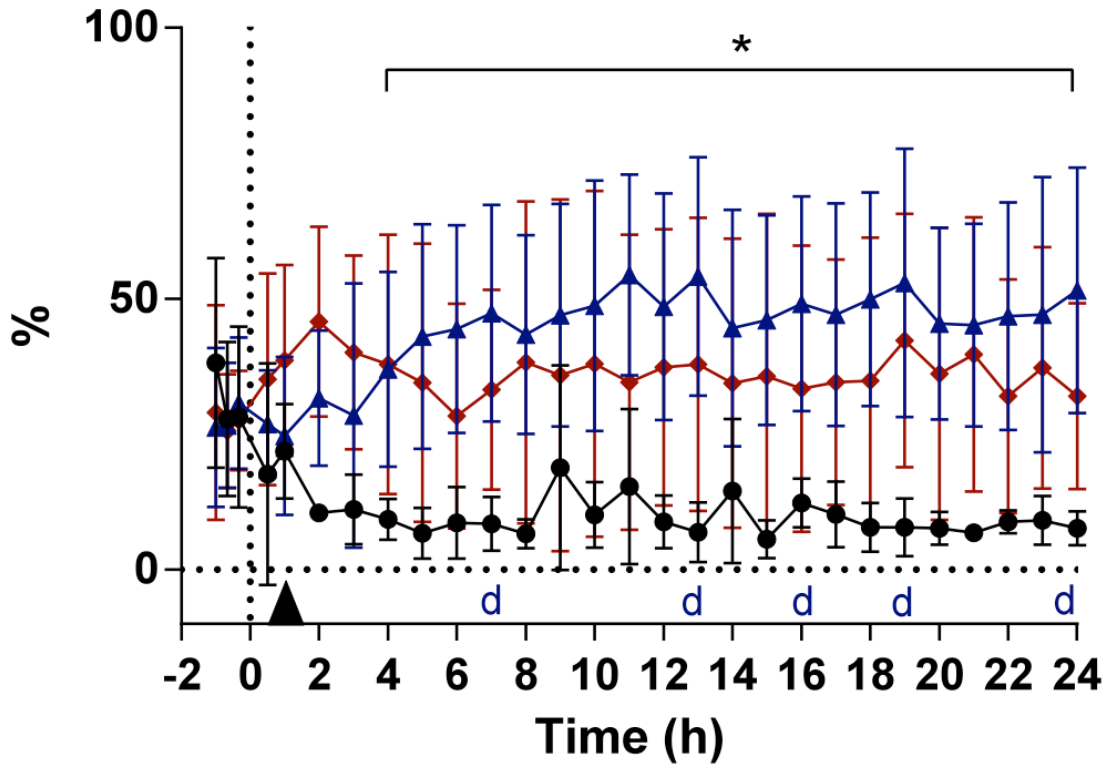

Figure 47. Change in shunt fraction observed over the course of the 24h monitoring period expressed as mean ± SD as provided by collaborators credited in ACKNOWLEDGEMENTS and explained in Section 2.3.2 (raw data not available for reanalysis). Air Controls (● n=4). CG Controls (▲ n= 10 decreasing to 5). CG CPAP (◆ n=8). ▲ denotes initiation of CPAP at 1h. d represents the death of an animal from the phosgene control group. ✱= significant difference between phosgene and air controls. CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.7.2 Cardiac Output

Cardiac output, stroke volume, contractility, stroke volume variation and systemic vascular resistance did not significantly differ between groups in the collaborator provided analysis. The application of PEEP did not adversely affect measured cardiovascular variables and differences in survival are

more likely attributable to pulmonary pathology. Cardiac output data are shown in Figure 48.

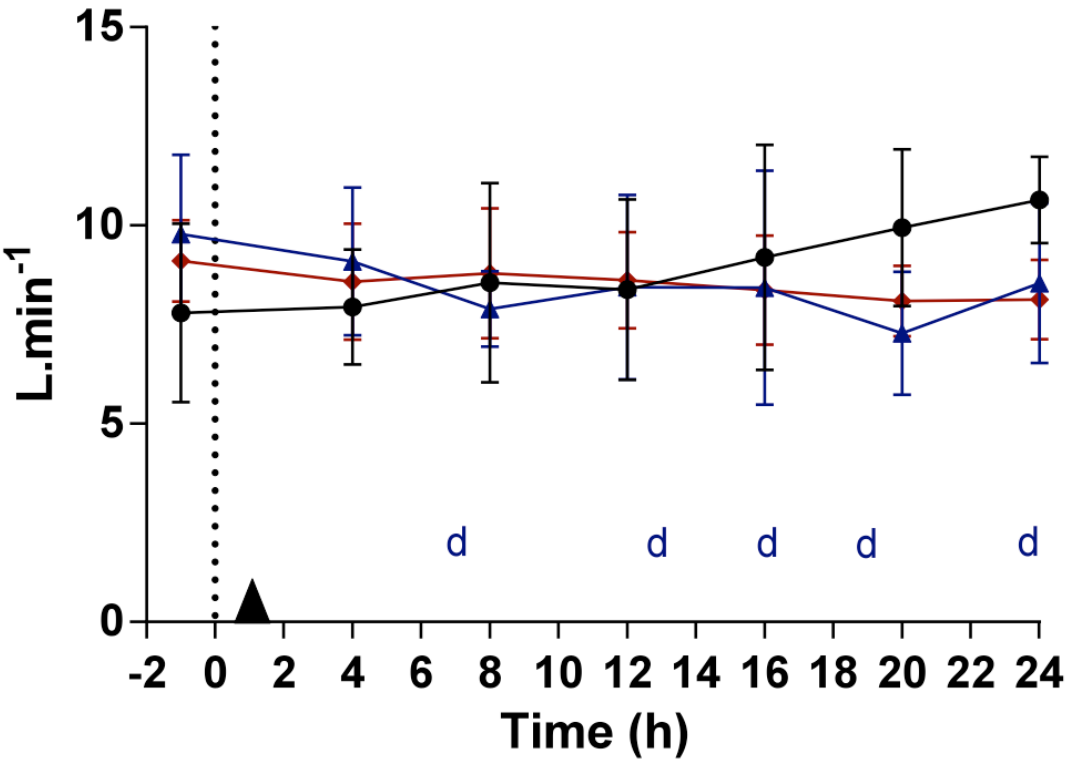

Figure 48. Cardiac output observed over the course of the 24h monitoring period expressed as mean ± SD as provided by collaborators credited in ACKNOWLEDGEMENTS and explained in Section 2.3.2 (raw data not available for reanalysis). Air Controls (● n=4). CG Controls (▲ n= 10 decreasing to 5). CG CPAP (◆ n=8). ▲ denotes initiation of CPAP at 1h. d represents the death of an animal from the phosgene control group. CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.7.3 Extravascular Lung Water

The calculated extravascular lung water (EVLW) did not significantly differ between the three groups and is shown in Figure 49. As discussed in Section 3.4.1, EVLW may be variably affected by PEEP or CPAP, and the mechanisms are not fully understood. By contrast, direct lung weight measurements showed a clear increase in extravascular lung water in CG

Controls, in Section 3.4.1. Transpulmonary thermodilution is known to have limitations in certain conditions (175) and these include:

- Heterogeneous lung injury such as caused by phosgene (114), where hypoxic pulmonary vasoconstriction redistributes blood flow away from oedematous regions of lung, results in an underestimate of EVLW.
- PEEP has an unclear relationship, most potential mechanisms predict a decrease, however if atelectatic alveoli are recruited and hypoxic pulmonary vasoconstriction is attenuated, then EVLW can be overestimated.

Confounding factors, especially the exclusion of affected lung regions from thermal signal sampling, and amplification of errors during the calculation may have produced results that conflict with the direct measurements of lung oedema presented in Section 3.4.1.

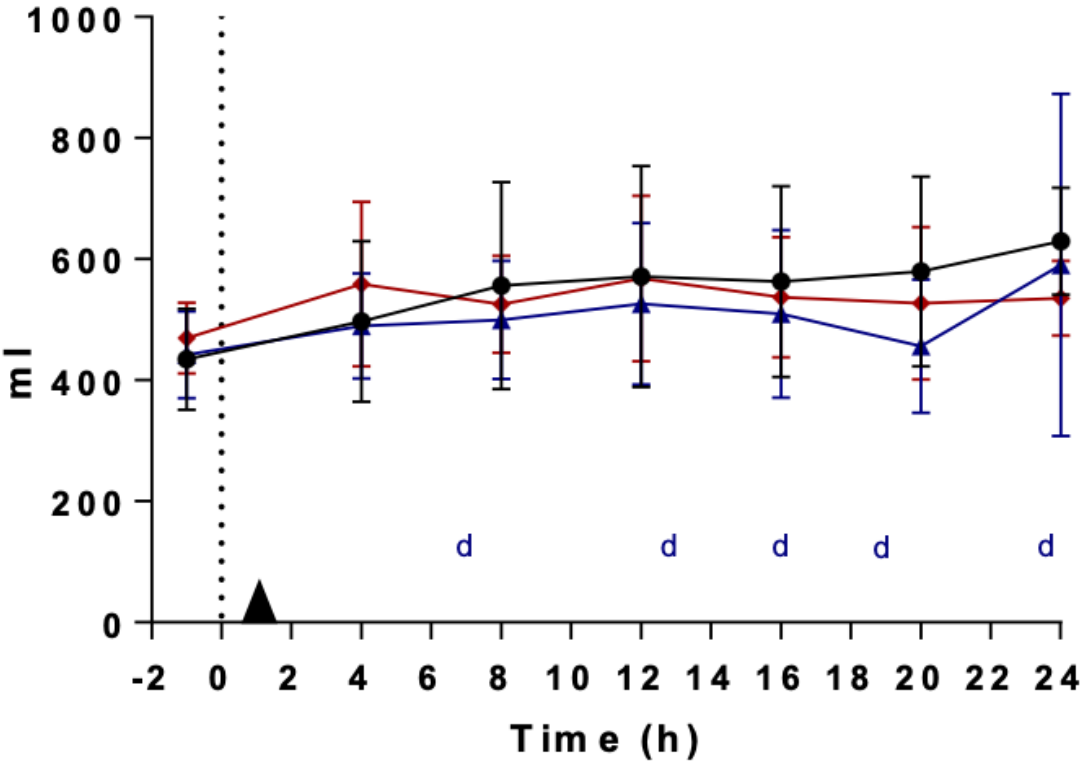

Figure 49. Change in extravascular lung water (EVLW) observed over the course of the 24h monitoring period expressed as mean ± SD as provided by collaborators credited in ACKNOWLEDGEMENTS and explained in Section 2.3.2 (raw data not available for reanalysis). Air Controls (● n=4). CG Controls (▲ n= 10 decreasing to 5). CG CPAP (◆ n=8). ▲ denotes initiation of CPAP at 1h. d represents the death of an animal from the phosgene control group. CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.8 Other Data

3.8.1 Cardiovascular

Heart rate, systolic, diastolic and mean arterial blood pressure were not significantly different between groups and remained stable throughout the monitoring period. Central venous pressure (CVP) fell in the phosgene

exposed control group which is at Figure 50. The CVP rose in the CPAP group on application of PEEP due to the rise in intrathoracic pressure. CVP fell significantly in the phosgene control group for reasons of major fluid losses described in Section 3.6.1. All Pulse Contour Continuous Cardiac Output (PiCCO) measurements were not significantly different between groups.

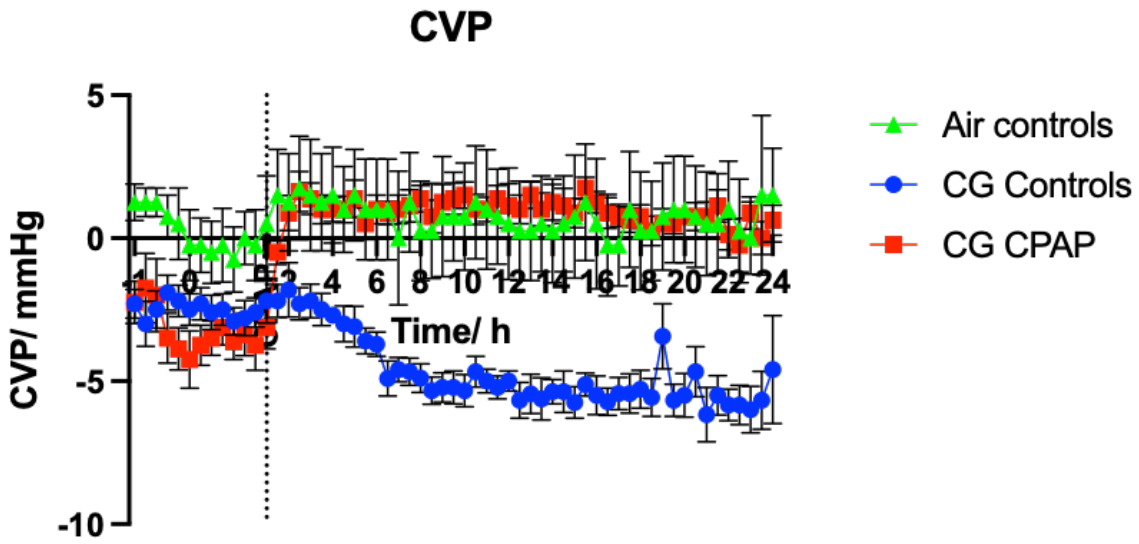

Figure 50. Change in central venous pressure (CVP) observed over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 102.7, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that CVP was significantly lower in CG Controls compared with both Air Controls ($p < 0.0001$) and CG CPAP ($p < 0.0001$), whereas Air Controls and CG CPAP did not differ significantly ($p > 0.9999$). These differences likely reflect the contribution of transmitted intrathoracic pressure due to PEEP and the loss of intravascular volume estimated in Section 3.6.1 . Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.8.2 Blood Chemistry

The following plasma values remained within normal range and with no significant difference between groups:

- Sodium
- Potassium
- Calcium
- Glucose

Arterial lactate concentration was higher in both phosgene exposed groups compared to air exposed controls, however the values remained within a modest range and were not indicative of severe metabolic derangement, they are shown in Figure 51.

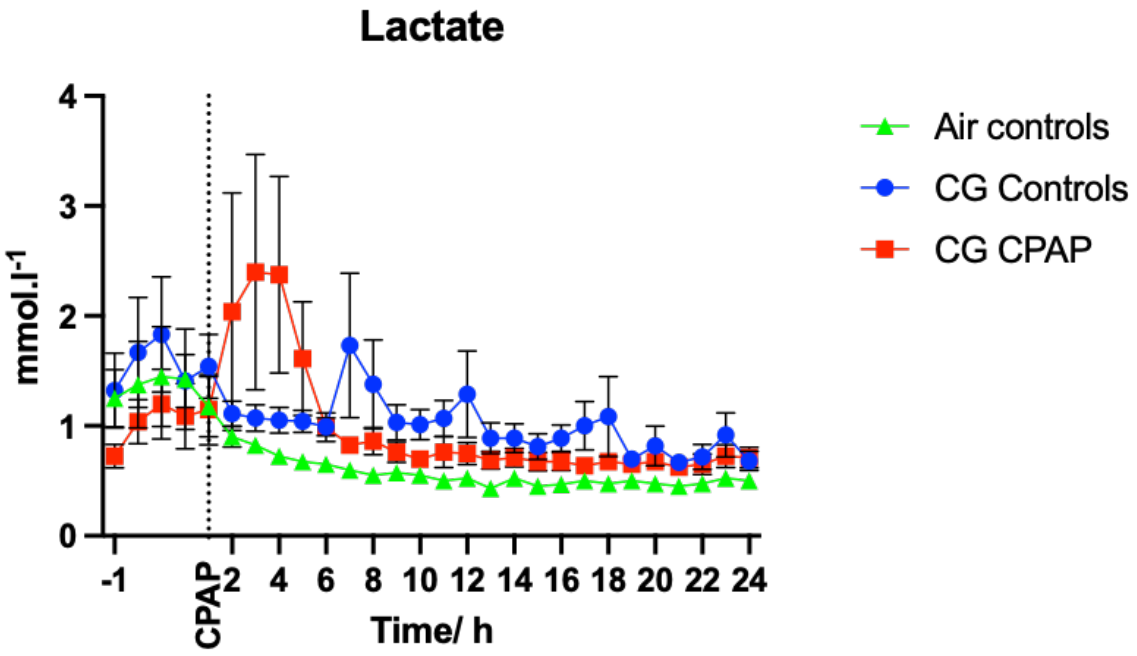

Figure 51. Arterial lactate concentration observed over the course of the 24h monitoring period expressed as mean ± SEM. CPAP denotes initiation of PEEP at 1h post exposure. Kruskal- Wallis test showed a significant difference among groups ($H = 24.23, df = 2, p < 0.0001$). Dunn's multiple comparisons confirmed that lactate was significantly higher in both phosgene exposed groups compared with Air Controls; Air Controls v CG Controls, $p < 0.0001$; Air Controls v CG CPAP, $p = 0.0097$, whereas CG Controls and CG CPAP did not differ significantly ($p = 0.156$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

3.8.3 Urine and Temperature

Urine output, pH and specific gravity remained constant throughout the 24h monitoring period and without a significant difference between groups. Rectal temperature was normal, constant and with no significant difference between groups for the duration of the 24h monitoring period.

3.8.4 Summary

These results show that survival after phosgene exposure to 24h was dramatically improved by the application of PEEP as a surrogate for CPAP 1h post exposure. CPAP lends stability to otherwise atelectatic alveoli and will probably have some effects on reducing lung oedema in a pig model. PEEP has been shown to reduce shunt and alveolar water in a dog model (176). The overall effects of this can be seen in improvements in oxygenation and ultimately survival. In addition to providing a more favourable Starling gradient, CPAP will normalise Pulmonary Vascular Resistance (PVR) by bringing collapsed lungs closer to Functional Residual Capacity (FRC) (177, 178). Thus the indirect effect of right heart failure and worsening pulmonary oedema is mitigated. Any contribution to pulmonary oedema made by lymph is lessened by the application of CPAP (179). However, the role played by pulmonary lymphatics in lung fluid homeostasis is not fully understood (180). It is not inconceivable that these effects on lymph redistribution are simply pressure related. CPAP can therefore be used to reduce the logistic burden of a mass exposure to phosgene by extending the timeline to onset of symptoms and reducing morbidity and mortality.

The exposure dose of $0.24 mgkg^{-1}$ is ideal. It caused death in half the phosgene exposed control group prior to 24h and demonstrated previously observed physiological and other measured variables in phosgene poisoning. These are most obviously the effects of severe non- cardiogenic pulmonary oedema and inflammatory response.

Lung wet weight to bodyweight ratio was higher in the phosgene exposed controls and lung wet weight to dry weight ratios were significantly improved by the application of CPAP. Likewise there were improvements in BAL protein concentration. The increase in haematocrit seen in the phosgene exposed control animals and associated fall in central venous pressure give an indication of the huge volume of lung oedema present. However, derived measures of extravascular lung water were not significantly different between groups.

P_aCO_2 was higher in the phosgene with CPAP group, most likely due to the increase in dead space. Shunt fraction was worse in the phosgene control group but other derived measures and acid- base were largely unchanged between groups.

The increase in circulating neutrophils in the PEEP exposed group is notable and is most easily explained by changes in neutrophil trafficking induced by PEEP in ALI. In short, PEEP may be reducing neutrophil migration into the alveolar space, consistent with the lower BAL counts observed. However, there is little published evidence to support this mechanism, and the effect may have been overlooked. The use of NLR as a biomarker for phosgene induced acute lung injury is not recommended, the findings do not match previous studies and the numbers may be confounded by changes in neutrophil distribution.

Overall CPAP is an effective intervention against phosgene induced acute lung injury and could quickly be instituted by the use of stockpiled commercial off the shelf devices.

4 - BRONCHOALVEOLAR LAVAGE LIPID FINDINGS

4.1 Bronchoalveolar Lavage

Terminal bronchoalveolar lavage (BAL) fluid was available to analyse from 4 air exposed controls, 10 phosgene exposed controls and 8 phosgene exposed treated with CPAP. This represents the end state of the lungs in the experiments. Samples could not be taken antemortem due to the clinical instability of the animal model. All air exposed study animals survived to 24h with stable cardiorespiratory physiology. 5 of the phosgene exposed controls died from anoxia before the 24h period elapsed.

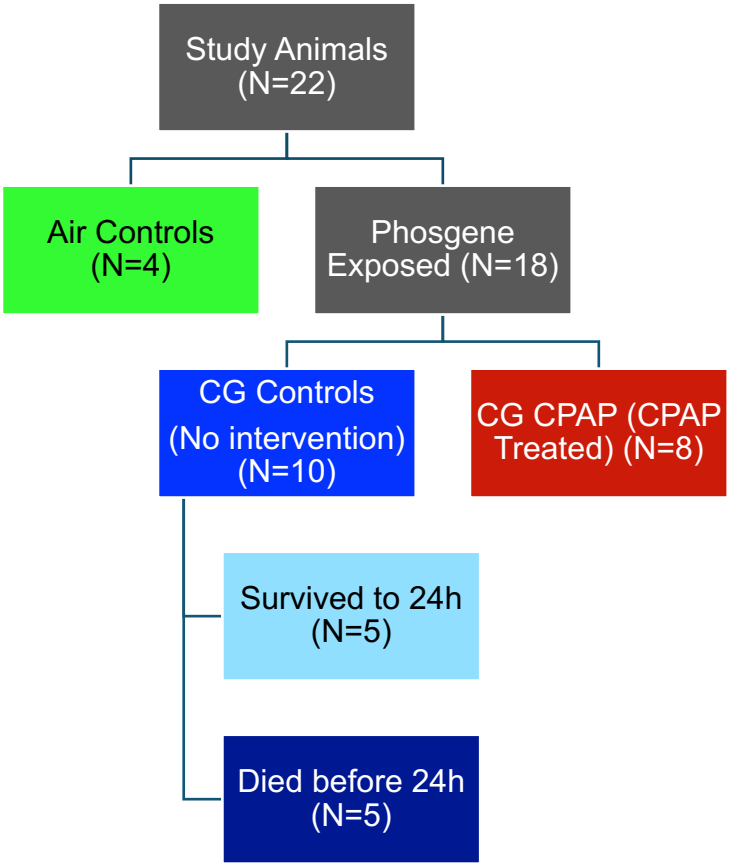

Figure 52. A flow diagram describing the study. The resulting outcome of the phosgene exposed control animals is also shown. Key colours match those presented graphically in this section.

BAL sample procedures are described in Section 2.1.7 and lipid analysis by mass spectrometry in Section 2.2.4. This chapter presents the lipid profile of BAL for the major glycerophospholipid classes; phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), phosphatidylinositol (PI) and sphingomyelin (SM).

Percentage proportions and actual amounts were analysed because the BAL was recovered using standardised means, tightly intubating the right median bronchus under a combination of direct and fibrescopic vision. This approach reduces the risk of recovery errors. *p* values are represented as $p \leq 0.05$ = ★, $p \leq 0.01$ = ★★, $p \leq 0.001$ = ★★★ and $p \leq 0.0001$ = ★★★★.

4.2 Glycerophospholipid Content

4.2.1 Major Glycerophospholipids in BAL

Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), phosphatidylinositol (PI) and sphingomyelin (SM) were analysed, the results are shown in Figure 53. The phospholipid fraction primarily consisted of PC (85%), followed by PI (6%) and PG (5%). SM proportion is less than <5%. All major glycerophospholipid fractions were unchanged following phosgene exposure with no significant difference in the CG Control and CG CPAP groups.

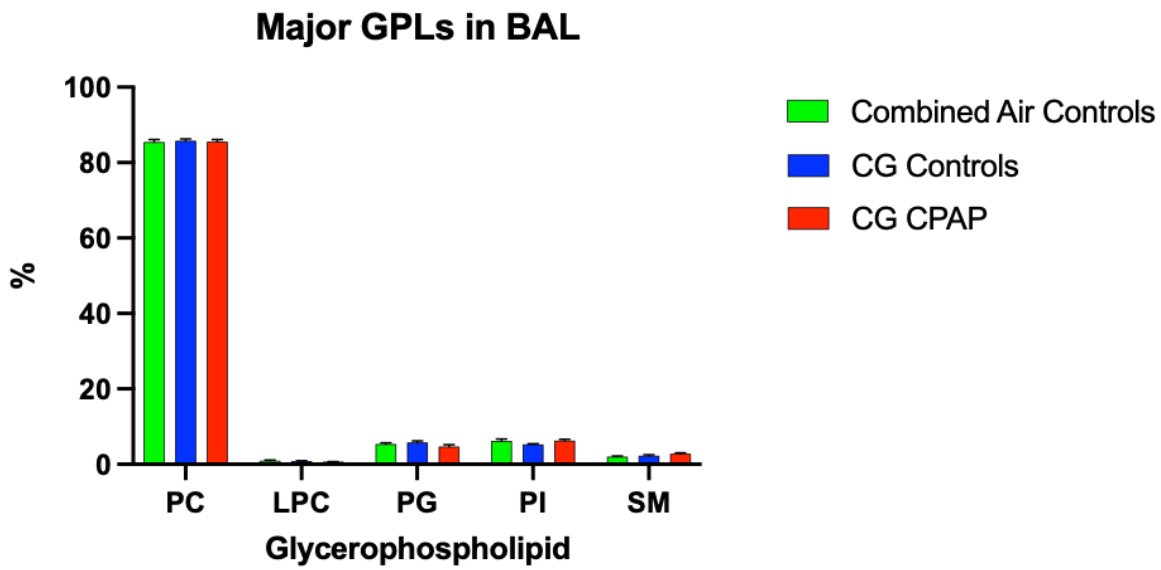

Figure 53. Fractional proportions (%) of major glycerophospholipid classes in terminal bronchoalveolar lavage. Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG) and sphingomyelin (SM) proportions were unchanged following phosgene exposure with no significant difference in the CG Control and CG CPAP groups. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

This phospholipid distribution is consistent with previous studies and the porcine surfactant composition. However, there are no longitudinal studies in pig models evaluating the effect of phosgene exposure on surfactant composition in pigs. Human studies have examined GPL content beyond 24h and found significant alterations in phospholipid composition (72, 80). A decrease in PC has been previously observed in examinations of ARDS patients, as has a decrease in PG and an increase in PI. LPC and SM both increase in ARDS beyond 24h and we are not replicating all of those findings here.

The application of PEEP did not change the fractions of the major GPL classes observed. However, the amounts of GPL recovered from the terminal BAL show a remarkable difference. This is shown in Figure 54. All classes of GPL increase by the same amount, approximately 3.5 times for CG Controls and 1.5 for the CG CPAP group. This indicates that all classes are increasing by the same factor, the relative proportions are unaltered. This situation could arise when type II alveolar cells necrose and release surfactant into the alveolar space or there may be increased synthesis due to tissue damage prior to cellular exhaustion. Reduced destruction or other loss of GPLs seems unlikely because different classes of GPL have distinct clearance kinetics (181). Therefore, if GPL loss or enhanced turnover were the drivers of the observed increase, non-uniform changes would be expected across GPL classes. If surfactant is stored prior to release, in the proportions seen in healthy alveoli, then there will not be a change in composition. The mechanism by which PEEP attenuates this response is uncertain, but may involve reduced epithelial injury or modulation of surfactant turnover. It is also possible that some GPLs could be leaking in from plasma, the more abundant plasma PC species increase in BAL however, the most abundant PC species in phosgene affected BAL is PC32:0 which was present in only very small concentrations in plasma. If there was leakage across a damaged alveolar capillary interface, it was selective and not bilateral.

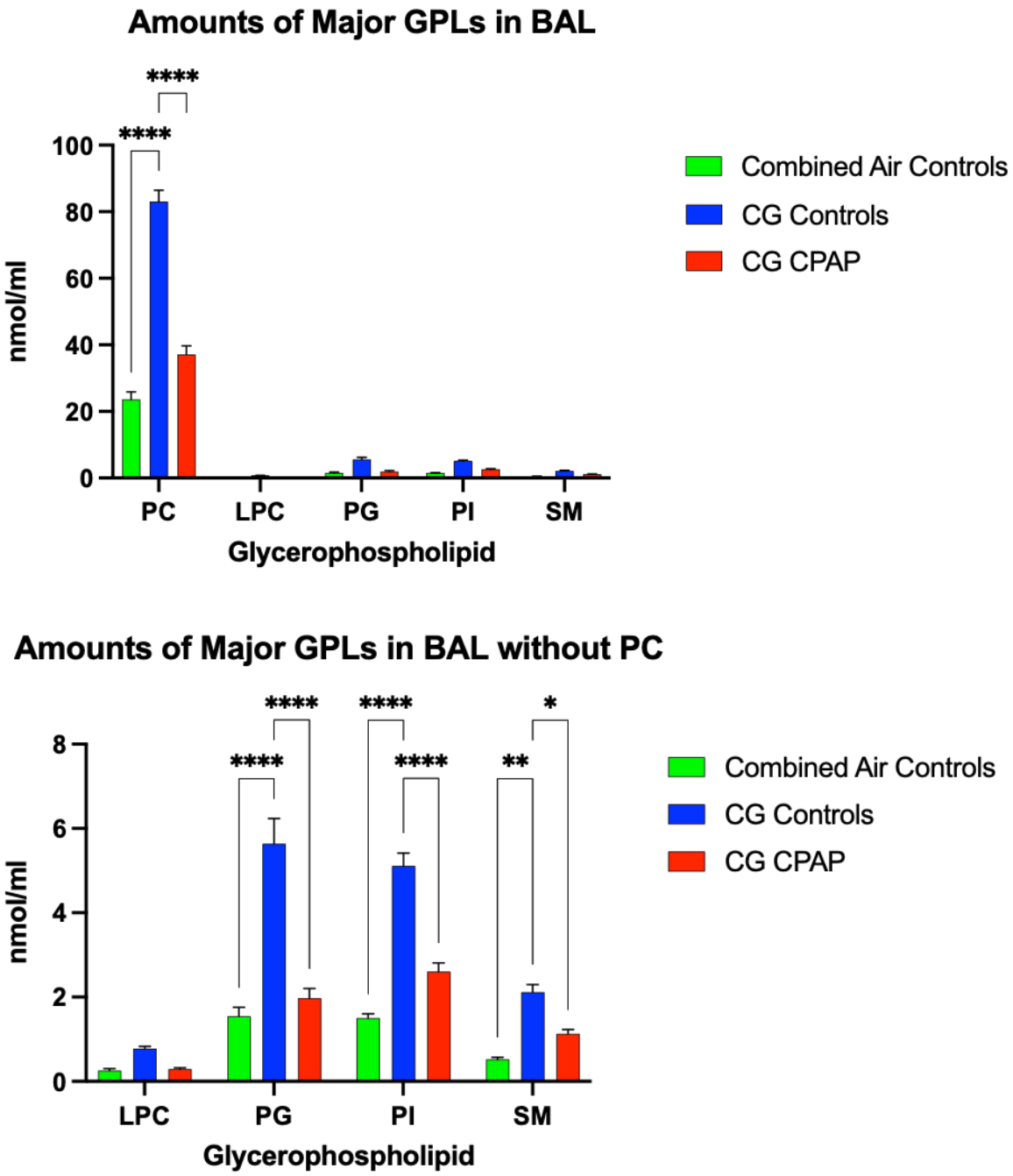

Figure 54. Absolute amounts of major glycerophospholipid (GPL) classes in terminal bronchoalveolar lavage. PC has been omitted from the lower graph for ease of interpretation. Significant increases were seen in all GPL classes in CG Controls and were significantly attenuated in CG CPAP, except for LPC. No significant differences were observed between Air Controls and CG CPAP groups. Data expressed as mean ± SEM. Statistical analysis by two-way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air

Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

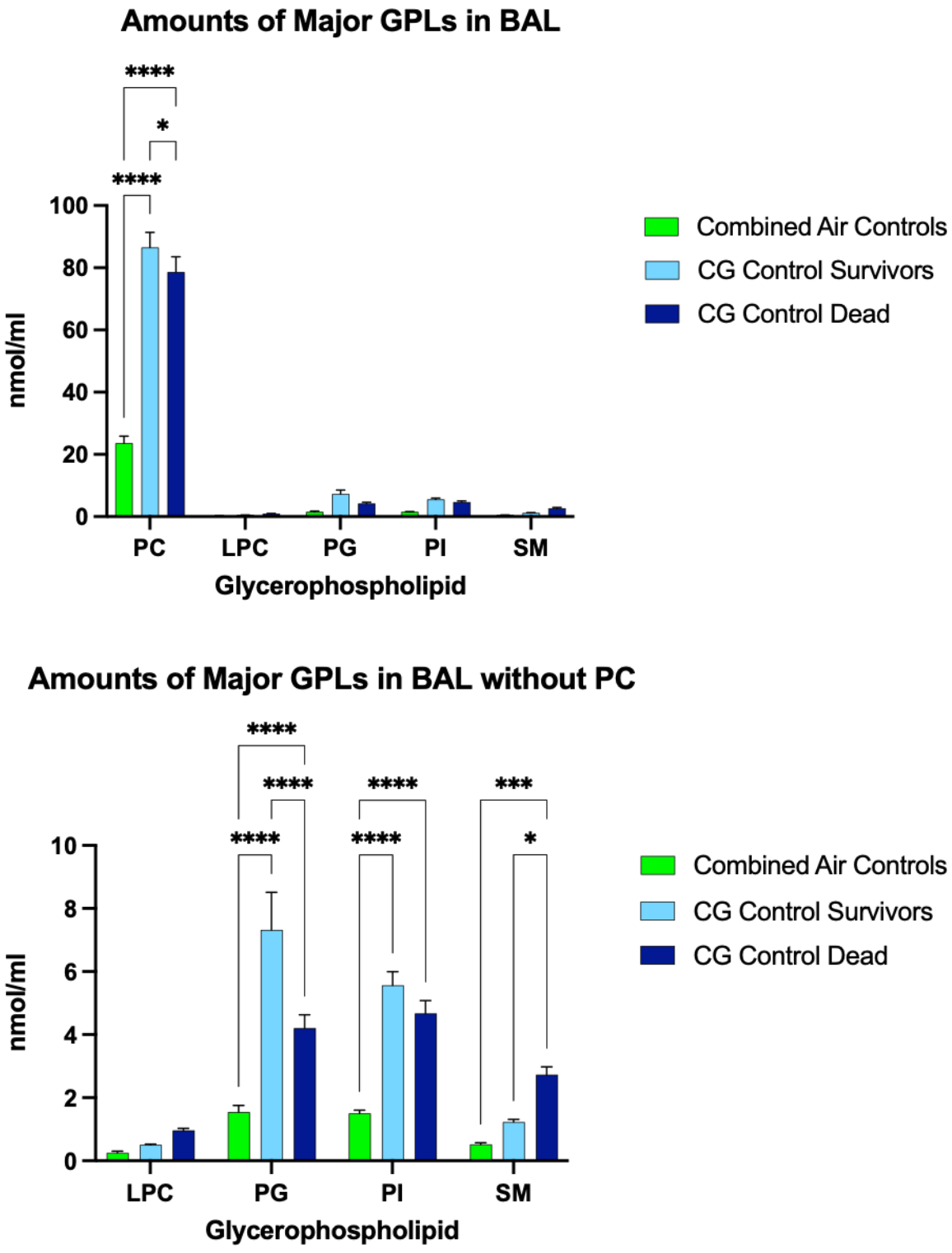

Figure 55. Absolute amounts of major glycerophospholipid (GPL) classes in terminal bronchoalveolar lavage according to survival outcome. Animals in CG Controls were divided into survivors to 24h and non-survivors dying before 24h; CG Control Survivor and CG Dead respectively. Phosgene

exposure increased PC, PG, PI and SM whereas LPC showed no significant differences between groups. PC and PG were both increased in CG Control Survivors when compared to CG Control Dead, whereas SM was significantly higher in CG Control Dead. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Control Survivors (n = 5), CG Control Dead (n = 5). CG = Phosgene.

Figure 55 splits the CG Control group into survivors to 24h and those that died before 24h. Survivors showed higher alveolar PC and PG in response to phosgene exposure compared with non- survivors. This may represent an active or protective secretory reaction. In contrast, alveolar SM was significantly higher in the animals which died before 24h, consistent with release of intracellular SM during necrosis or inflammatory cell turnover. LPC and PI showed no significant differences between survivors and non-survivors.

The increment in all surfactant specific phospholipids such as PC and PG is much less in those that died. This blunted increment in surfactant associated phospholipids PC and PG in non- survivors suggests an exhausted type II alveolar cell response or increased surfactant clearance. Whereas, the disproportionate rise in SM may indicate an enhanced inflammatory response including neutrophil infiltration.

4.2.2 Phosphatidylcholine

Fractions of phosphatidylcholine species are presented in Figure 56 in two parts for ease of interpretation of the less abundant species. PC32:0, commonly 16:0/16:0 or DPPC, contributed a lower fraction in phosgene exposed animals for both CG CPAP and CG Controls groups. The monounsaturated PC34:1, commonly 16:0/18:1, showed reciprocal elevation in fraction in the injured group. This mirrors the findings in ARDS, where

disaturated PC, principally PC32:0, decrease, and unsaturated species increase in proportion in ARDS and ALI (80, 182). The application of PEEP attenuated the decrease in PC32:0. However, the absolute amounts of all PC species are increased following phosgene exposure, and this was limited by PEEP (Figure 57). The primary PC responsible for surface tension reduction characteristics of surfactant is DPPC, PC16:0/16:0. The decrease in proportion of disaturated PCs and greater increases in other unsaturated PC species suggests dysfunctional surfactant in the injured group. These changes may result from destruction, accelerated breakdown or reduced synthesis induced by phosgene. Using isotopically labelled choline, turnover of disaturated PC is demonstrably higher in ARDS (80, 182) and the same may be in phosgene induced acute lung injury. In effect, this means that although production is increased, it does not match the rate at which disaturated PC is lost. Neither of the aforementioned studies measured LPC so it is not known whether the Lands cycle was influenced. In this study's data there is an increment in diunsaturated and polyunsaturated PC species in the injured group. Species likely to include arachidonate (20:4), such as PC36:4, PC38:5 and PC38:4, all showed proportional increments in CG Controls, which may reflect neutrophilic inflammatory cellular infiltrate rather than resulting from actual surfactant synthesis.

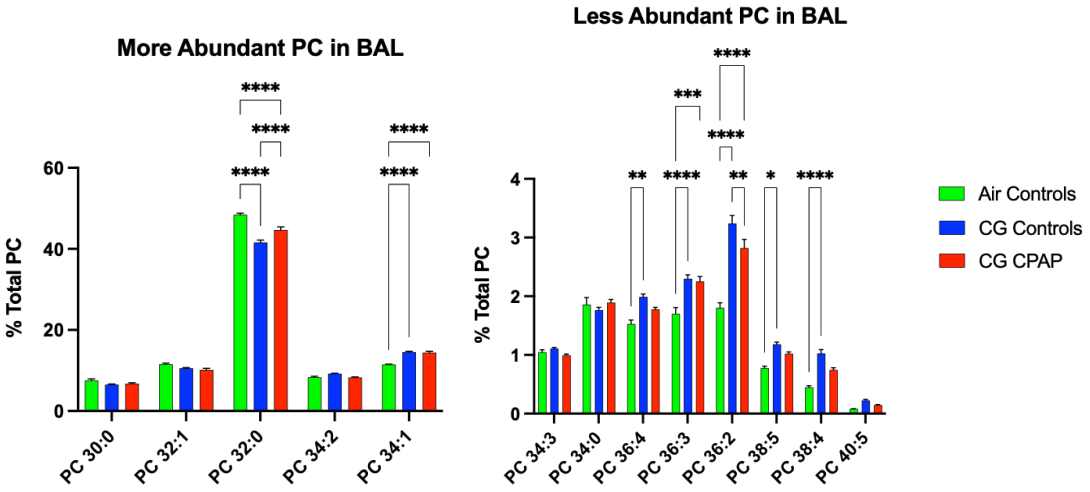

Figure 56. Fractional proportions (%) of PC species in terminal bronchoalveolar lavage. Data expressed as mean ± SEM. PC32:0 (commonly 16:0/16:0) decreased after phosgene exposure, with this reduction attenuated by the application of PEEP. Other saturated species also tended to decrease but not significantly. Diunsaturates and polyunsaturates tended to increase. Species likely to include arachidonate (20:4), (PC 36:4, PC38:5, PC 38:4) all significantly increased, and PEEP lessened that increase. Labels denote sum composition (carbons: double bonds); acyl isomers and sn- positions are not resolved. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

Leakage of cellular material into the alveoli occurs, as discussed in Section 4.2.1, but this does not appear to influence the fractional composition of PC. For example, PC32:0 (commonly 16:0/16:0) is virtually absent from plasma, so its reduction in fraction cannot be attributed to plasma influx, particularly when the absolute increase is considered. 18:2 PC species have been shown to increase in the BALF of triggered asthma patients and assumed to derive from plasma (183). In this phosgene study, these species, likely to be PC34:2, PC36:2 and PC 36:3, were not *all* seen to rise in proportion significantly. When absolute amounts of PC species were measured, they showed marked increases in CG Controls, with a moderated response in the CG CPAP group, Figure 57.

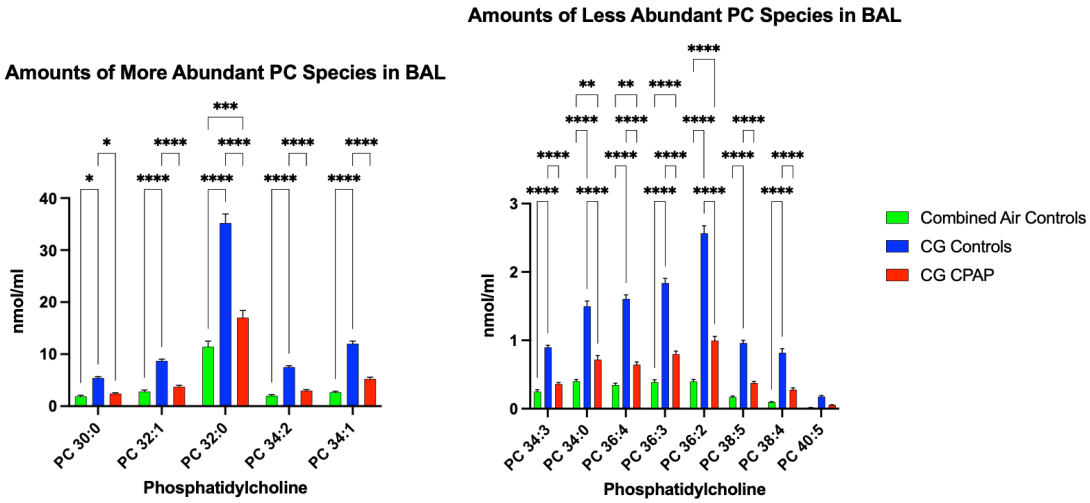

Figure 57. Absolute amounts (nmol/ml) of PC species in terminal bronchoalveolar lavage. All species increased significantly following phosgene exposure, with the exception of PC40:5 (commonly PC18:0/22:5). The increases were significantly blunted by the application of PEEP. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

The increases in amounts seen are due to increased synthesis or release, reduced catabolism, reduced elimination, or a combination of these mechanisms. Given the highly reactive nature of phosgene, it is likely that type II alveolar cells are killed, and release packaged surfactant as part of their necrosis. Impaired clearance may also contribute, since reduced elimination by alveolar macrophages has been observed in alveolar proteinosis (184) and reduced catabolism occurs in association with Surfactant Protein-D (SP-D) knockout mice (65). PEEP may act to preserve alveolar macrophage function or promote lipid catabolism, thereby attenuating these changes. Secondary analysis by survival status, showed that PC32:0 (commonly PC16:0/16:0) was significantly increased in survivors when compared to the non-survivors in the CG Control group, again this could represent a protective effect and is seen in other saturated species. By contrast, the diunsaturates, PC36:2 (commonly PC18:0/18:2) and PC38:4

(commonly PC18:0/20:4) were significantly increased in the non-survivors (Figure 58). These findings are likely to reflect plasma and cellular composition from inflammatory cellular infiltrate in the alveolar milieu rather than functional surfactant production.

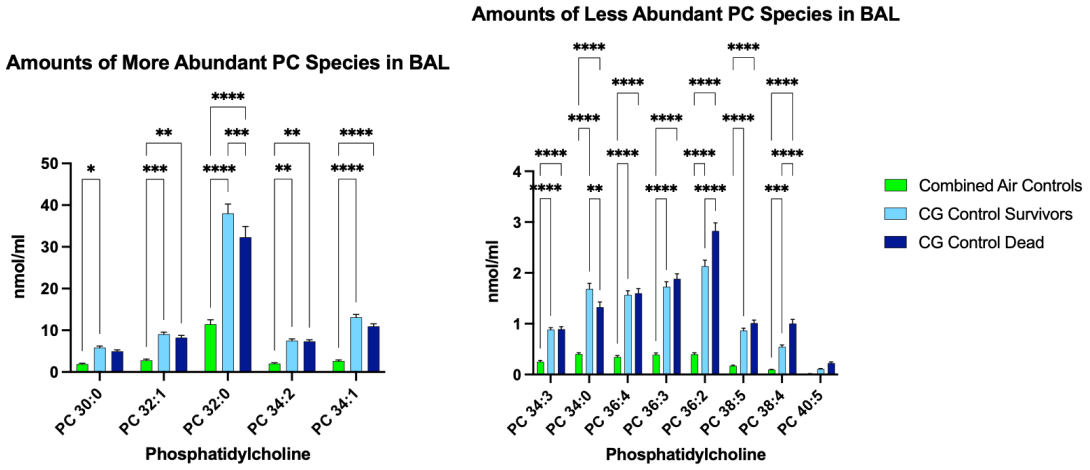

Figure 58. Absolute amounts of phosphatidylcholine (PC) species in terminal bronchoalveolar lavage according to survival outcome. Animals in CG Controls group were divided into survivors to 24h and non-survivors dying before 24h; CG Control Survivor and CG Dead respectively. Across species, phosgene exposure increased PC amounts v Air Controls. Between CG subgroups, saturated species were higher in CG Control Survivors (PC32:0 and PC 34:0), whereas PCs likely to contain 18:2 or 20:4 were higher in CG Control Dead (PC36:2 and PC38:4). Other species showed no significant differences between CG Control Survivor and CG Control Dead. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Control Survivors (n = 5), CG Control Dead (n = 5). CG = Phosgene.

4.2.3 Lysophosphatidylcholine

There are no major changes in fractional composition of LPC. The most abundant LPC species is 16:0 and its fraction decreases following phosgene

exposure, although the absolute amount increases markedly. This suggests that PC16:0/16:0 is not substantially eliminated via the Lands pathway. The other saturated species, LPC18:0 was unchanged in proportion. The largest fractional change was an increase in the arachidonate LPC20:4 and this may be due to the modulation of inflammatory mediator pathways. The changes are illustrated in Figure 59. Total LPC increases in phosgene induced lung injury, in contrast to reports in ARDS (72) and this may be due to comparison between the acute stage of phosgene induced acute lung injury and the later stages of ARDS. There are notably large increases in absolute amounts of LPC16:0 which is representative of the metabolism of abundant PC16:0/16:0 and LPC20:4 which may reflect the role of arachidonic acid in inflammatory mediation. Large increases in LPC species are seen in non-survivors when compared to survivors in the CG Control group, the largest difference occurring with 20:4, Figure 60.

Since both total PC and total LPC increased in CG Controls, the rise in PC cannot be explained simply by enhanced hydrolysis of PC into LPC. Instead, these concurrent increases suggest impaired PC catabolism, with accumulation of intact PC alongside increased LPC. The disproportionate increase in LPC20:4 likely reflects activation of arachidonate dependent inflammatory pathways rather than bulk surfactant turnover.

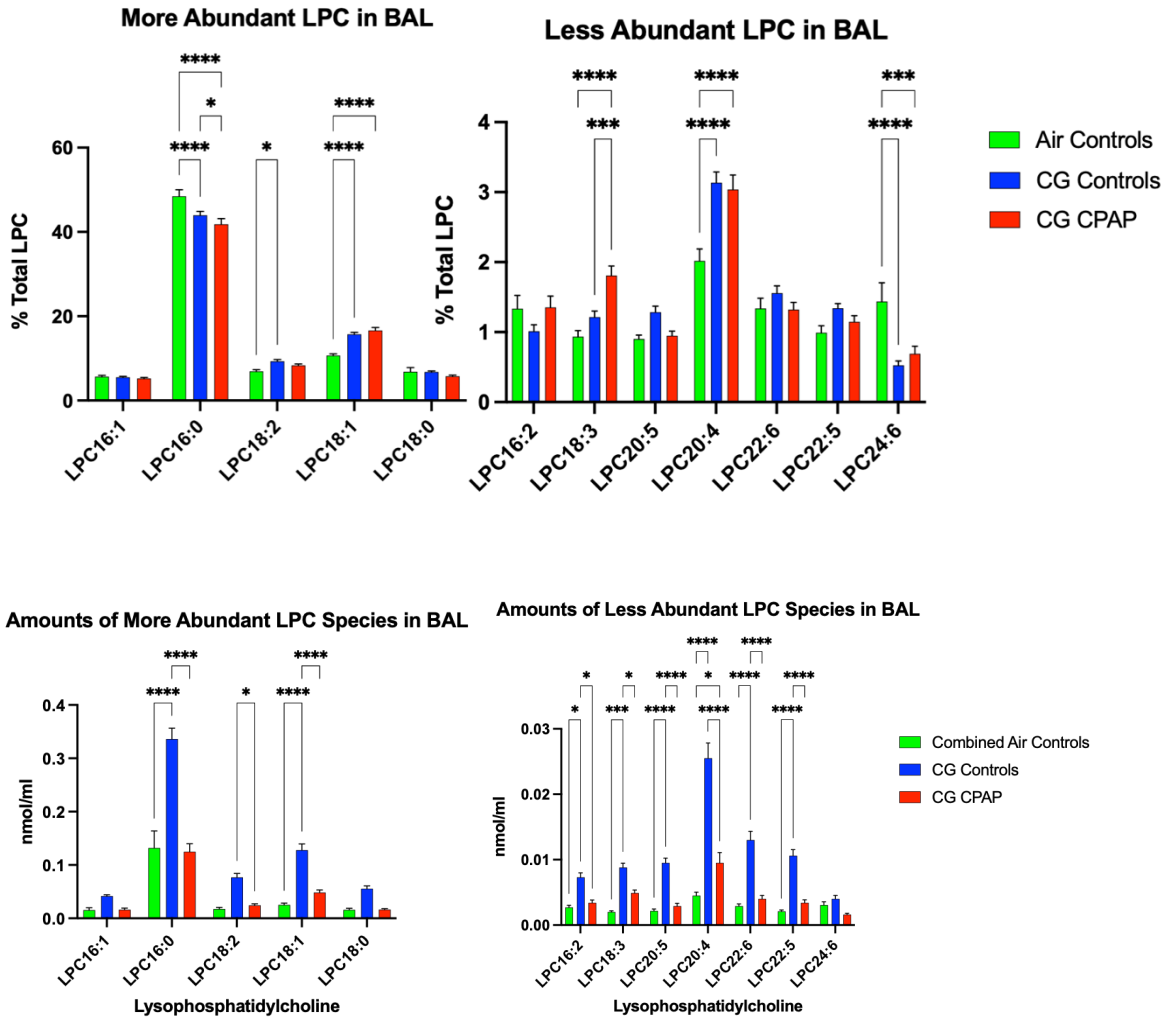

Figure 59. Fractional composition and absolute amounts of lysophosphatidylcholine (LPC) species in terminal bronchoalveolar lavage. Fractional shifts were modest overall. Phosgene exposure increased LPC amounts, most prominently LPC16:0, LPC18:1, LPC18:2 and LPC18:0, with additional increases in unsaturated longer chain species, LPC20:4, LPC22:5, LPC22:6 and LPC20:5. Many of these increases were attenuated in the CG CPAP group. Data expressed as mean ± SEM. Statistical analysis by two-way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

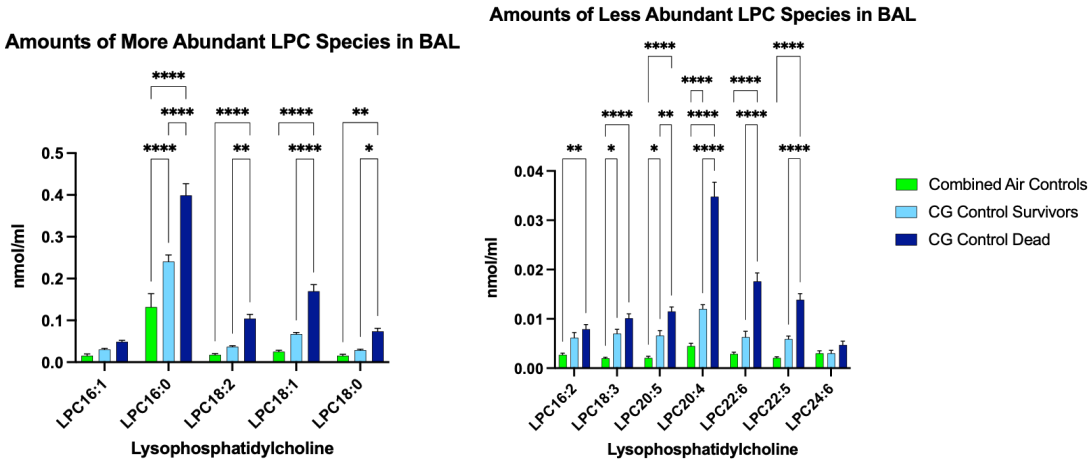

Figure 60. Absolute amounts of lysophosphatidylcholine (LPC) species in terminal bronchoalveolar lavage according to survival outcome. Animals in CG Controls group were divided into survivors to 24h and non-survivors dying before 24h; CG Control Survivor and CG Dead respectively. LPC16:0 increased in both CG subgroups and was higher in CG Control Dead than CG Control Survivors. The polyunsaturated LPCs; LPC20:5, LPC20:4, LPC22:6 and LPC22:5 were elevated and significantly higher in CG Control Dead than CG Control Survivors, whereas LPC16:1 showed no group differences. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Control Survivors (n = 5), CG Control Dead (n = 5). CG = Phosgene.

4.2.4 Phosphatidylglycerol

The total PG fraction is known to decrease in established ARDS, but this was not observed in the first 24h of phosgene induced acute lung injury, where the overall fraction remained stable. However, the absolute amount of PG does rise. Individual species have not previously been reported in phosgene induced acute lung injury, and are presented in Figure 61. The predominant species was PG34:1 (commonly PG16:0/18:1) and this is consistent with previous descriptions of surfactant composition. There were changes in the fractional PG composition between groups. In CG Controls there were fractional increases in PG34:1 (commonly PG16:0/18:1) and falls in fractions

of PG34:3 (commonly PG16:1/18:2) and PG36:5 (commonly PG16:1/20:4). These changes were attenuated in the CG CPAP group. CG Control data shows that all species of PG are increased in survivors to 24h, although not all species show a significant difference.

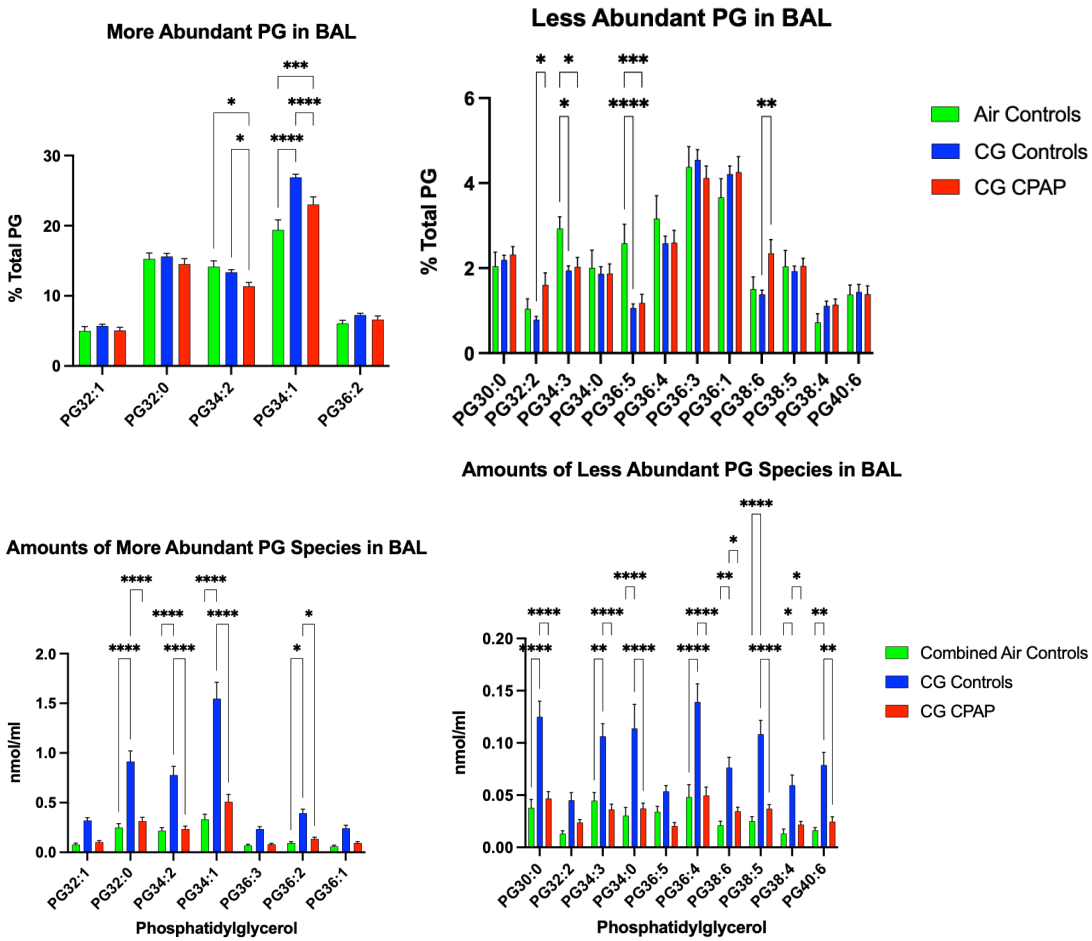

Figure 61. Fractional composition of phosphatidylglycerol (PG) species in terminal bronchoalveolar lavage. Phosgene exposure altered PG composition. Fractions of PG34:1 (commonly PG16:0/18:1) were higher, and PG34:2 (commonly PG16:0/18:2) were lower. The increase in PC34:1 was attenuated In the CG CPAP group and the decrease in PC34:2 was exacerbated in the CG CPAP group. The absolute amounts are all increased in CG Controls, with the increase attenuated in the CG CPAP group. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

4.2.5 Phosphatidylinositol

The most abundant PI species was PI34:1 (commonly PI16:0/18:1) followed by PI34:2 (commonly PI16:0/18:2). PI fraction increases in established ARDS but the effect on individual PI species has not previously been described in phosgene induced acute lung injury. Overall fractional composition was only modestly altered by phosgene. PI34:2 and PI34:3 (commonly PI16:1/18:2) were the only ones to decrease and the reduction is exacerbated by PEEP in the CG CPAP group. Other increases in fraction accentuated by PEEP were due to differences in the relative amounts of species whose absolute increases PEEP appeared to limit. Absolute amounts of all measured PI species increased after phosgene exposure and were significantly attenuated by the application of PEEP. See Figure 62. There were no significant differences in PI species, fractions or amounts, between survivors and non-survivors in the CG Control Group.

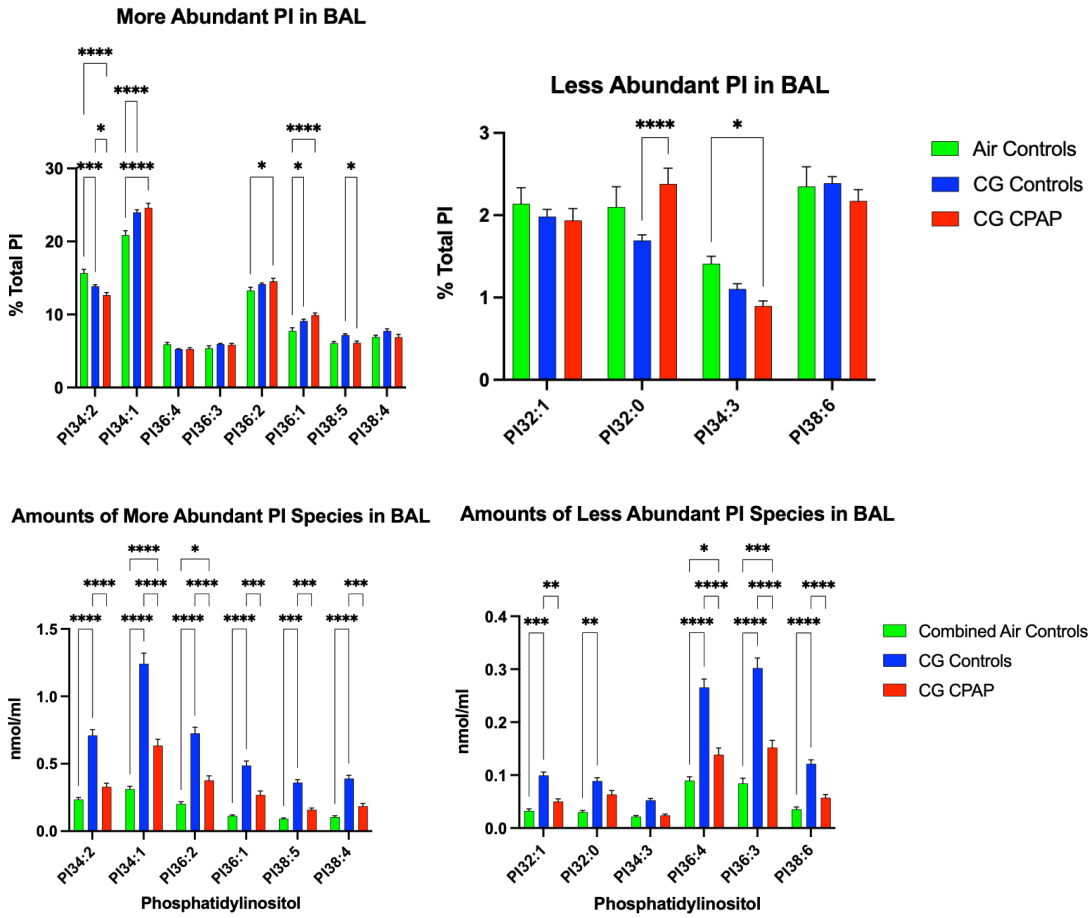

Figure 62. Phosphatidylinositol (PI) species in terminal bronchoalveolar lavage. Fractions are less affected by phosgene. PI34:1 (commonly PI16:0/18:1) increased after phosgene exposure and that was increased by the application of PEEP in the CG CPAP group. PI34:2 (commonly PI16:0/18:2) fraction decreased and that decrease was more notable in the CG CPAP group. Smaller fractional increases were detected in PI36:1 (commonly PI18:/18:1) and PI36:2 (commonly PI18:0/18:2). Most other PI fractions were stable. In contrast, absolute amounts of all measured PI species increased after phosgene exposure and PEEP attenuated the increase. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

4.2.6 Sphingomyelin

Sphingomyelin (SM) is a minor component of pulmonary surfactant. SM is known to increase in ARDS and this is due to its localisation to the cell and organelle membranes which are broken down during cell injury and necrosis (185). It represents less than 1% of lung lavage phospholipid content and will rise as cell necrosis releases cellular contents, including damaged cellular and organelle membranes, during the inflammatory process. See Figure 63. The arachidonate species, SM20:4 was detected at <1% of total SM, and therefore excluded from the analysis. The rise in SM is greater in those CG Controls that died before 24h compared with survivors (Figure 64). Six of the more abundant SM species increased following phosgene exposure, and these changes were attenuated by the application of PEEP, suggesting a protective effect on respiratory epithelium.

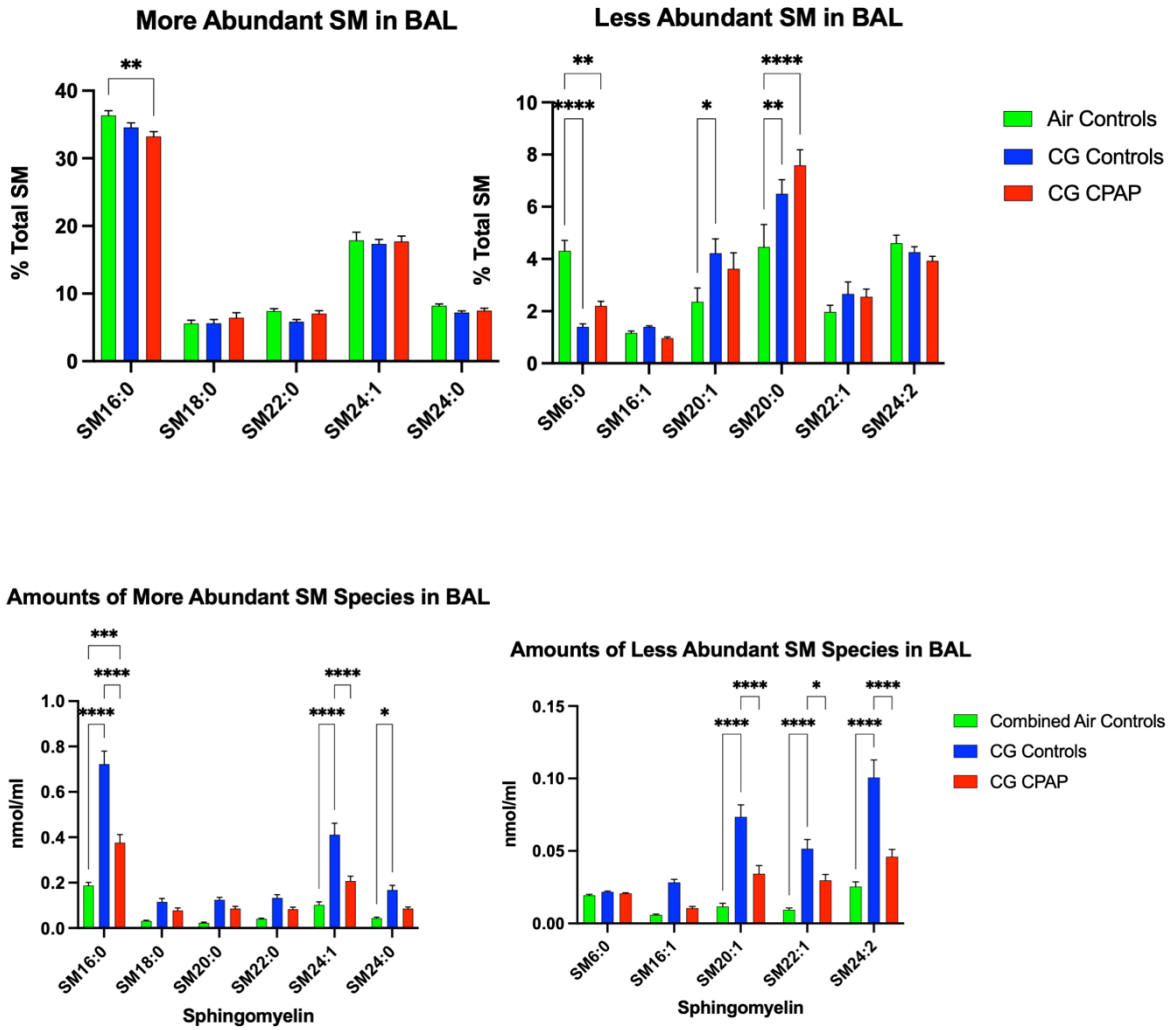

Figure 63. Sphingomyelin (SM) in terminal bronchoalveolar lavage. Fractional composition demonstrated a shift towards long chain species; SM20:1, SM22:1 and SM 24:2 after phosgene exposure and these changes were attenuated in the CG CPAP group. After phosgene exposure, specific increases were seen in absolute amounts of some sphingomyelin species derived from cellular membranes and these were blunted in the CG CPAP group. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

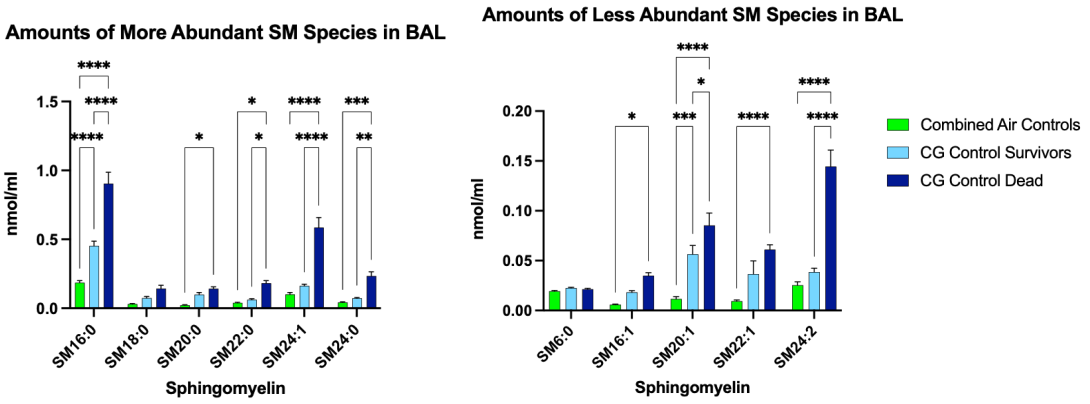

Figure 64. Absolute amounts of sphingomyelin (SM) species in terminal bronchoalveolar lavage according to survival outcome. Animals in CG Controls group were divided into survivors to 24h and non-survivors dying before 24h; CG Control Survivor and CG Dead respectively. SM16:0 increased in both CG subgroups and was higher in CG Control Dead than CG Control Survivors. Longer chain mono- and diunsaturated SMs; SM24:1, SM20:1, SM22:1 and SM24:2 were elevated after phosgene exposure and significantly higher in CG Control Dead than CG Control Survivors. Data expressed as mean ± SEM. Statistical analysis by two- way ANOVA with Tukey's multiple comparisons test. Sample sizes were Air Controls (n = 4), CG Control Survivors (n = 5), CG Control Dead (n = 5). CG = Phosgene.

4.2.7 Surfactant Lipids are not Translocated to Plasma

PEEP limited the increase in absolute amounts of many surfactant lipids; one simple hypothesis is that these lipids are displaced by hydrostatic mechanisms into plasma. To evaluate the effect of PEEP in augmenting plasma lipid content by hydrostatic mechanisms, an abundant surfactant lipid that is scarce in plasma was examined. PC32:0 (commonly PC16:0/16:0) showed little in proportion, and absolute amounts were non-significantly lower in the CPAP group in Figure 65. This is observed in the context of concentration of plasma solutes described in Section 3.6.1. Reduction in surfactant GPLs by CPAP is not due to leakage into plasma.

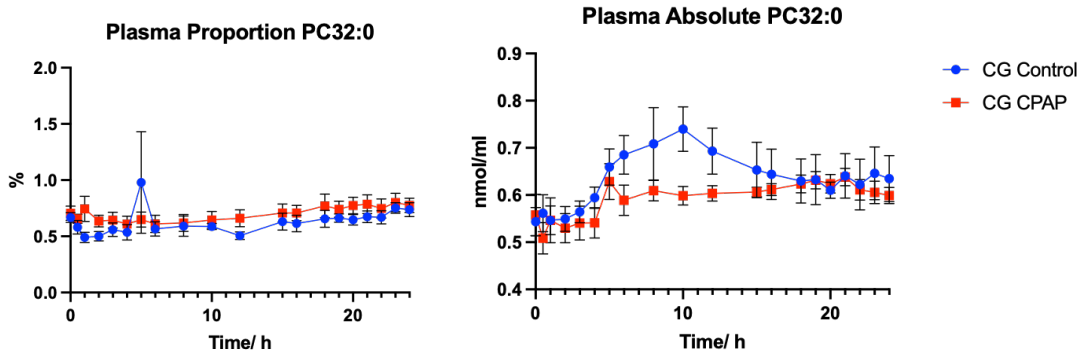

Figure 65. Low concentrations of PC32:0 (commonly PC16:0/16:0) in plasma were not increased by the application of CPAP. Two-way ANOVA revealed no significant interaction between groups over time. While a small overall difference in absolute amounts between groups was observed, this was not related to CPAP application and opposite to the expected effect if PEEP were driving surfactant lipids into the circulation. Data expressed as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

4.3 Linking Glycerophospholipids in BAL to Clinical Findings

Comparing individual animal data provides greater insight into the degree of inflammation and the severity of ALI. This type of analysis has not previously been conducted. Examination of the relationships between Total PC and Respiratory Rate or Oxygenation did not reveal any significant associations, and no consistent trends were observed.

4.3.1 Total PC and Neutrophils in BAL

The relationship between PC and lung inflammation was explored using individual animal data. A negative correlation was observed in the CG CPAP group, indicating that at higher neutrophil counts, there is an associated lower total PC. This represents a treatment related effect; in air or phosgene exposed controls there is no significant relationship. Higher neutrophil counts in BAL of CG CPAP animals were associated with lower PC. If PEEP was preserving neutrophil function, they could utilise PC, for example as a substrate for inflammatory mediator synthesis. The CG Control group has the highest neutrophil count and therefore inflammatory response, along with the highest concentration of total PC as noted previously. Pooling the data is not appropriate, as the 3 groups are under distinct biological influences. The relationship is displayed in Figure 66.

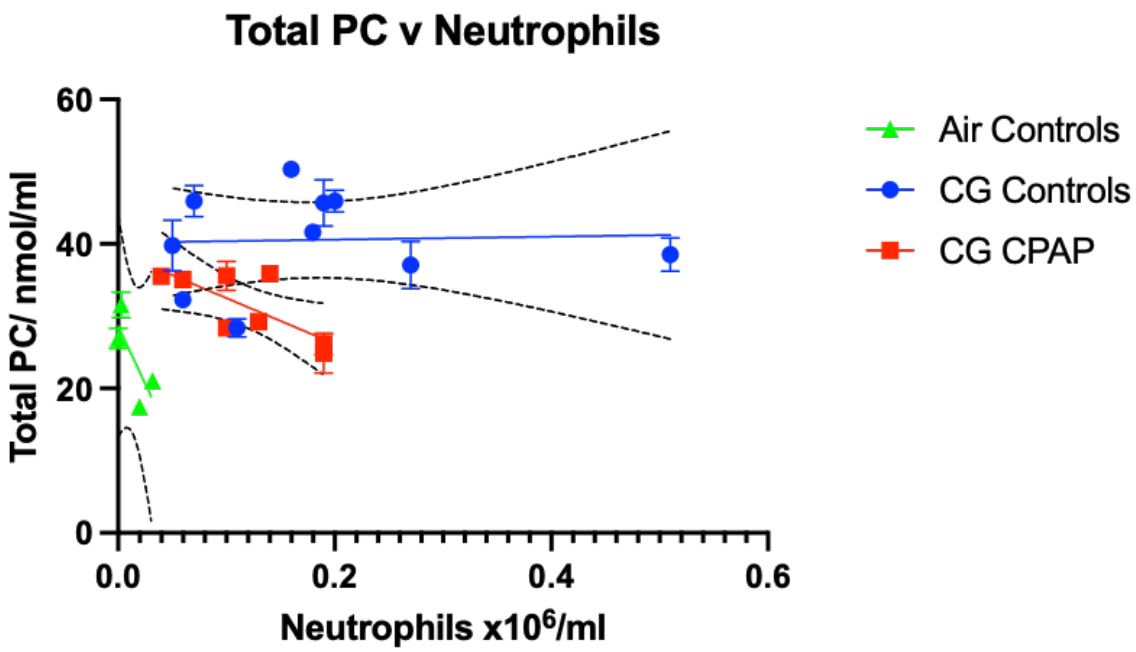

Figure 66. Relationship between total phosphatidylcholine (PC) concentration and absolute neutrophil count in terminal bronchoalveolar lavage (BAL) across experimental groups. Data are presented as scatter plots and linear regression lines with dashed lines representing the 95% confidence intervals. The null hypothesis of zero slope was tested using an F-test. The slopes of the regression lines represent the relationship between Total PC and neutrophil count in each group. Only the CG CPAP group demonstrated a significant negative correlation ($slope - 63.3, R^2 = 0.56, p = 0.0339$), suggesting an inverse relationship between Total PC and neutrophil count. No significant correlations were observed for Air Controls ($R^2 = 0.59, p = 0.2324$) or CG Controls ($R^2 = 0.002, p = 0.9091$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

4.3.2 Total PC and Alveolar Macrophages in BAL

As noted in Section 3.4.3 there was a non-significant increase in alveolar macrophages in BAL in the PEEP treated group. While an increase in alveolar macrophages may provide a mechanism for the lower concentration of PC seen in BAL due to enhanced clearance, this was not supported by

significant effects in the data. To explore the relationship further, individual animal data is compared in Figure 67.

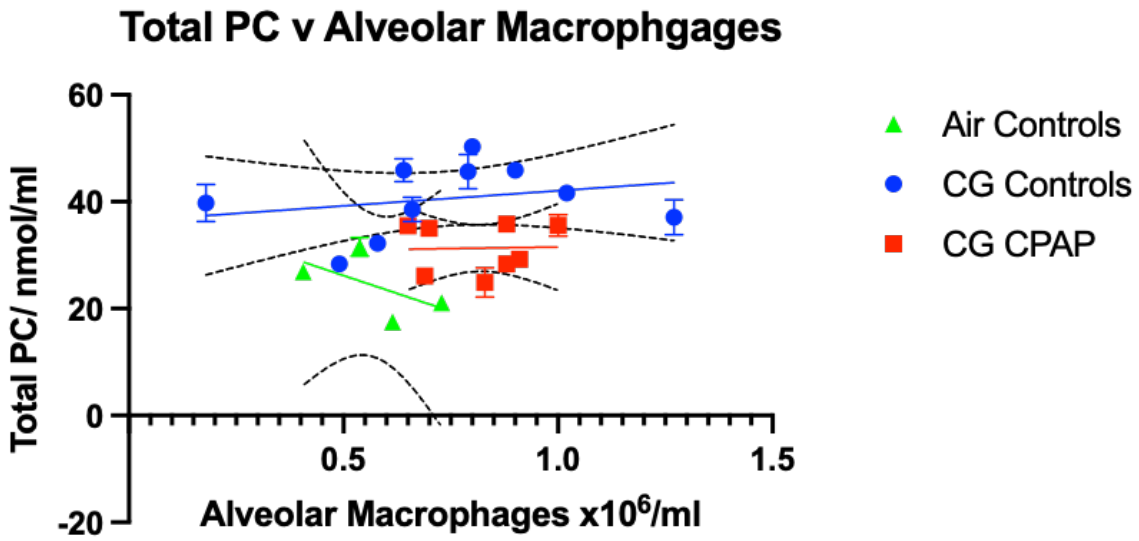

Figure 67. Relationship between total phosphatidylcholine (PC) concentration and alveolar macrophage counts in terminal bronchoalveolar lavage (BAL) across experimental groups. Data points represent individual measurements, and trendlines are derived from linear regression analysis with dashed lines representing the 95% confidence intervals. The null hypothesis of zero slope was tested using an F-test. The slopes of the lines indicate no significant correlation in Air Controls ($slope = -26.8, R^2 = 0.34, p = 0.416$), CG Controls ($slope = 5.67, R^2 = 0.063, p = 0.485$), or CG CPAP groups ($slope = 1.18, R^2 = 0.001, p = 0.941$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

While the data does not show significant correlations, the pattern was consistent with other findings such as PEEP limiting surfactant PC accumulation. This supports the concept of PEEP contributing to protective mechanisms. There is a trend towards preserved surfactant recycling, and macrophage function with PEEP.

4.3.3 Saturated PC and Neutrophils in BAL

To gain further insight into surfactant's functional state, saturated PC was examined and compared in BAL from individual animals. The CG CPAP group showed a significant inverse relationship while the control groups showed no clear trends. The data is plotted in Figure 68.

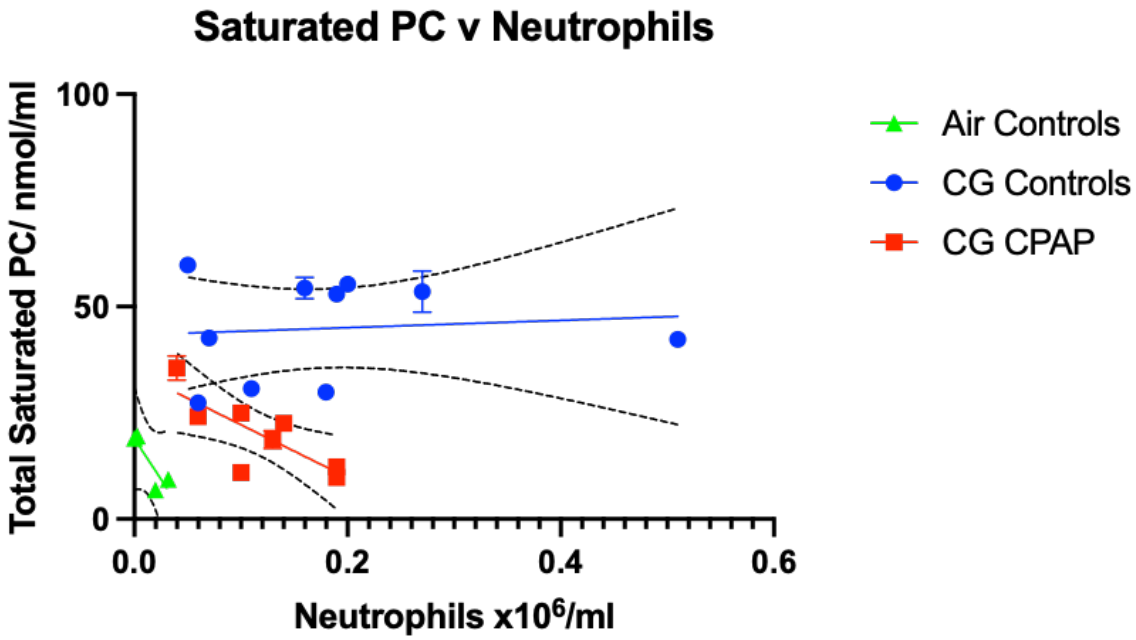

Figure 68. Relationship between saturated phosphatidylcholine (PC) concentration and neutrophil count in terminal bronchoalveolar lavage (BAL) across study groups. Data points represent individual animals, with linear regression lines for each group, with dashed lines indicating 95% confidence intervals. Only the CG CPAP group showed a significant inverse relationship ($slope = -124.5, R\text{^}2 = 0.61, p = 0.0220$) between saturated PC and neutrophil count. No significant relationships were observed in Air Controls ($slope = -382.5, R^2 = 0.77, p = 0.123$), or CG Controls ($slope = 8.54, R^2 = 0.009, p = 0.791$). Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

The CPAP group demonstrated a significant inverse relationship, again suggesting a potential protective role in surfactant metabolism and modulation of neutrophilic inflammation. Higher neutrophil counts in BAL of

CG CPAP animals were associated with lower saturated PC. If PEEP was preserving neutrophil function, they could consume PC, for example as a substrate for cell membrane repair. The higher concentrations of PC observed in the CG Control group could partly reflect stimulation by inflammatory cells (186). However, the role of Inflammatory cells in surfactant clearance could have become apparent beyond the 24h monitoring period (187, 188). Measurements of BAL could not be taken antemortem so trends over time could not be assessed. It is possible that PC concentrations were higher at 12 hours and that clearance processes subsequently reduced concentrations to the observed levels.

4.3.4 Total PC and Total LPC in BAL

Exploring surfactant metabolism further, the relationship between total PC and total LPC was examined in individual animals. As discussed in Section 1.2.3, PC can undergo deacylation to LPC as part of surfactant homeostasis. Figure 69 illustrates this relationship, including the effect of PEEP on PC clearance and its conversion to LPC.

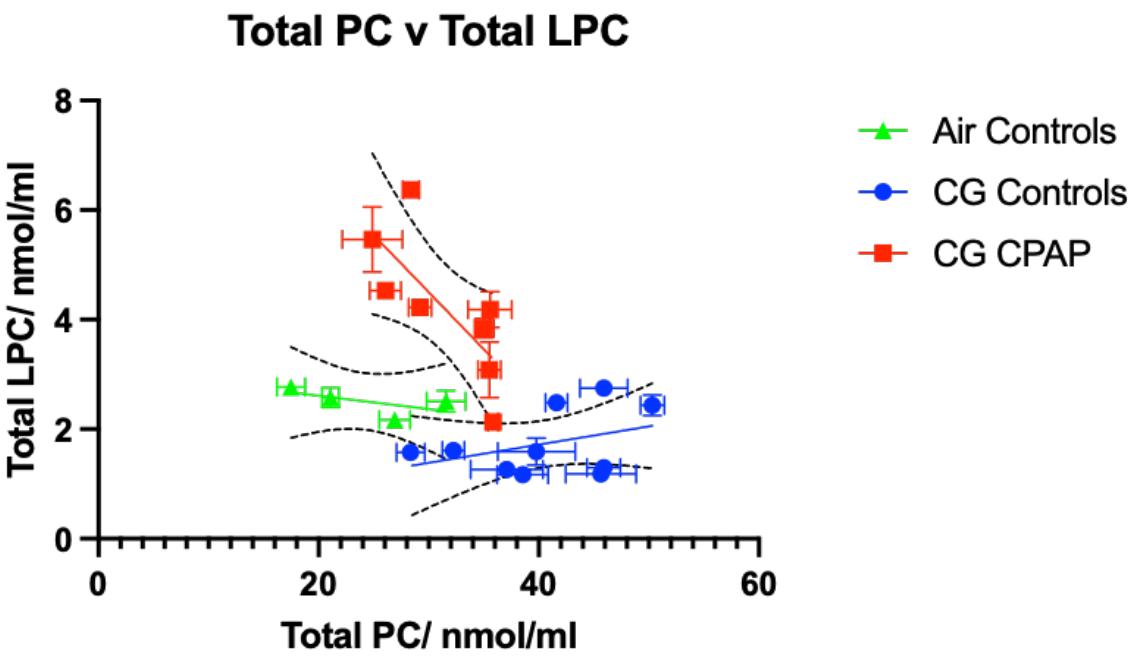

Figure 69. Relationship between Total phosphatidylcholine (PC) and Total lysophosphatidylcholine (LPC) in terminal bronchoalveolar lavage (BAL) across experimental groups. Data are presented as scatter plots with linear regression lines and dashed 95% confidence intervals. The null hypothesis of zero slope was tested using an F-test. A significant inverse relationship is observed in the CG CPAP group ($slope = -0.208, R^2 = 0.55, p = 0.0356$), indicating reduced conversion of PC to LPC and suggesting an effect of PEEP on surfactant metabolism. Air Controls ($slope = -0.024, R^2 = 0.38, p = 0.387$) and CG Controls ($slope = 0.033, R^2 = 0.14, p0.281$) showed weak, non-significant correlations, suggesting LPC levels in these groups are influenced by factors beyond direct PC metabolism. Sample sizes were Air Controls (n = 4), CG Controls (n = 10), CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

The application of PEEP in the CG CPAP group may have limited the activity of PLA_2 which converts PC to LPC as discussed in Section 1.2.3. This mechanism could have explained the lower, albeit non- significant LPC levels observed in the CG CPAP group BAL. The reduced conversion of PC to LPC in BAL supports the hypothesis that PEEP attenuated surfactant secretion early in phosgene induced lung inflammation.

4.4 Summary of Glycerophospholipids in BAL

In phosgene induced acute lung injury, glycerophospholipids in BAL increased across all classes examined. This was likely due to a combination of potential mechanisms including increased release during cellular injury and necrosis, hyperstimulation of type II alveolar cells and altered surfactant recycling, induced by phosgene. The remarkable consistency of the rise in every class, evidenced by the identical fractional composition between study groups, suggests that surfactant lipids are packaged together and stored intracellularly prior to release in the ratios found extracellularly. Interpretation is best focused on actual concentrations when recovery errors are minimised, as in this study.

The application of PEEP was associated with attenuation of these changes. This may reflect stabilisation of alveolar conditions and reduced inflammatory responses. Limitation of type II alveolar cell necrosis, modulation of surfactant recycling, or preservation of alveolar macrophage clearance functions are other potential mechanisms. Reduced inflammatory signalling may have lessened the stimuli for excessive surfactant release.

A substantial fraction of alveolar surfactant glycerophospholipids are cleared by alveolar macrophages or recycled by type II alveolar cells (34, 62, 189). PEEP appears to moderate this effect when applied 1h post exposure. Although the exact mechanisms are unclear, possible explanations are:

- Limiting ongoing type II alveolar cell necrosis and therefore release
- Modulating type II alveolar cell surfactant recycling function
- Modulating alveolar macrophage function for elimination of excess GPLs

LPC, LPG and LPI are not significantly altered in fraction or amount, and certainly not markedly elevated post-phosgene exposure suggesting minimal hydrolysis by PLA_2. However, in the CG CPAP group, an inverse relationship between PC and LPC suggests a regulatory effect on PLA_2, limiting excessive hydrolysis under these conditions. In this study, observations have shown that PC is increased in more severe intoxication, this regulatory effect

could result from disruption of the Lands cycle with increased toxicity. Nonetheless, the consistently elevated PC levels across the other two groups indicate that hydrolysis via this pathway is not the predominant mechanism overall.

As shown in Section 3.4.3 alveolar macrophages in BAL were (non-significantly) increased by the application of CPAP post phosgene exposure. The overall proportion of alveolar macrophages was significantly decreased in the CG Control group, this is a result of the non-significant increase in number, in combination with a rise in absolute neutrophil count occurring in that group. This finding supports a role for elimination of excess GPLs by preservation of alveolar macrophages.

When the CG Control Group was stratified by survival outcome, the differences in some lipid groups were associated with altered survival. Increases in PC and PG were associated with improved survival, while increased SM was associated with death. Increased saturated PC was favourable while increased disaturated PC was not. This highlighted that the increases in all surfactant glycerophospholipids were a compensatory mechanism during lung injury and the absence of compensation was associated with negative outcomes.

Higher PC levels in the CG Control group could be attributed to a combination of increased synthesis, reduced clearance, and other inflammatory contributions. The application of PEEP in the CG CPAP group, by mitigating inflammation and supporting more efficient surfactant metabolism, likely prevented the accumulation of PC observed in the CG Control group. This suggests CPAP played a role in maintaining surfactant homeostasis. Taken together, these findings indicate that elevated glycerophospholipids in BAL resulted from compensatory and pathological processes, and that PEEP, as a surrogate for CPAP moderated these changes, supporting surfactant homeostasis and improving outcome.

5 - PLASMA LIPID FINDINGS

5.1 Introduction

Detailed analysis of the systemic changes in the lipidomics of phosgene induced acute lung injury has not been done before. This kind of analysis could reveal a marker of phosgene exposure prior to the development of signs and symptoms. Even if not diagnostic, changes in composition or ratios of plasma lipids may provide useful mechanistic or prognostic information.

Plasma samples from 8 phosgene exposed (CG Control) and 8 phosgene exposed treated with PEEP (CG CPAP) animals were available to analyse at the following time points: baseline at -40min (annotated as 0), 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 16, 18, 19, 20, 21, 22, 23 and 24 hours. No plasma from air exposed controls could be released from DSTL Porton Down for analysis. Typical mass spectra are shown in Figure 70. As detailed in Section 2.2.4, extracted samples were analysed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS). Collision induced fragmentation was used to identify glycerophospholipid species, namely PC, LPC, SM (m/z 184^{+}), PI (m/z 241^{-}) and PG (m/z 153). Neutral loss scans identified PE (m/z 141^{+}) and PS (m/z 87^{-}). This data was then imported into GraphPad Prism10 for statistical analysis and graphical illustration. *p* values are represented as $p \leq 0.05$ = ★, $p \leq 0.01$ = ★★, $p \leq 0.001$ = ★★★ and $p \leq 0.0001$ = ★★★★.

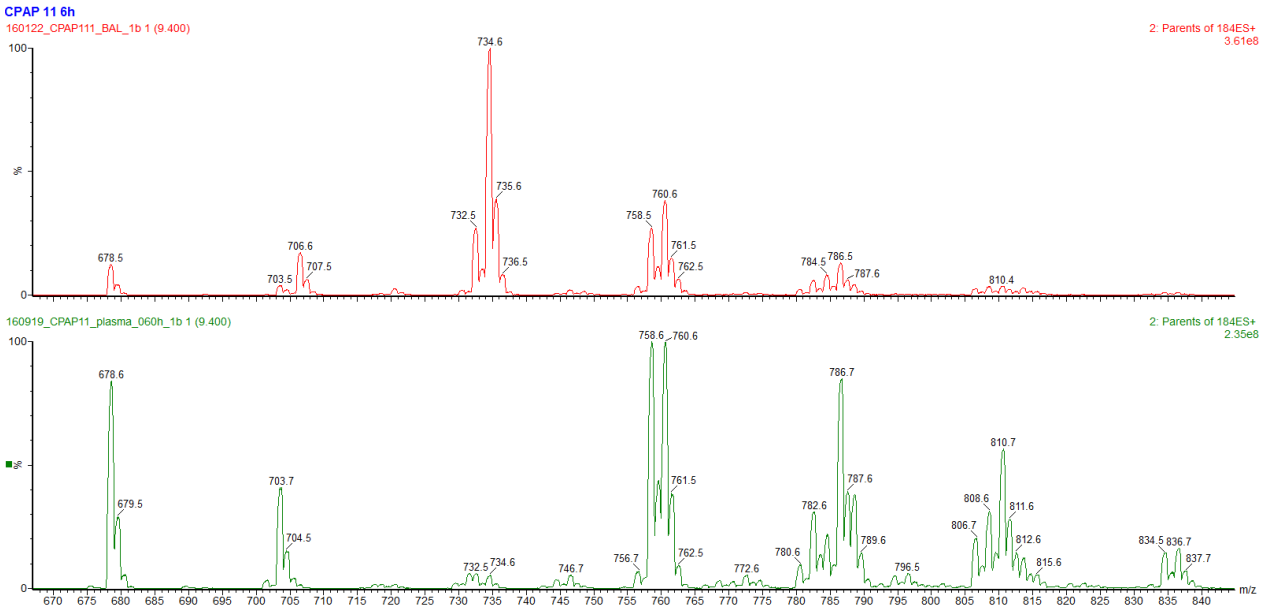

Figure 70. Typical scans of m/z184+ of bronchoalveolar lavage (BAL) fluid (top) and plasma (bottom).

5.2 Plasma Findings

Animals assigned to the phosgene control arm are denoted CG Control and animals assigned to the phosgene but treated with PEEP are denoted CG CPAP. All results are expressed as mean ± standard error of the mean (SEM). Only species occurring at >1% abundance are reported.

5.2.1 Major Glycerophospholipids in Plasma

Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) concentrations were significantly higher in the CG CPAP group, while phosphatidylserine (PS) was significantly higher in CG Controls. Lysophosphatidylcholine (LPC) and phosphatidylglycerol (PG) showed no significant differences between CG Controls and CG CPAP groups. The changes are summarised in Figure 71. Analysis of survivors and non-survivors in the CG Control group revealed no significant differences. The application of PEEP to the lungs appears to influence the regulation of glycerophospholipids in plasma, particularly PC and PE where the largest differences occurred.

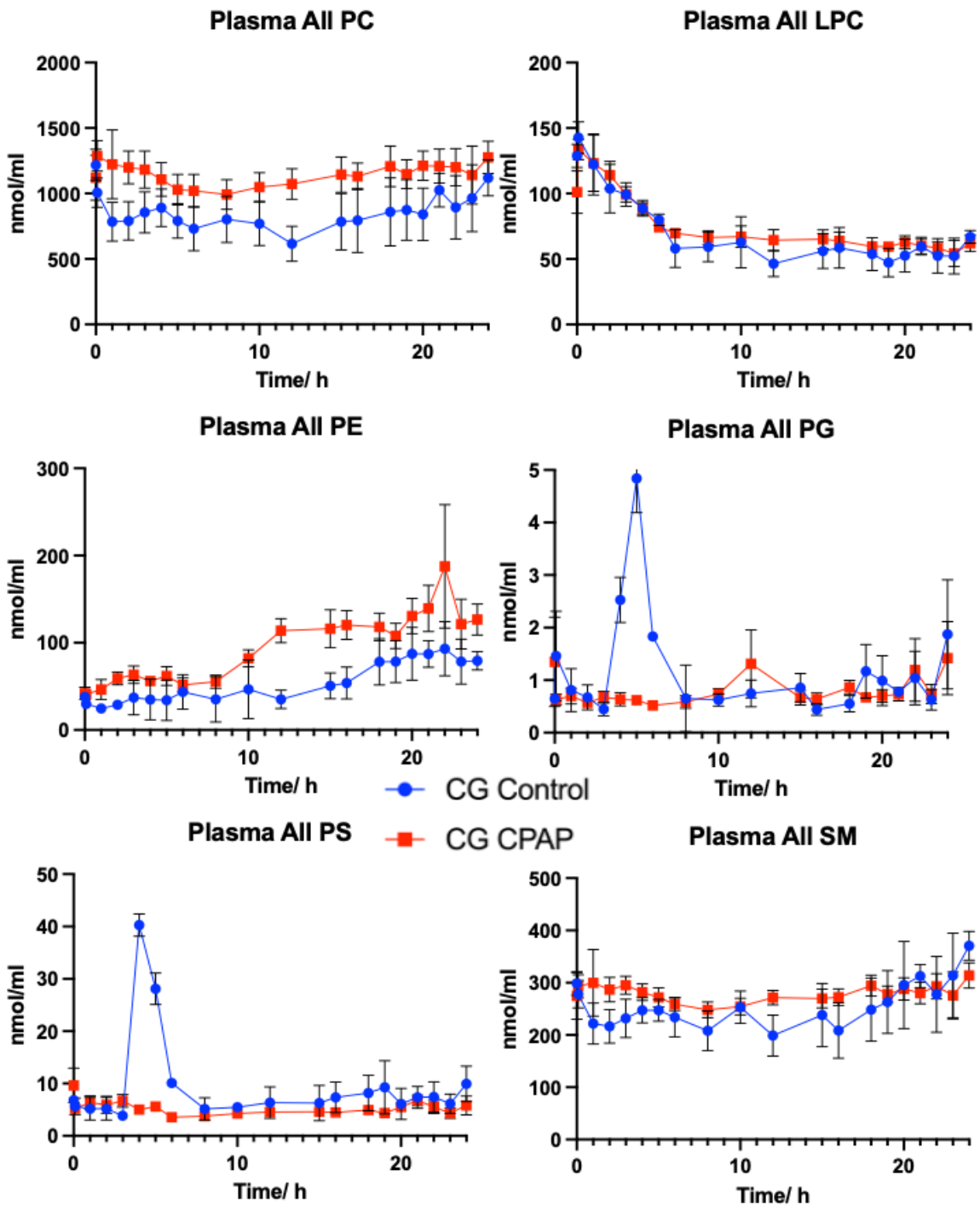

Figure 71. Absolute amounts of major glycerophospholipid classes in plasma measured during the experiments. There was a significant overall increase in plasma phosphatidylcholine (PC) and phosphatidylethanolamine (PE) concentrations in CG CPAP compared with CG Controls (Mann-Whitney $U = 26\ and\ 78, p < 0.0001\ and\ p = 0.0007$, respectively). Lysophosphatidylcholine (LPC) concentration decreased over time but

showed no significant group difference ($p = 0.1274$). Because PC is metabolised to LPC, the temporal variation in LPC reflected upstream changes in PC metabolism. Phosphatidylglycerol (PG) showed no significant group difference ($p = 0.3013$). Phosphatidylserine (PS) concentrations were lower in the CG CPAP group ($U = 84, p = 0.0013$). Differences at time points 4h and 5h in PG and PS were likely artefactual. Sphingomyelin (SM) concentration increased modestly ($U = 117, p = 0.0245$) in CG CPAP compared with CG Controls. Median (nmol.ml^{-1}) values for CG Controls and CG CPAP were respectively: PC★★★★ 848 and 1146; LPC 59.3 and 65.8; PE★★★ 45.0 and 94.9; PG 0.79 and 0.69; PS★★ 6.60 and 5.14; SM★ 248 and 279. Statistical comparisons used two-tailed Mann-Whitney U tests. Data are presented as mean ± SEM. Sample sizes were CG Controls (● n = 8 decreasing to 5) and CG CPAP (■ n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

When expressed as a proportion of total glycerophospholipids, fractional differences were evident between groups, shown in Figure 72. The relative abundance of PC and PE was significantly higher in the CG CPAP group. In contrast, PS and PG comprised a smaller fraction in CG CPAP. LPC and SM fractions showed no significant difference. These findings indicate that PEEP was associated with an enrichment of PC and PE and a relative depletion of PS and PG. This suggests a selective modulation of lipid trafficking or secretion.

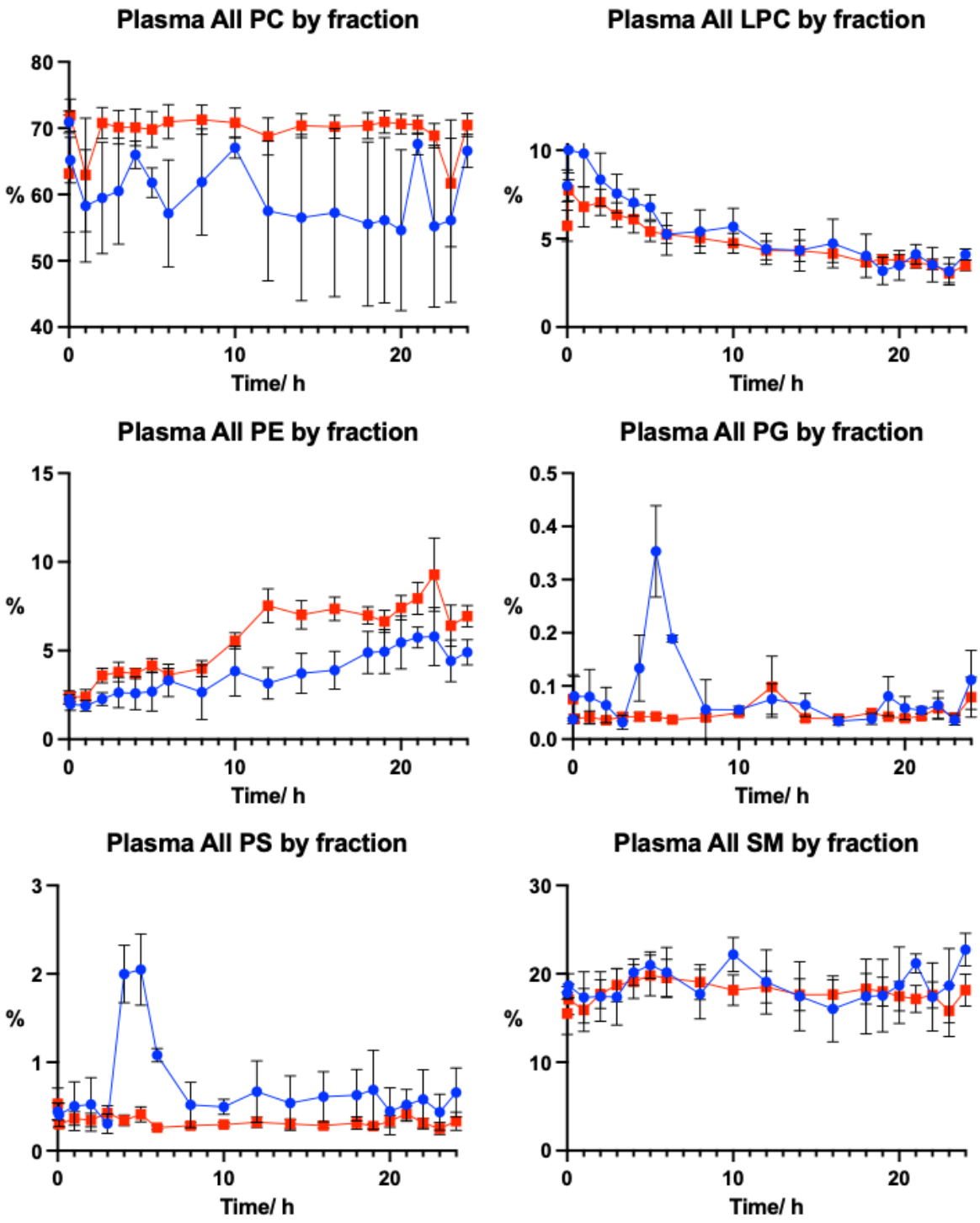

Figure 72. Fractional composition of major glycerophospholipid classes in plasma. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) comprised a larger fraction in CG CPAP than CG Controls (Mann-Whitney $U = 33\ and\ 103, p < 0.0001\ and\ p = 0.0080$, respectively). Phosphatidylglycerol (PG) and phosphatidylserine (PS) and represented

smaller fractions in CG CPAP ($U = 124\ and\ 24, p = 0.0402\ and\ p < 0.0001$, respectively. Differences at time points 4h and 5h in PG and PS were likely artefactual. Lysophosphatidylcholine (LPC) and sphingomyelin (SM) fractions did not differ significantly ($p = 0.4291\ and\ p = 0.2888$, respectively). Statistical comparisons used two-tailed Mann-Whitney U tests. Data are presented as mean ± SEM. Sample sizes were CG Controls (● n = 8 decreasing to 5) and CG CPAP (■ n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

5.2.2 Phosphatidylcholine

Phosphatidylcholine species of interest were analysed, the results are plotted as absolute concentrations and fractions when compared to the total amount of species of interest. Saturated PC species occur at <1% of total PC in plasma and are not displayed. During the course of the experiments the plasma concentration of the PC species of interest fluctuated.

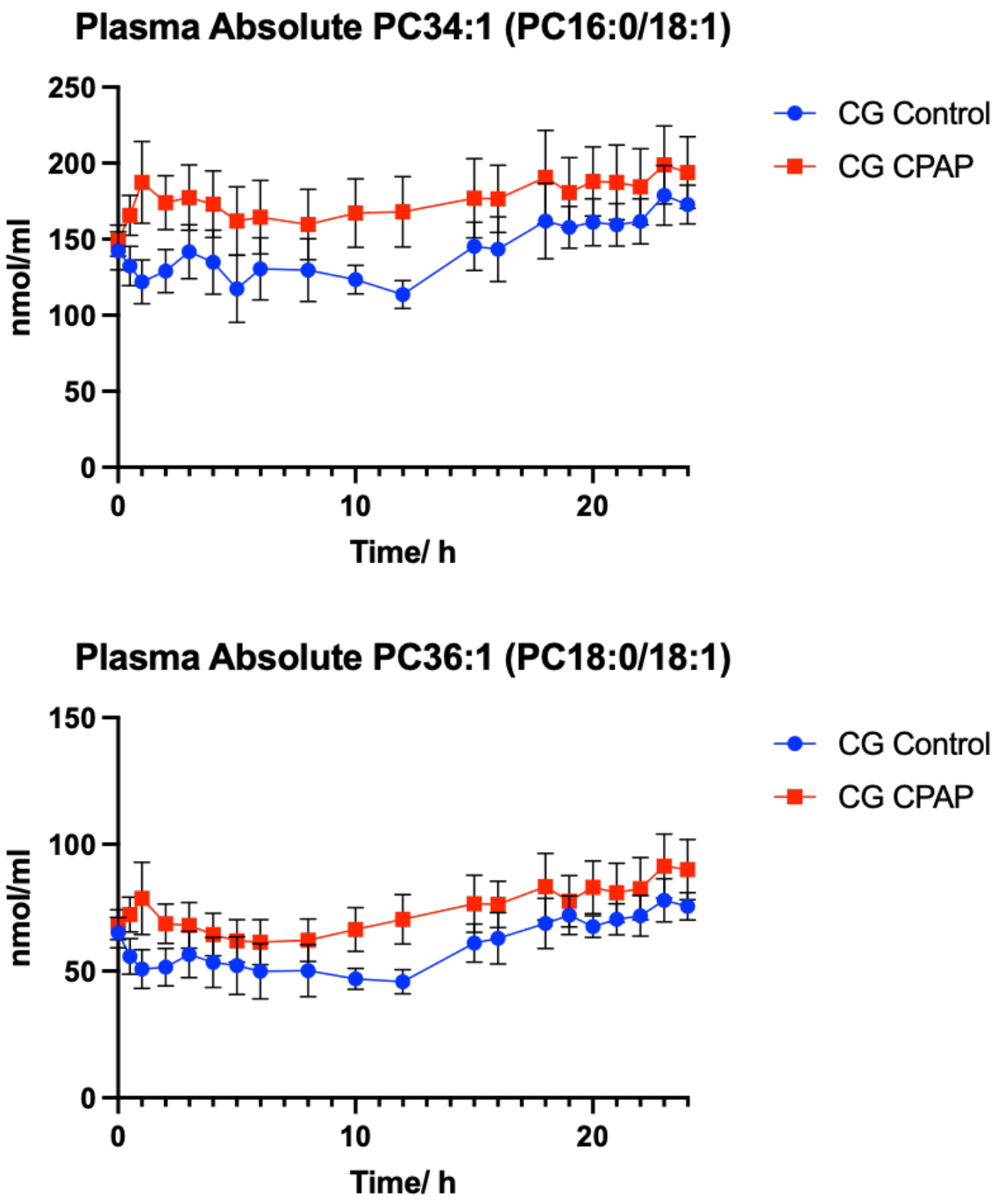

Figure 73. Plasma absolute concentrations of monounsaturated phosphatidylcholine species PC34:1 (commonly PC16:0/18:1) and PC36:1 (commonly PC18:0/18:1) over time in CG Control and CG CPAP groups. Mann-Whitney U tests revealed a significant effect between groups for both PC34:1 ($U = 27, p < 0.0001$) and PC36:1 ($U = 69, p = 0.0002$). Median concentrations (nmol.ml^{-1}) for CG Controls and CG CPAP were PC34:1= 14.21 and 17.68 respectively and for PC36:1= 5.88 and 7.43 respectively.

CG CPAP demonstrated consistently higher concentrations, compared with CG Control, of these monounsaturated PC species throughout the monitoring period. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

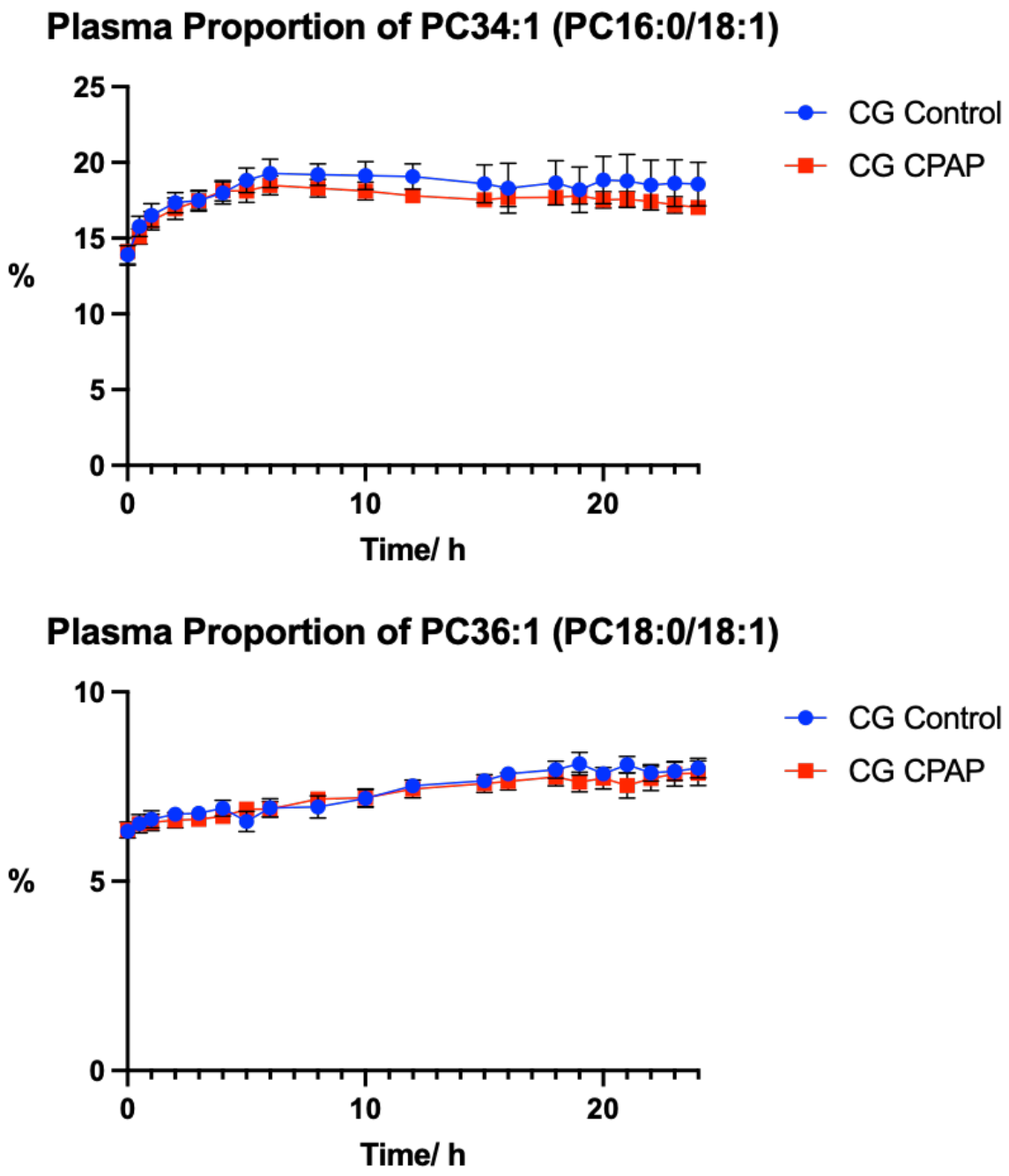

Figure 74. Plasma proportions of monounsaturated phosphatidylcholine (PC) species as a proportion of all measured PC over time. Mann-Whitney U tests revealed that PC34:1 (commonly PC16:0/18:1) comprised a slightly smaller fraction of total PC in CG CPAP compared with CG Controls ($U = 90, p = 0.0024$). No group effect was observed for PC36:1 (commonly PC18:0/18:1) ($U = 162, p = 0.3141$). Median values (%) for CG Controls and CG CPAP were 18.58 and 17.55 for PC34:1, and 7.35 and 7.32 for PC36:1 respectively. Statistical comparisons used two-tailed Mann-Whitney U tests. Data are

presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

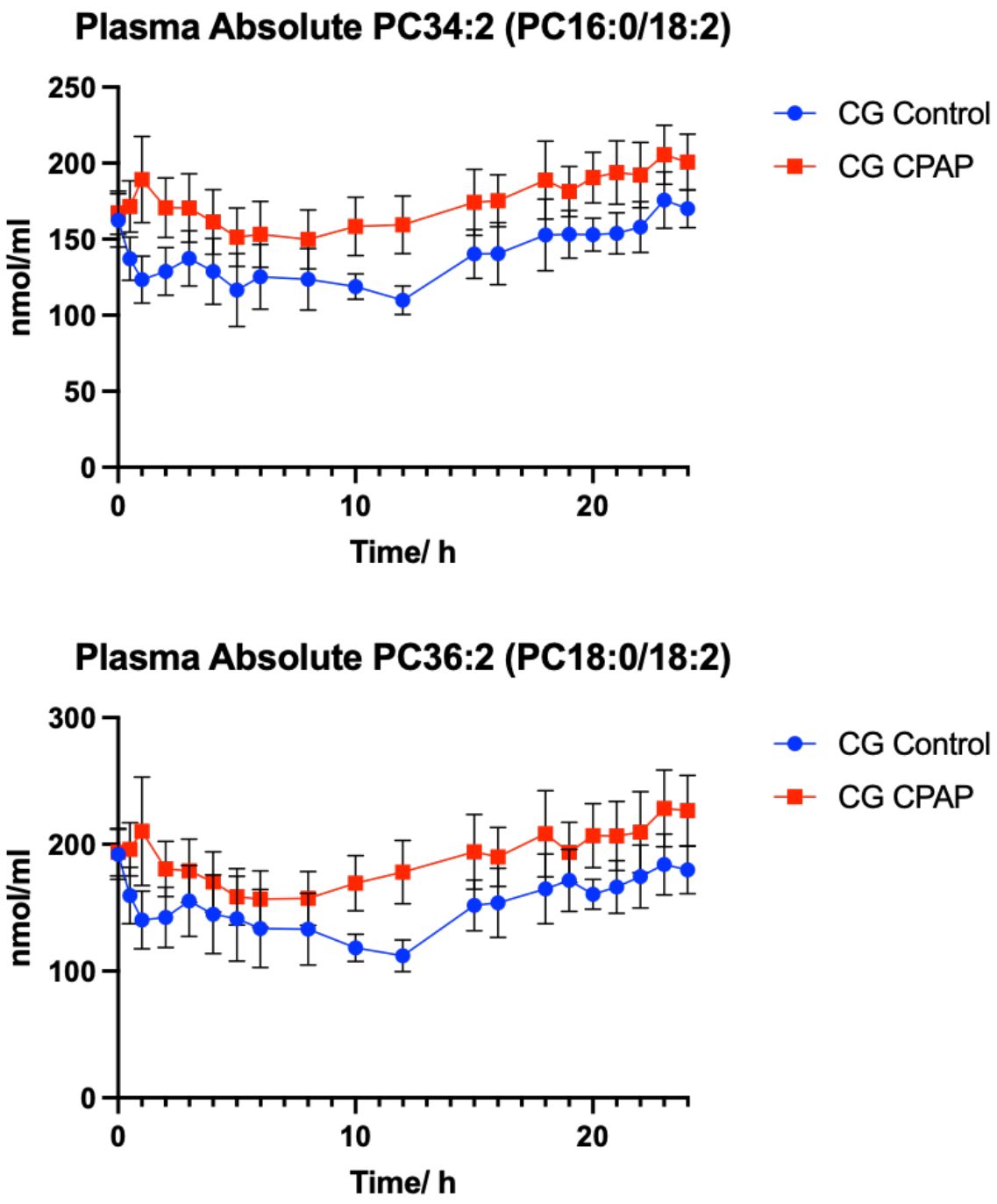

Figure 75. Plasma absolute concentrations of diunsaturated phosphatidylcholine (PC) species; PC34:2 (commonly PC16:0/18:2) and PC36:2 (commonly PC18:0/18:2), in CG Control and CG CPAP groups. Mann-Whitney U tests revealed significantly higher concentrations in CG CPAP compared with CG Controls for both species (PC34:2 $U = 37, p < 0.0001$ and PC36:2 $U = 46, p < 0.0001$).Median concentrations (nmol.ml^{-1}) for CG Controls and CG CPAP were 13.88 and 17.29 for PC34:2, and 15.47

and 19.38 for PC36:2 respectively. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

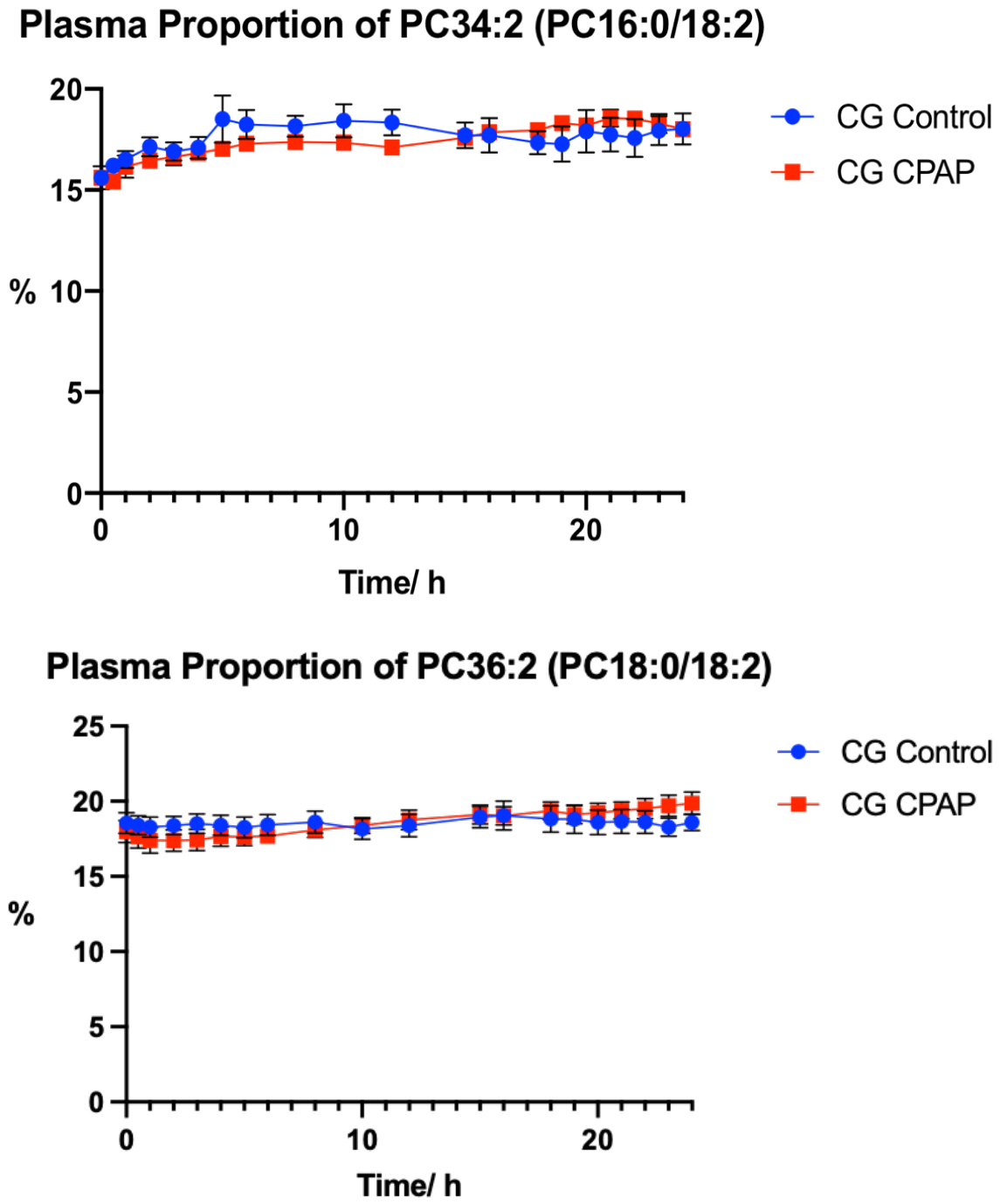

Figure 76. Plasma relative proportions of diunsaturated PC species; PC34:2 (commonly PC16:0/18:2) and PC36:2 (commonly PC18:0/18:2, in CG Control and CG CPAP groups. Mann-Whitney U tests revealed no significant differences between CG Control and CG CPAP groups for either PC34:2 ($U = 179, p = 0.5831$) or PC36:2 ($U = 199, p = 0.9893$). Median proportions (%) for CG Controls and CG CPAP were 17.71 and 17.35 for PC34:2, and18.52 and 18.58 for PC36:2 respectively. Statistical comparisons used

two-tailed Mann-Whitney U tests. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

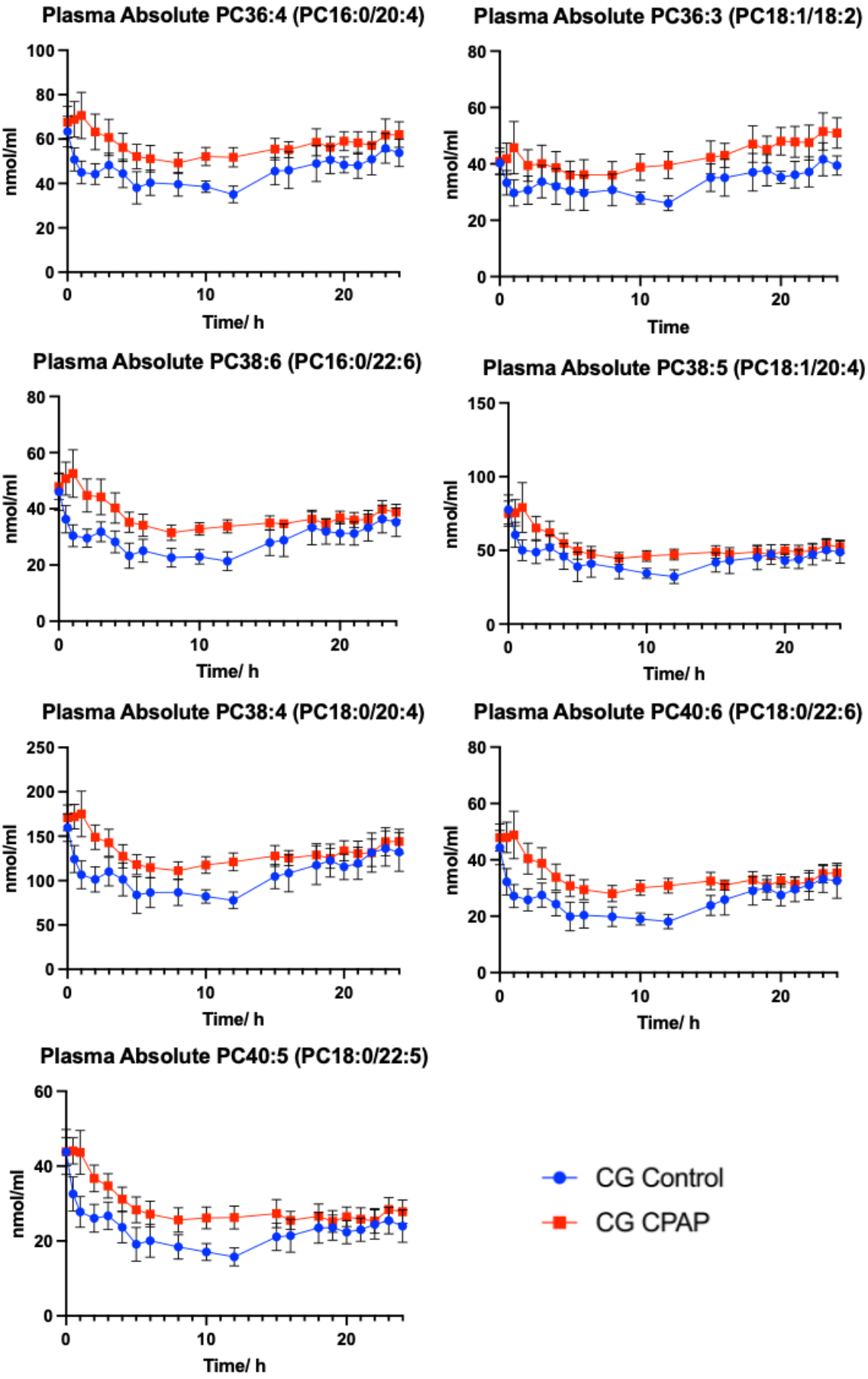
Plasma Absolute PC36:4 (PC16:0/20:4)
nmol/ml
Time/ h
Plasma Absolute PC36:3 (PC18:1/18:2)
nmol/ml
Time
Plasma Absolute PC38:6 (PC16:0/22:6)
nmol/ml
Time/ h
Plasma Absolute PC38:5 (PC18:1/20:4)
nmol/ml
Time/ h
Plasma Absolute PC38:4 (PC18:0/20:4)
nmol/ml
Time/ h
Plasma Absolute PC40:6 (PC18:0/22:6)
nmol/ml
Time/ h
Plasma Absolute PC40:5 (PC18:0/22:5)
nmol/ml
Time/ h
CG Control
CG CPAP

Figure 77. Plasma concentrations of polyunsaturated phosphatidylcholine (PC) species in CG Control and CG CPAP groups across multiple species for the observation period. Mann-Whitney U tests revealed significantly higher concentrations in CG CPAP for all species: PC36:4 ($U = 32, p < 0.0001$), PC36:3 ($U = 33, p < 0.0001$), PC38:6 ($U = 51, p < 0.0001$), PC38:5 ($U = 90, p = 0.0024$), PC38:4 ($U = 72, p = 0.0003$), PC40:6 ($U = 60, p < 0.0001$) and PC40:5 ($U = 60, p < 0.0001$). Median concentrations were consistently higher in CG CPAP than in CG Controls. Therefore, CG CPAP exhibited elevated levels of all polyunsaturated PC species throughout the monitoring period. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

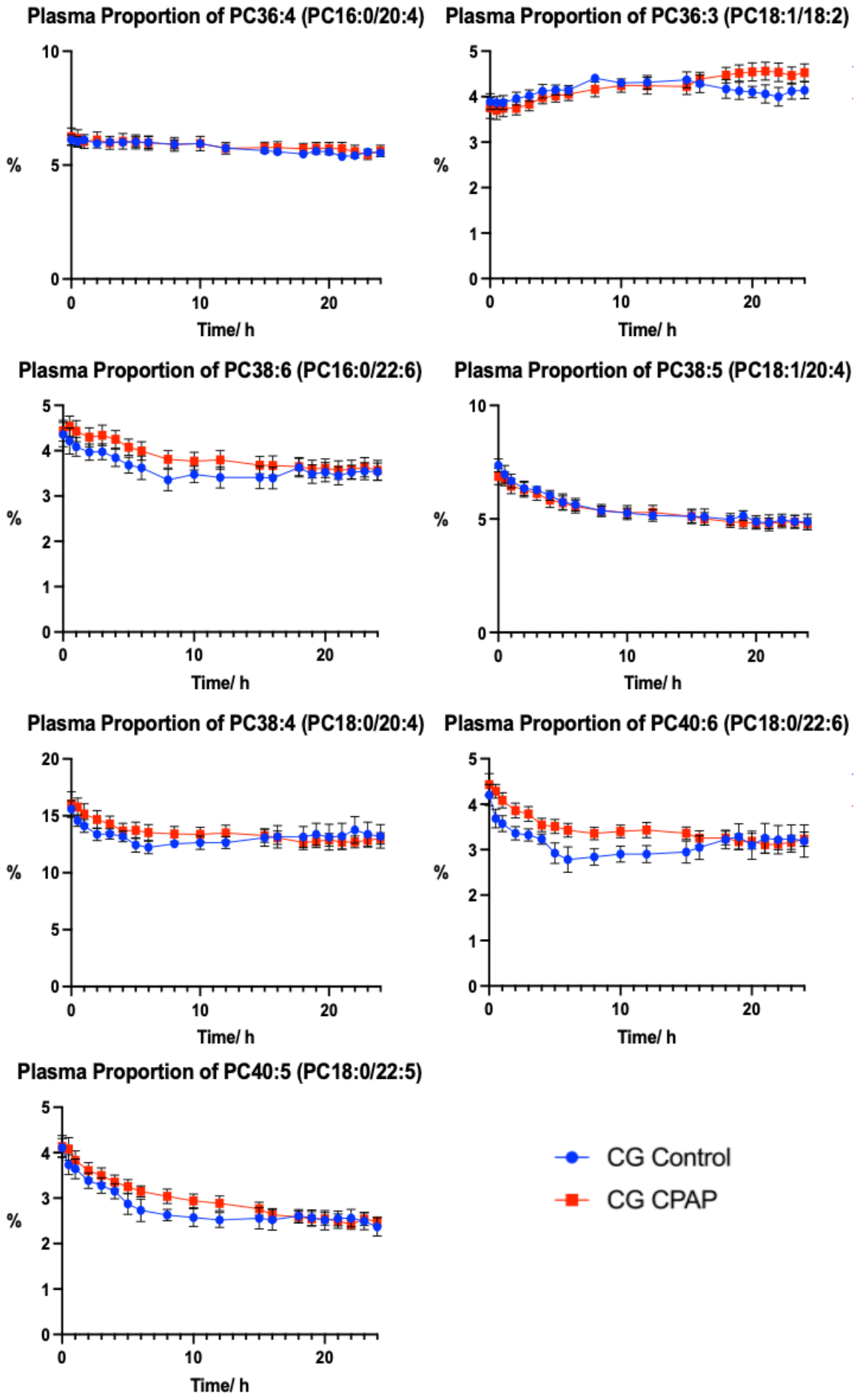
Plasma Proportion of PC36:4 (PC16:0/20:4)
Plasma Proportion of PC36:3 (PC18:1/18:2)
Plasma Proportion of PC38:6 (PC16:0/22:6)
Plasma Proportion of PC38:5 (PC18:1/20:4)
Plasma Proportion of PC38:4 (PC18:0/20:4)
Plasma Proportion of PC40:6 (PC18:0/22:6)
Plasma Proportion of PC40:5 (PC18:0/22:5)
%
Time/ h
CG Control
CG CPAP

Figure 78. Plasma relative proportions of polyunsaturated phosphatidylcholine (PC) species in CG Control and CG CPAP for the observation period. Mann-Whitney U tests revealed no significant group differences for PC36:4 (commonly PC16:0/20:4) ($U = 160, p = 0.2888$),PC36:3 (commonly PC18:1/18:2) ($U = 167, p = 0.3834$), PC38:5 (commonly 18:1/20:4) ($U = 177, p = 0.5468$), PC38:4 (commonly PC18:0/20:4) ($U = 156, p = 0.2423$) or PC40:5 (commonly PC18:0/22:5) ($U = 167, p = 0.3834$). Significantly higher proportions were seen in CG CPAP for PC38:6 (commonly PC16:0/22:6) ($U = 99, p = 0.0056$) and PC40:6 (commonly PC18:0/22:6) ($U = 107, p = 0.0112$). Median proportions (%) for CG Controls and CG CPAP were: PC36:4 = 5.83 and 5.84, PC36:3 = 4.13 and 4.23, PC38:6★★ = 3.54 and 3.78, PC38:5= 5.21 and 5.29, PC38:4 = 13.19 and 13.40, PC40:6★ = 3.22 and 3.38, PC40:5 = 2.59 and 2.91, respectively. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8 decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

5.2.3 Summary of Phosphatidylcholine Results

Monounsaturated and diunsaturated species showed either stability or increases in their absolute concentrations, with no decrements observed, values were higher in the PEEP treated group; CG CPAP. In contrast, polyunsaturated species generally decreased in both absolute concentrations and proportions.

Notably, an initial spike in absolute amounts was seen in all the PEEP treated groups but thereafter a decline or failure to increment in polyunsaturated species was seen in both arms. Mono- and diunsaturated species tended to recover or remain stable, with some increases seen in the CG CPAP group. Overall, PEEP tended to preserve the concentration of most measured plasma lipid species and attenuated their depletion, in the CG CPAP group.

In patients with ARDS, principally of pneumonic origin, phosphatidylcholine kinetics of $CD15^+$ leucocytes and $CD3^+$ T-lymphocytes have been examined (190). In $CD15^+$ cells there is an influx of polyunsaturated PC species as part of the inflammatory response. Further losses from plasma result from the formation of inflammatory mediators such as eicosanoids, derived from arachidonate containing species (PC36:4, PC38:4 and PC38:5), and this is likely to be mediated, in part by $CD15^+$ cells. Mono and diunsaturated PC species have a reduced role in inflammatory signalling which could explain their relative stability or increase in proportion during systemic inflammation. The chemical nature of none, or single double bond renders them more stable.

The observed changes in plasma PC as a result of the application of PEEP to the lungs supports the concept of the hepatopulmonary axis. This lung-liver axis is capable of orchestrating rapid changes in concentration of PC and has been dynamically studied in the context of alveolar proteinosis (191). The reduction in polyunsaturated PC associated with reduced liver PEMT activity seen in ARDS patients (88), aligns with the findings. However, whereas PC34:2 (commonly PC16:0/18:2) decreased in ARDS patients, it remained stable in phosgene induced acute lung injury. This likely reflects early sampling in the experiment, before systemic inflammation and hepatic PEMT impairment are established. Direct comparison with later presentation human ARDS patients may not be appropriate.

5.2.4 Lysophosphatidylcholine

The concentration of all plasma LPC species analysed fell over the 24h time period. Unsaturated species exhibited a spike in the first hour, whereas the saturated species, LPC16:0 and LPC18:0 remained stable. This early peak may reflect a common inflammatory response mechanism as a result of damage to cell membranes. PC degradation by PLA_2 tends to favour unsaturated PC species (192). The overall proportions were similar, indicating a common pattern of reduction in concentration. After the initial

spike, the subsequent fall is consistent with acyl remodelling by the liver and not due to degradation of PC. The corresponding absolute and fractional data are presented in Figure 79 and Figure 80.

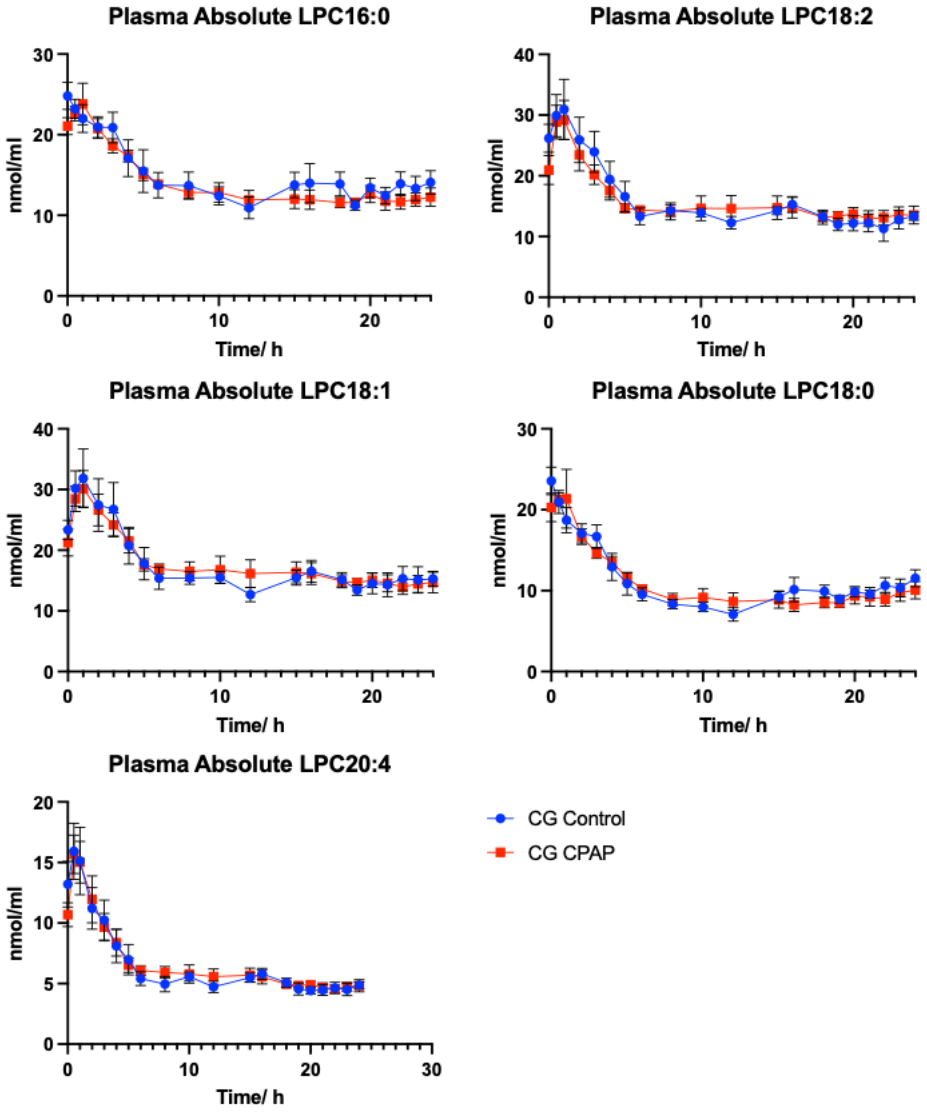

Figure 79. Plasma concentrations of lysophosphatidylcholine (LPC) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant differences between groups for any of the LPC species analysed: LPC16:0 ($U = 151, p = 0.1918$), LPC18:2 ($U = 169, p = 0.4135$), LPC18:1 ($U = 190, p = 0.7994$), LPC18:0 ($U = 176, p = 0.5291$), and LPC20:4 ($U = 173, p = 0.4777$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP were respectively: LPC16:0 = 13.90 and 12.74, LPC18:2 = 14.09 and 14.64, LPC18:1 = 15.44 and 16.44,

LPC18:0 = 10.24 and 9.55, LPC20:4 = 5.46 and 5.74. Although unsaturated LPC species showed early transient increases within the first hour, no group differences were detected. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

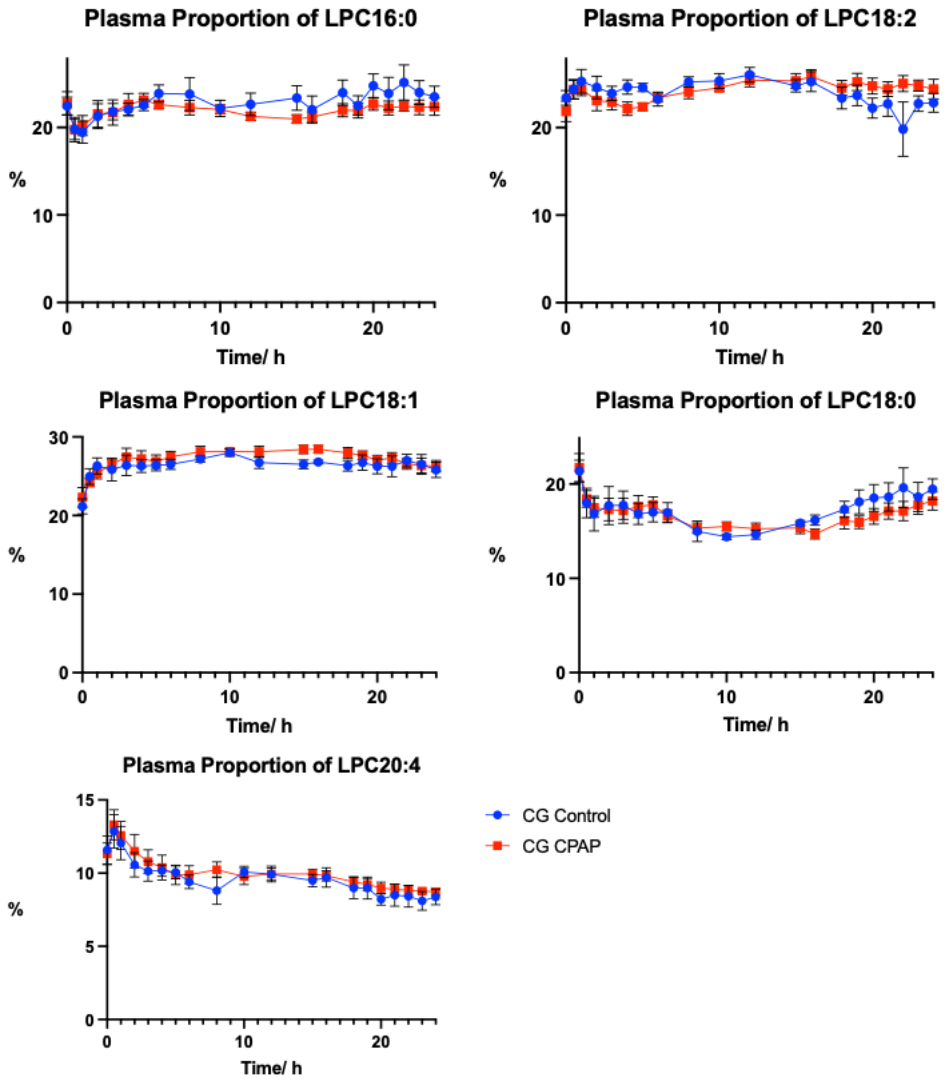

Figure 80. Plasma proportions of lysophosphatidylcholine (LPC) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significant differences between groups for LPC16:0 ($U = 117, p = 0.0245$) and LPC18:1 ($U = 104, p = 0.0087$). No significant differences were observed for LPC18:2 ($U = 189, p = 0.7788$), LPC18:0 ($U = 160, p = 0.2888$) or LPC20:4 ($U = 164, p = 0.3408$). Median proportions (%) for CG Control and CG CPAP were respectively: LPC16:0 = 22.60 and

22.14, LPC18:2 = 24.16 and 24.40, LPC18:1 = 26.39 and 27.28, LPC18:0 = 17.52 and 17.13, LPC20:4 = 9.60 and 9.89. Minor shifts in LPC16:0 and LPC18:1 fractions suggest subtle changes in lipid remodelling in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene; CPAP = Continuous Positive Airway Pressure.

5.2.5 Phosphatidylethanolamine

Saturated PE species were present at concentrations <1% of total PE and therefore were not analysed. Concentrations of all unsaturated species present at >1% rise, with higher concentrations observed in the CG CPAP group. This pattern is consistent with the overall rise in glycerophospholipids seen in association with PEEP and suggests a coordinated regulation of PE alongside PC in response to this. In fractional terms, shorter chain species (PE34:1, PE34:2, PE36:4 and PE36:1) accounted for a smaller proportion of total PE in the CG CPAP group, whereas longer chain polyunsaturated species (PE40:7 and PE 40:5) were either preserved or relatively enriched. Absolute concentrations are presented in Figure 81 to Figure 83. Fractions are presented in Figure 84 to Figure 86.

The increase in plasma PE concentrations in the CG CPAP group may result from a coordinated hepatic response to improved oxygenation and reduced systemic inflammation when compared to the CG Control group. PE and PC are linked through the PEMT pathway described in Section 1.2.3, an increase in both suggests enhanced synthesis rather than redistribution (48). The relative reduction of shorter chain PE species, with preservation or enrichment of longer chain polyunsaturated forms indicates selective consumption of the more labile mono- and diunsaturated species as part of the inflammatory response, consistent with PLA_2 mediated degradation (192). The longer chained highly unsaturated species such as PE40:7 and PE40:5 were retained in the CG CPAP group, these PE species have greater membrane stabilising and anti-inflammatory potential (193), and their

maintenance in PEEP exposed animals suggests attenuation of oxidative stress and PLA_2 activity. Collectively, the pattern supports the concept of a hepatopulmonary axis regulating systemic phospholipid composition in response to more favourable lung physiology and reduced inflammatory signalling.

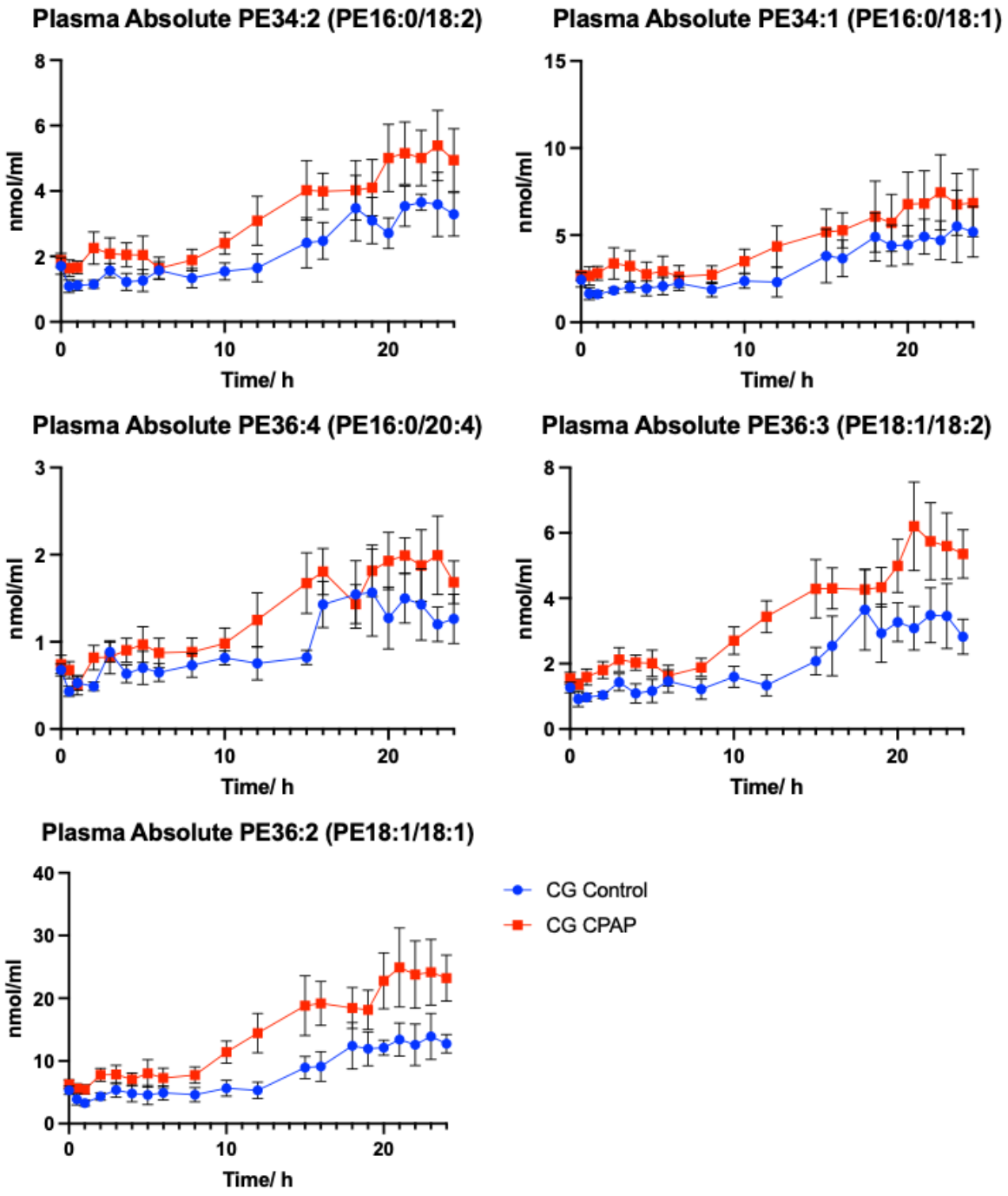

Figure 81. Plasma concentrations of phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher concentrations in the CG CPAP group for all PE species in this figure: PE34:2 (commonly PE16:0/18:2) ($U = 101, p = 0.0067$), PE34:1 (commonly PE16:0/18:1) ($U = 100, p = 0.0061$), PE36:4 (commonly PE16:0/20:4) ($U = 116, p = 0.0227$), PE36:3 (commonly PE18:1/18:2) ($U = 95, p = 0.0039$) and PE36:2 (commonly PE18:1/18:1) ($U =$

$90, p = 0.0024$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP were respectively: PE34:2 = 1.68 and 2.75, PE34:1 = 2.41 and 3.93, PE36:4 = 0.82 and 1.12, PE36:3 = 1.53 and 3.07, PE36:2 = 5.51 and 12.95. All these PE species were elevated in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

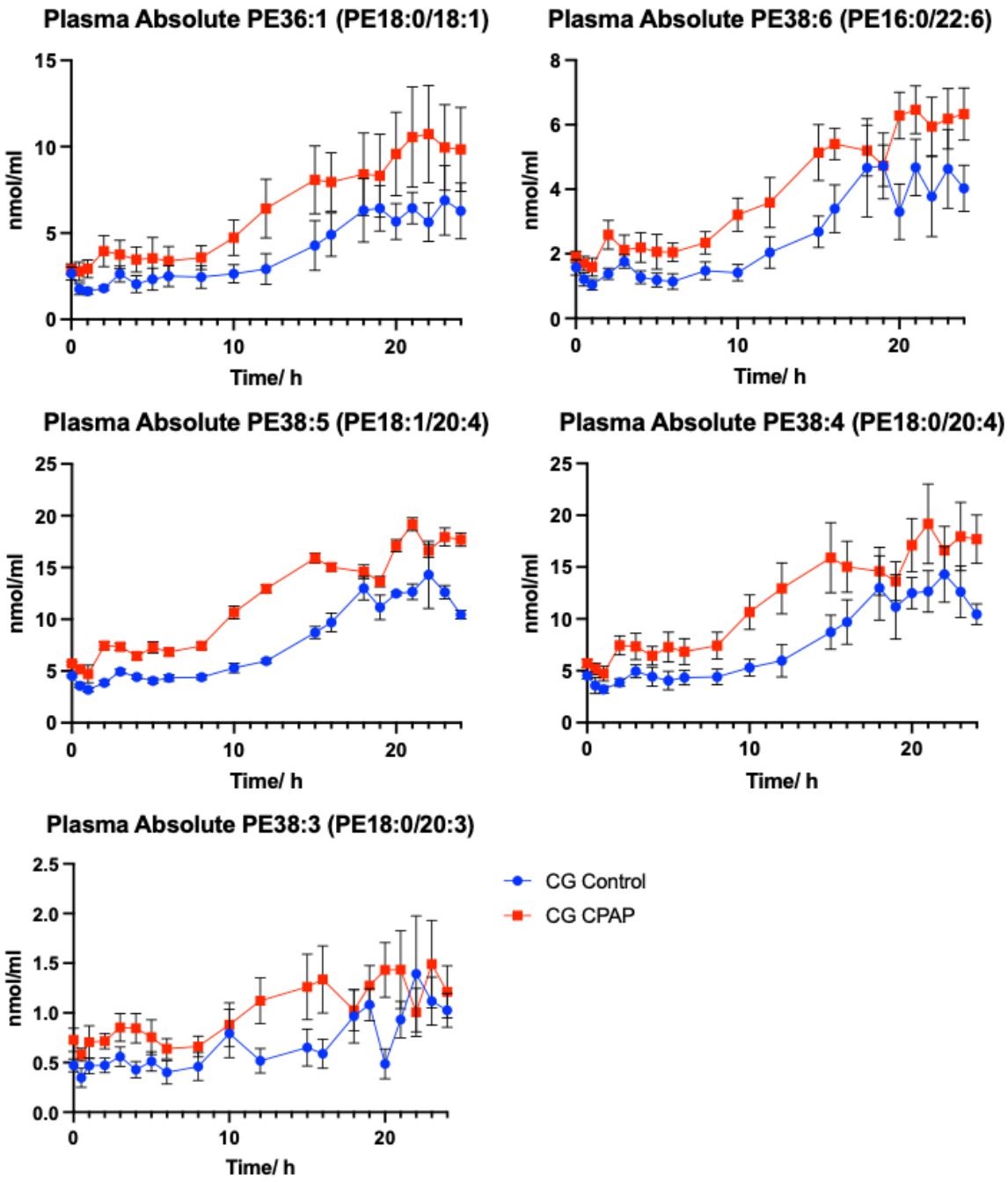

Figure 82. Plasma concentrations of polyunsaturated and longer-chain phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher concentrations in the CG CPAP group for all PE species in this figure: PE36:1 (commonly PE18:0/18:1) ($U = 94, p = 0.0035$), PE38:6 (commonly PE16:0/22:6) ($U = 100, p = 0.0061$), PE38:5 (commonly PE18:1/20:4) ($U = 96, p = 0.0043$), PE38:4 (commonly PE18:0/20:4) ($U = 96, p = 0.0043$) and

PE38:3 (commonly PE18:0/20:3) ($U = 83, p = 0.0012$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP were respectively: PE36:1 = 2.79 and 5.58, PE38:6 = 1.90 and 3.40, PE38:5 = 5.64 and 11.80, PE38:4 = 5.64 and 11.80, PE38:3 = 0.54 and 0.94. All these PE species were elevated in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

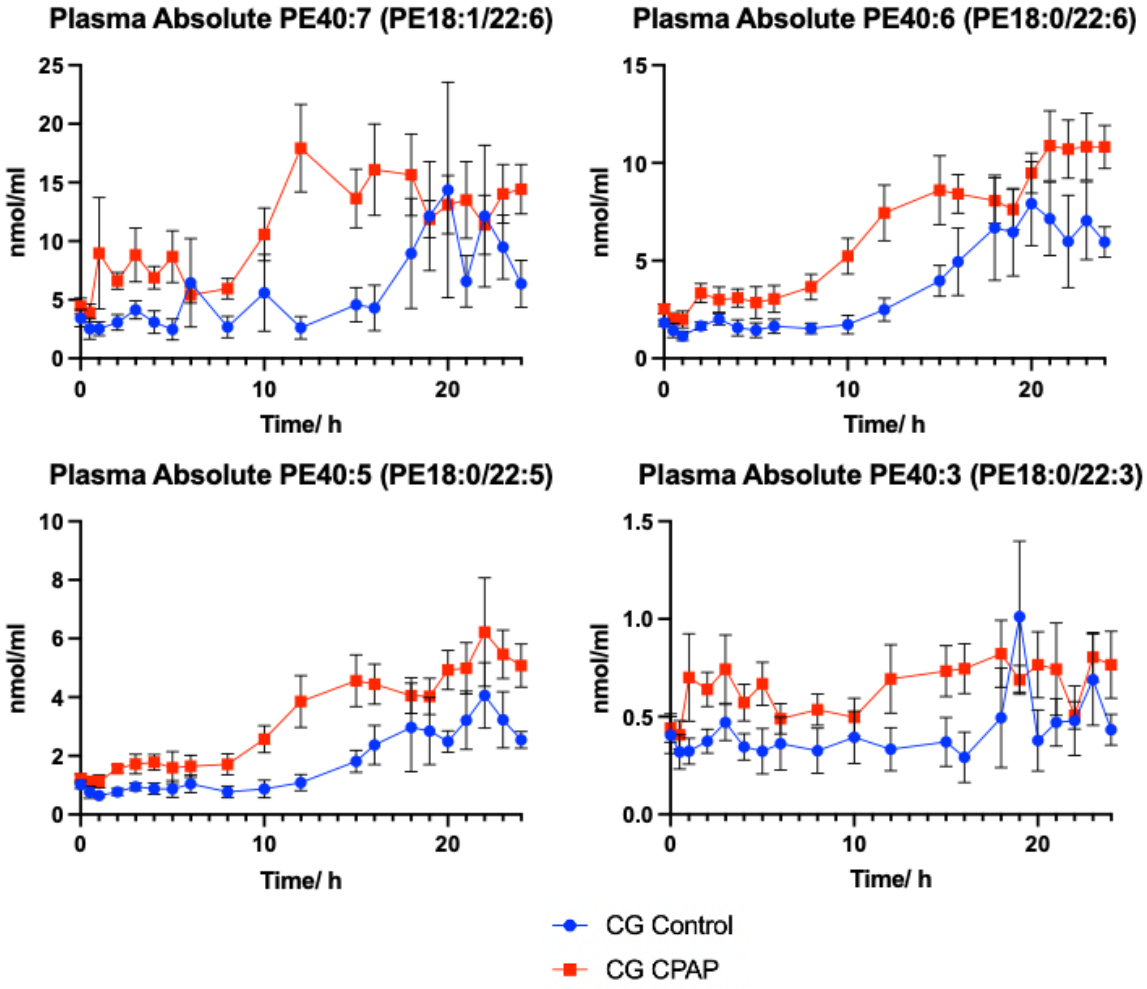

Figure 83. Plasma concentrations of long chain and very long chain phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher concentrations in the CG CPAP group for all PE species in this figure: PE40:7 (commonly PE18:1/22:6) ($U = 76, p = 0.0005$), PE40:6 (commonly PE18:0/22:6) ($U = 93, p = 0.0032$), PE40:5 (commonly PE18:0/22:5) ($U = 89, p = 0.0022$) and PE40:3 (commonly PE18:0/22:3) ($U = 40, p < 0.0001$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP were respectively: PE40:7 = 4.45 and 10.97, PE40:6 = 2.25 and 6.34, PE40:5 = 1.07 and 3.21, PE40:3 = 0.38 and 0.69. All these PE species were significantly elevated in the CG CPAP, indicating a consistent increase

across the higher molecular weight PE species. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

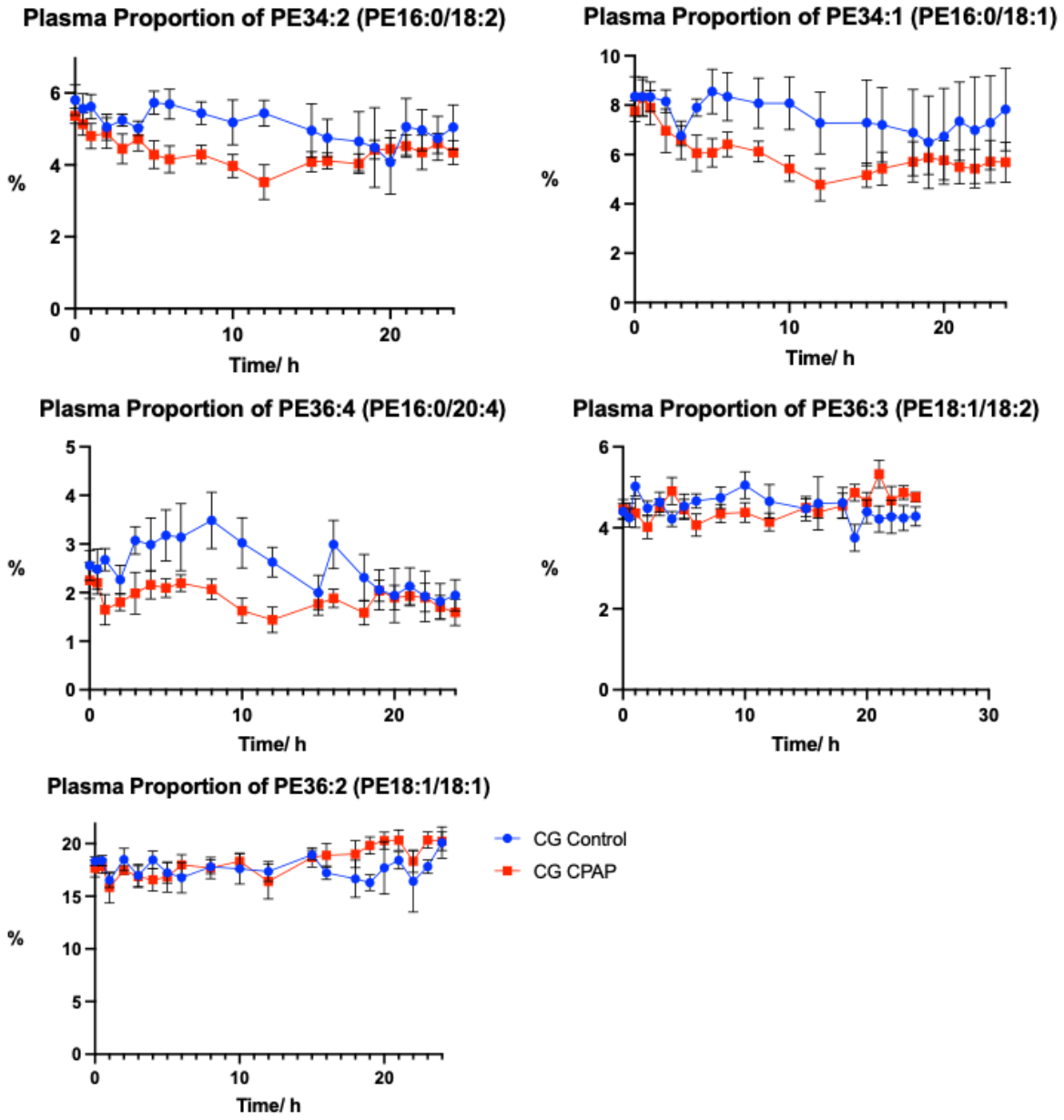

Figure 84. Plasma proportions of phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly lower proportions in the CG CPAP group for PE34:2 (commonly PE16:0/18:2) ($U = 5, p < 0.0001$), PE34:1 (commonly PE16:0/18:1) ($U = 41, p < 0.0001$) and PE36:4 (commonly PE16:0/20:4) ($U = 54, p < 0.0001$). No significant differences were detected for PE36:3 (commonly PE18:1/18:2) ($U = 185, p = 0.6980$) or PE36:2 (commonly PE18:1/18:1) ($U = 149, p = 0.1738$). Median proportions (%) for CG Control and CG CPAP were respectively: PE34:2 = 5.05 and 4.39, PE34:1 = 7.59 and 5.82, PE36:4 = 2.52 and 1.90, PE36:3 = 4.48 and 4.48, PE36:2 = 17.66 and 18.15. Shorter-chain PE species accounted for a smaller fraction of total PE in the CG CPAP group, whereas overall species distribution remained largely preserved. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

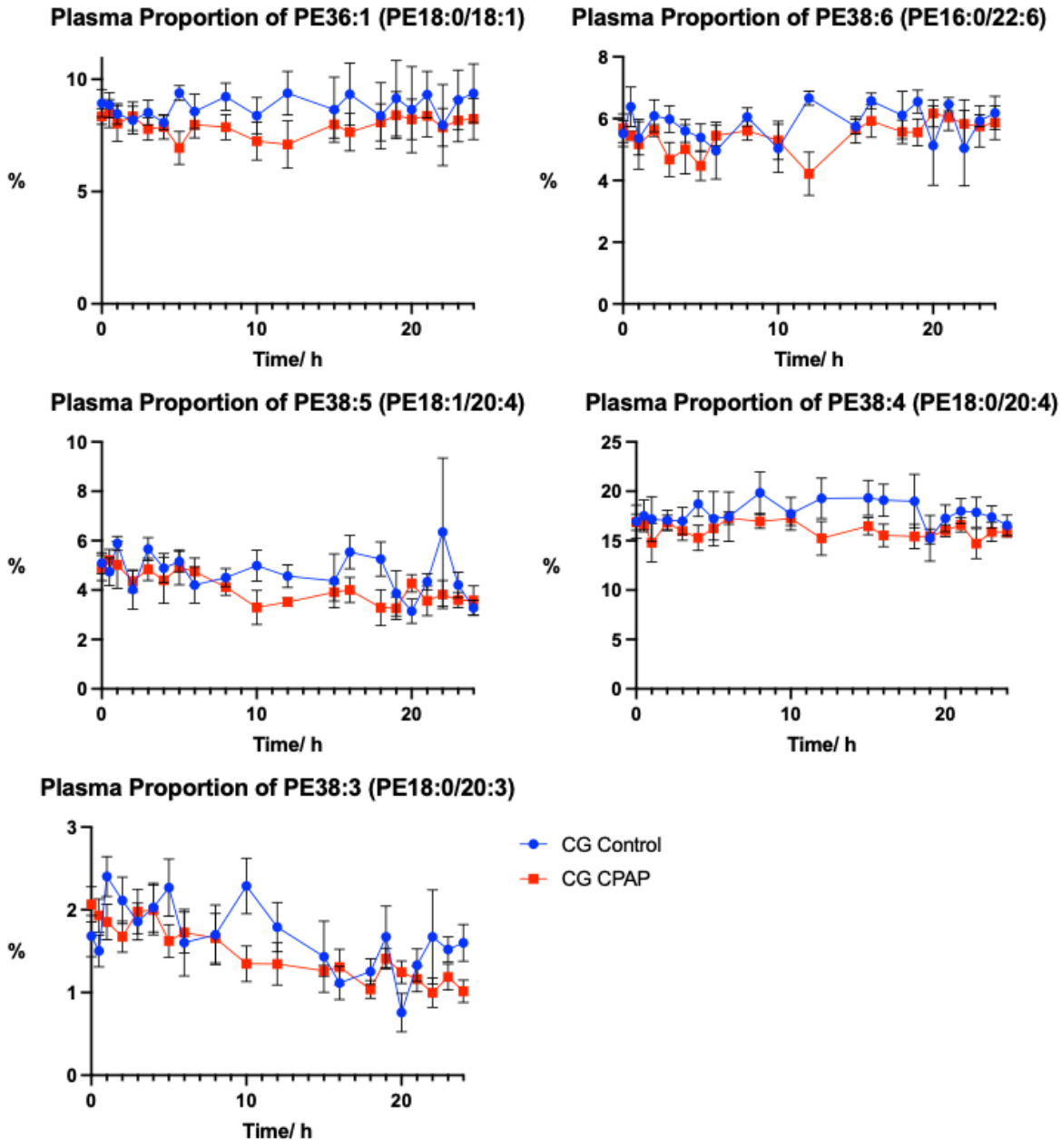

Figure 85. Plasma proportions of phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly lower proportions in the CG CPAP group for PE36:1 (commonly PE18:0/18:1) ($U = 34, p < 0.0001$), PE38:5 (commonly PE18:1/20:4) ($U = 120, p = 0.0304$) and PE38:4 (commonly PE18:0/20:4) ($U = 36, p < 0.0001$). No significant differences were detected for PE38:6 (commonly PE16:0/22:6) ($U = 128, p = 0.0524$) or PE38:3 (commonly

PE18:0/20:3) ($U = 144, p = 0.1344$). Median proportions (%) for CG Control and CG CPAP were, respectively: PE36:1 = 8.75 and 8.02; PE38:6 = 5.96 and 5.59; PE38:5 = 4.65 and 4.06; PE38:4 = 17.47 and 16.00; PE38:3 = 1.67 and 1.38. There was a small but consistent reduction in the fractional contribution of several longer-chain PE species in the CPAP-treated group, while the overall class distribution remained largely stable. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene; CPAP = Continuous Positive Airway Pressure.

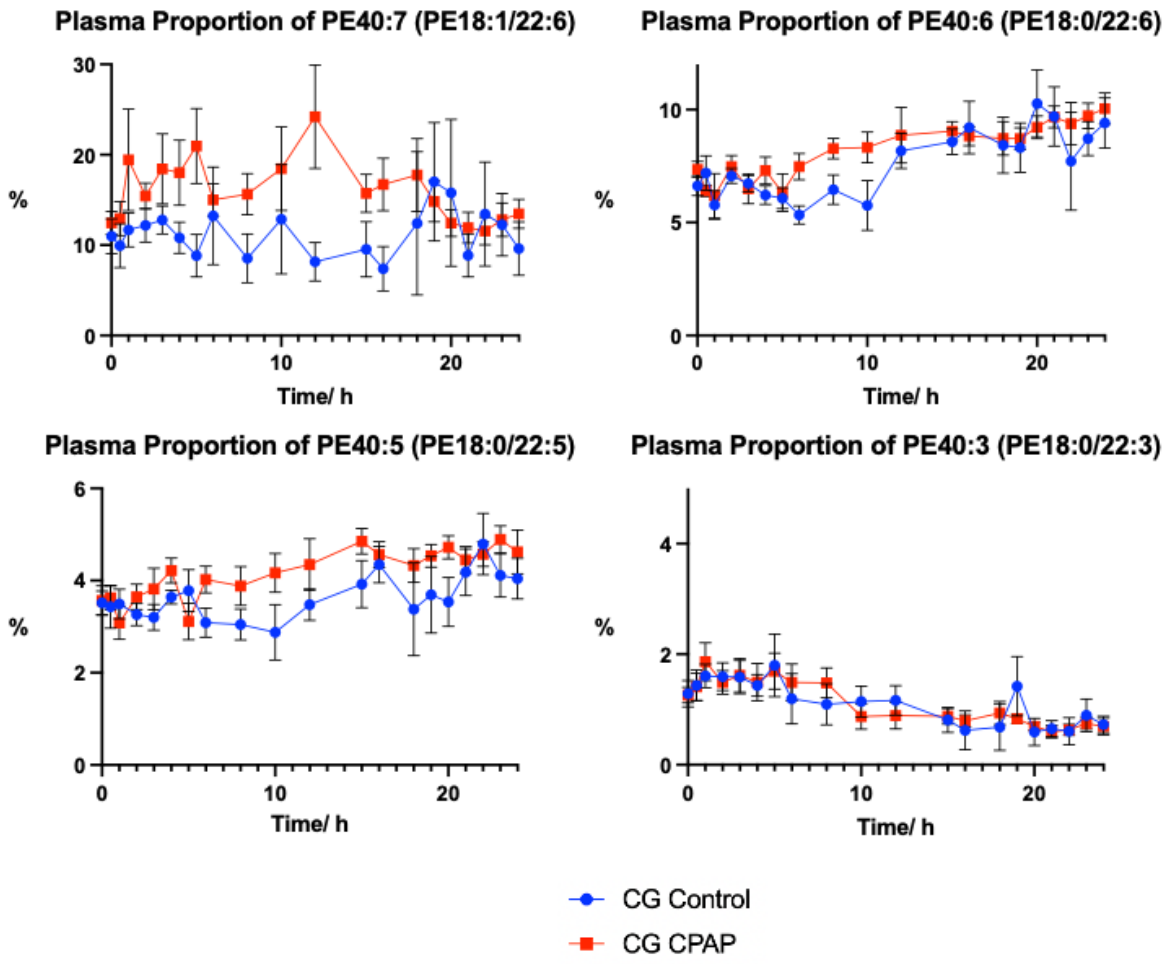

Figure 86. Plasma proportions of very-long-chain phosphatidylethanolamine (PE) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher proportions in CG CPAP for PE40:7 (commonly PE18:1/22:6) ($U = 53, p < 0.0001$) and PE40:5 (commonly PE18:/222:5) ($U = 92, p = 0.0029$). No significant differences were detected for PE40:6 (commonly PE18:0/22:6) ($U = 145, p = 0.1417$) or PE40:3 (commonly PE18:0/22:3) ($U = 191, p = 0.8201$). Median proportions

(%) for CG Control and CG CPAP were respectively: PE40:7 = 11.34 and 15.53, PE40:6 = 7.46 and 8.52, PE40:5 = 3.53 and 4.27, PE40:3 = 1.16 and 0.91. The longest chain PE species were either preserved or relatively enriched in the CG CPAP group, suggesting selective maintenance of highly unsaturated PE species. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

5.2.6 Phosphatidylserine

Plasma phosphatidylserine (PS) represented <1% of total plasma glycerophospholipids, however, its localisation to the interior leaflet of cell membranes and externalisation as part of cell activation or death (38), may make it an indicator of cellular integrity. Total plasma PS concentration was significantly lower in animals treated with PEEP. Shorter chain species such as PS20:2 (commonly PS10:1/10:1), PS26:0 (PS14:0/12:0), PS30:3 (commonly PS20:3/10:0) and PS30:0 (commonly PS16:0/14:0) were reduced in animals exposed to PEEP, both in absolute and fractional terms. PS36:2 (commonly PS18:0/18:2) was absolutely and proportionally higher in animals treated with PEEP, suggesting selective maintenance or preservation of mid chain diunsaturated species. Among the polyunsaturated forms, PS38:5 (commonly PS18:1/20:4) decreased by fraction but not absolute amount, whereas PS38:4 (commonly PS18:0/22:4) increased by absolute amount and fraction in animals exposed to PEEP. The overall pattern is that shorter species decreased, and di- and polyunsaturated species were relatively preserved by PEEP. Very long chain, unsaturated PS species were reduced by PEEP. Absolute concentrations are presented in Figure 87 to Figure 90, fractions are presented in Figure 91 to Figure 94. A transient rise at 4 h was observed in several PS species but is likely artefactual, reflecting sampling variability rather than a biological effect.

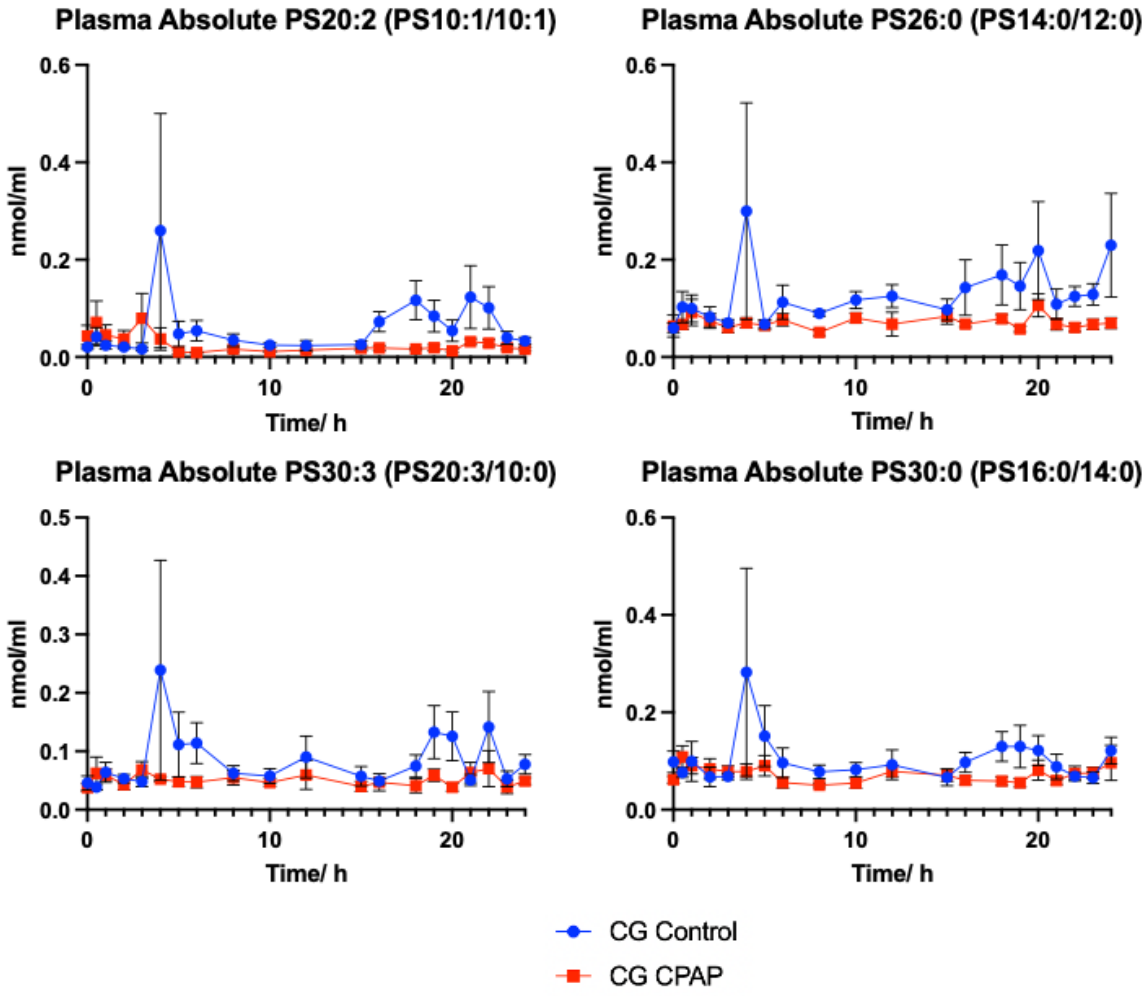

Figure 87. Plasma concentrations of shorter chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly lower concentrations in the CG CPAP group for all PS species shown: PS20:2 (commonly PS10:1/10:1) ($U = 88, p = 0.0020$), PS26:0 (commonly PS14:0/12:0) ($U = 43, p < 0.0001$), PS30:3 (commonly PS20:3/10:0) ($U = 98, p = 0.0051$), and PS30:0 (commonly PS16:0/14:0) ($U = 93, p = 0.0032$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP groups were respectively: PS20:2 = 0.0406 and 0.0190, PS26:0 = 0.1148 and 0.0677, PS30:3 = 0.0632 and 0.0484, PS30:0 = 0.0944 and 0.0750. All PS species were lower in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

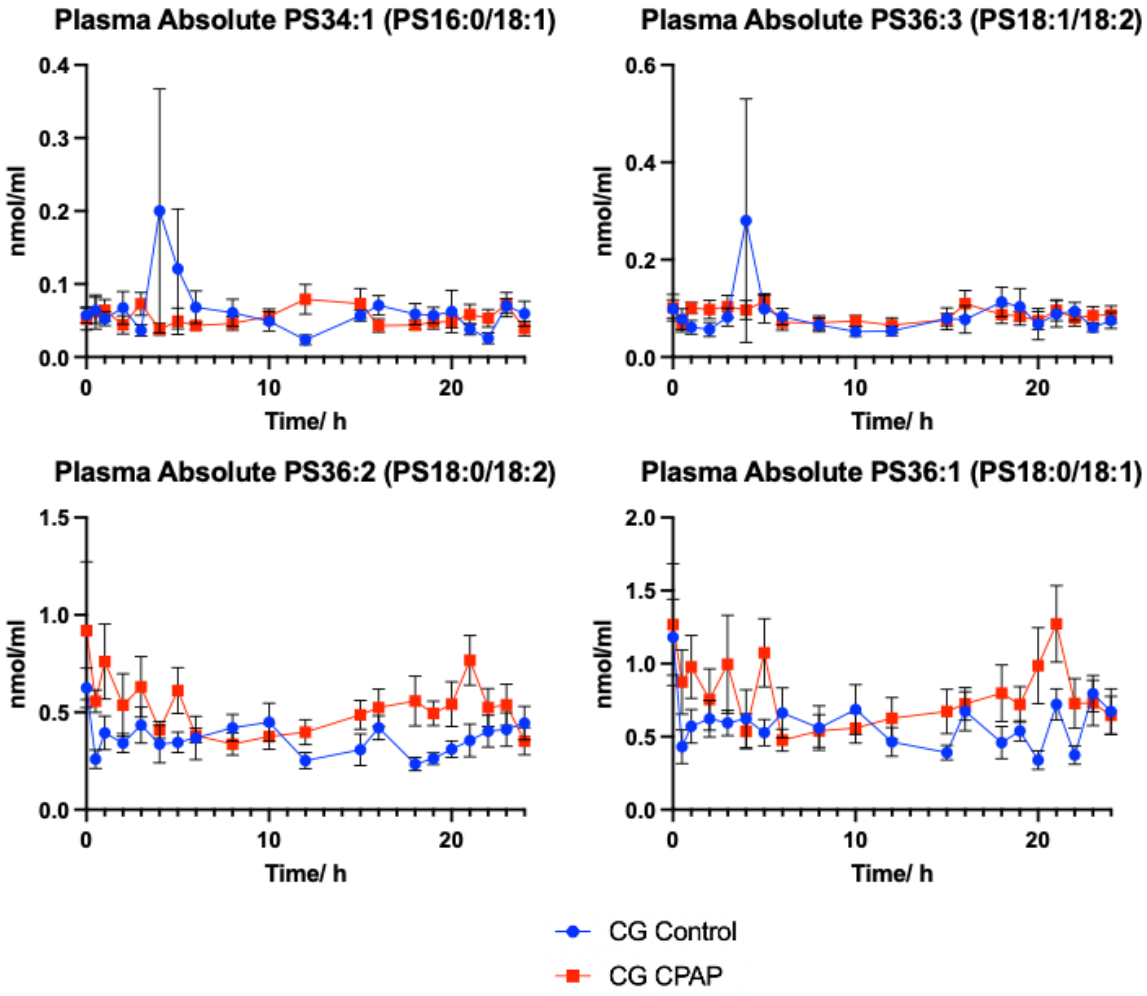

Figure 88. Plasma concentrations of longer chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant differences for PS34:1 (commonly PS16:0/18:1) ($U = 164, p = 0.3408$) and PS36:3 (commonly PS18:1/18:2) ($U = 155, p = 0.2315$). There were significantly higher concentrations in the CG CPAP group for PS36:2 (commonly PS18:0/18:2) ($U = 67, p = 0.0002$) and PS36:1 (commonly PS18:0/18:1) ($U = 86, p = 0.0016$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP groups were respectively: PS34:1 = 0.059 and 0.051, PS36:3 = 0.078 and 0.086, PS36:2 = 0.362 and 0.531, PS36:1 = 0.583 and 0.729. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

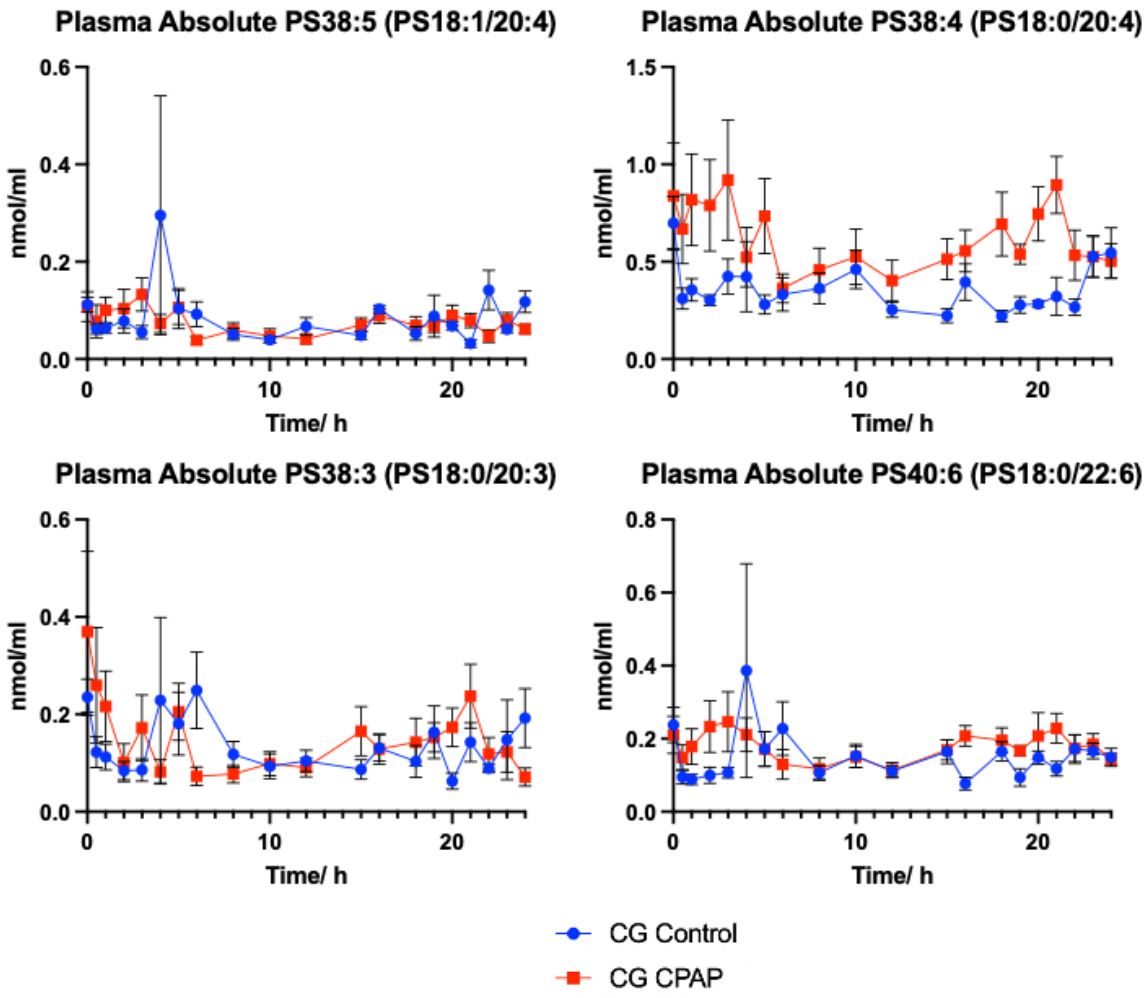

Figure 89. Plasma concentrations of polyunsaturated phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant differences for PS38:5 (commonly PS18:1/20:4) ($U = 195, p = 0.9042$) or PS38:3 (commonly PS18:0/20:3) ($U = 183, p = 0.6588$). There were significantly higher concentrations in the CG CPAP group for PS38:4 (commonly PS18:0/20:4) ($U = 39, p < 0.0001$) and PS40:6 (commonly PS18:0/22:6) ($U = 108, p = 0.0122$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP groups were respectively: PS38:5 = 0.0678 and 0.0757, PS38:4 = 0.327 and 0.547, PS38:3 = 0.120 and 0.136, PS40:6 = 0.149 and 0.176. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

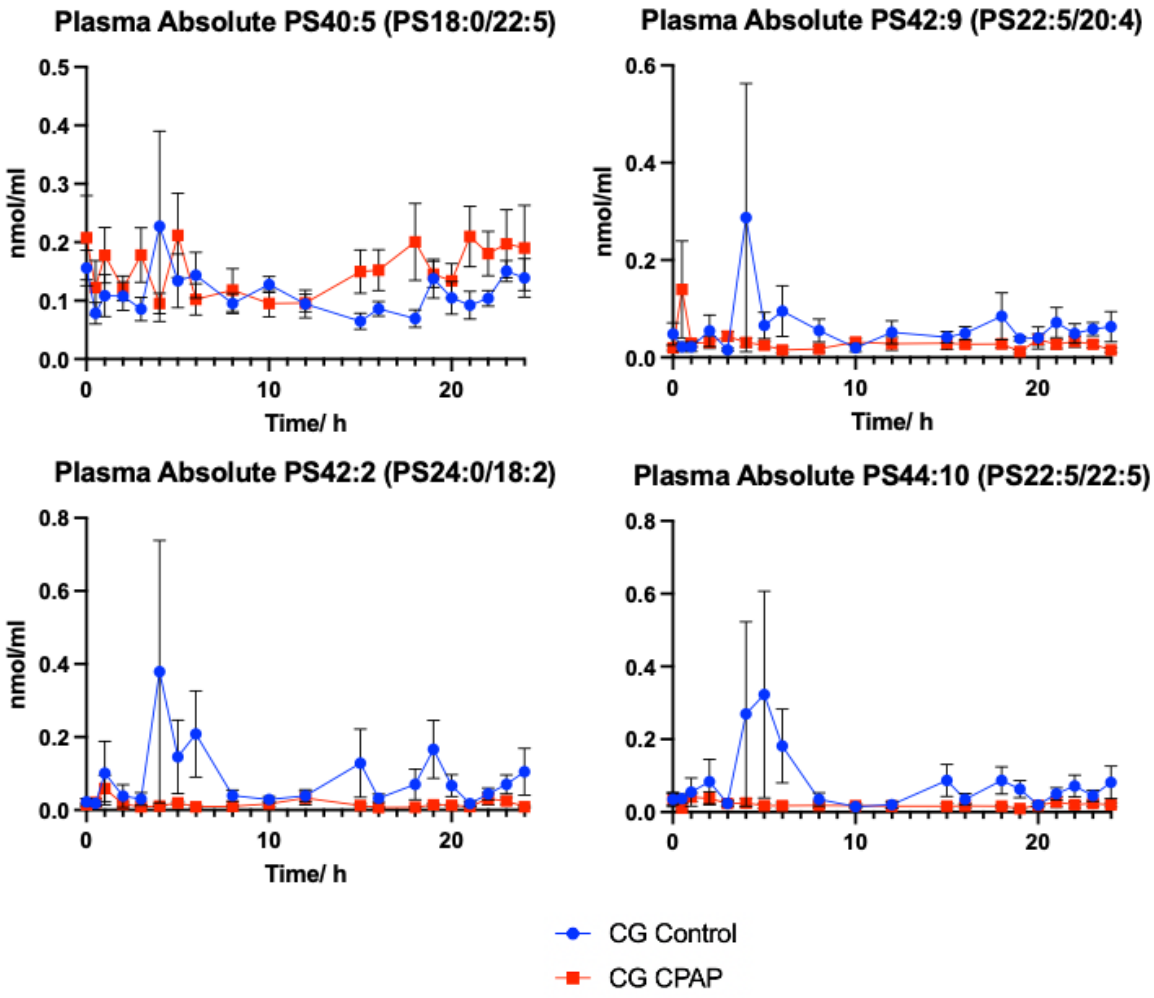

Figure 90. Plasma concentrations of very-long-chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher concentrations in the CG CPAP group for PS40:5 (commonly PS18:0/22:5) ($U = 97, p = 0.0047$) and significantly lower concentrations for PS42:9 (commonly PS22:5/20:4) ($U = 80, p = 0.0008$), PS42:2 (commonly PS24:0/18:2) ($U = 28, p < 0.0001$) and PS44:10 (commonly PS22:5/22:5) ($U = 50, p < 0.0001$). Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP were respectively: PS40:5 = 0.106 and 0.151, PS42:9 = 0.0507 and 0.0279, PS42:2 = 0.0555 and 0.0138, PS44:10 = 0.0525 and 0.0182. This indicates differing pathways for PS species when PEEP was applied, with shorter-chain PS40:5 increased and longer-chain, highly unsaturated PS species reduced. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8,

decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

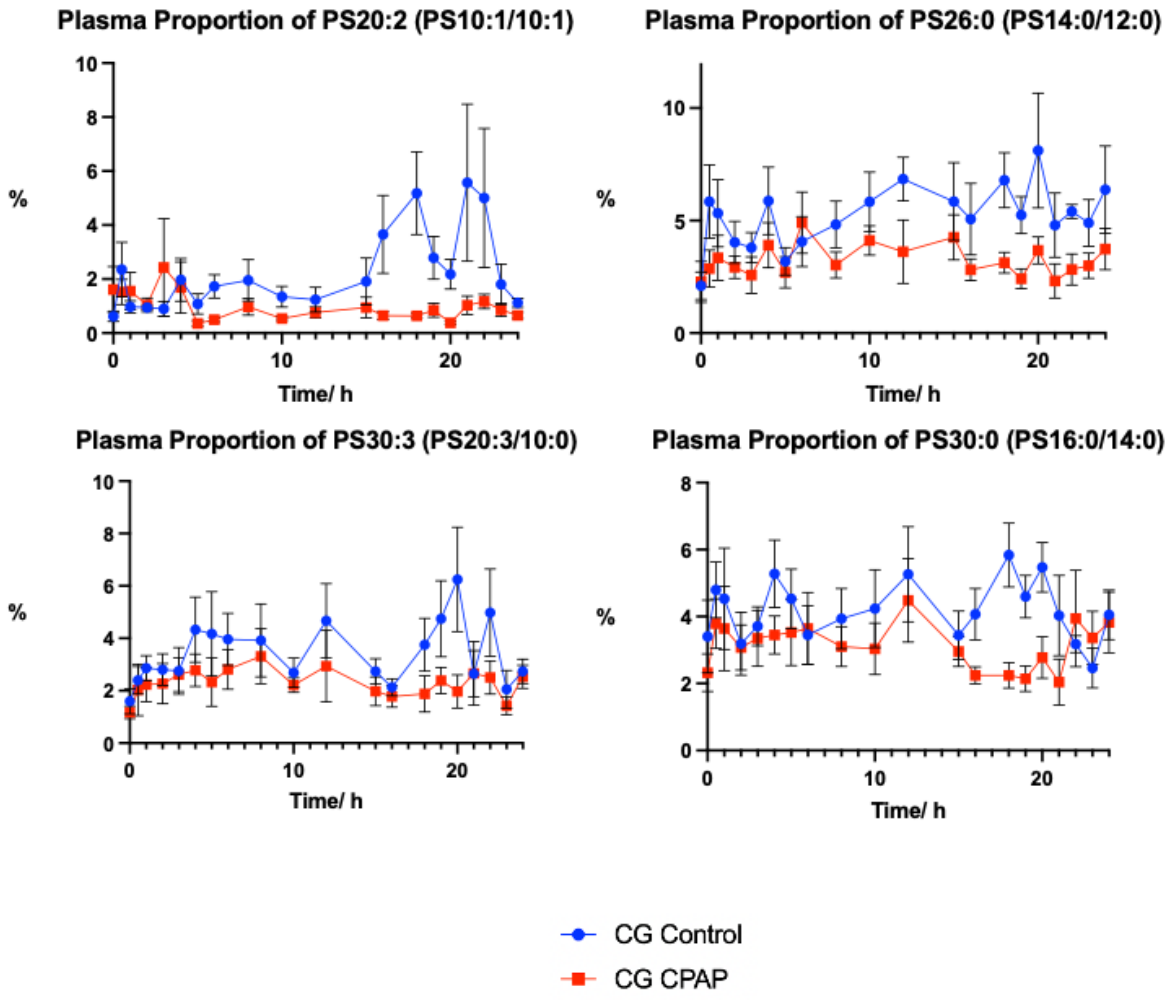

Figure 91. Plasma proportions of shorter-chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly lower proportions in the CG CPAP group for all PS species shown: PS20:2 (commonly PS10:1/10:1) ($U = 74, p = 0.0004$), PS26:0 (commonly PS14:0/12:0) ($U = 41, p < 0.0001$), PS30:3 (commonly PS20:3/10:0) ($U = 78, p = 0.0007$) and PS30:0 (commonly PS16:0/14:0) ($U = 69, p = 0.0002$). Median proportions (%) for CG Control and CG CPAP were respectively: PS20:2 = 1.86 and 0.90, PS26:0 = 5.29 and 3.01, PS30:3 = 2.83 and 2.30, PS30:0 = 4.06 and 3.23.

There was a general reduction in the relative abundance of lower molecular weight PS species in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

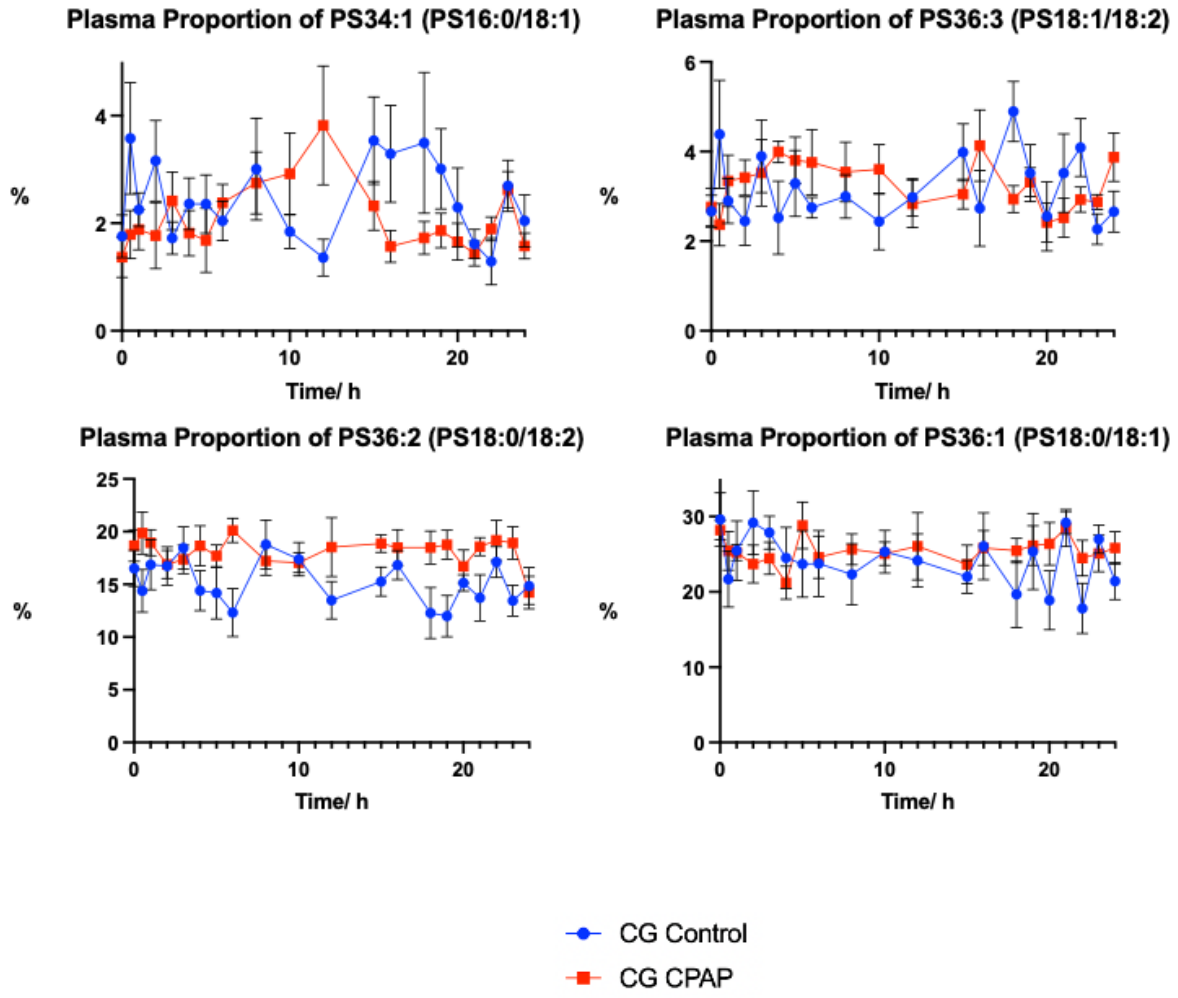

Figure 92. Plasma proportions of longer chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant differences between groups for PS34:1 (commonly PS16:0/18:1) ($U = 143, p = 0.1274$), PS36:3 (commonly PS18:1/18:2) ($U = 172, p = 0.4612$), or PS36:1 (commonly PS18:0/18:1) ($U = 154, p = 0.2211$). There was a significantly higher proportion of PS36:2 (commonly PS18:0/18:2) ($U = 43, p < 0.0001$) in the CG CPAP group. Median proportions (%) for CG Control and CG CPAP were respectively: PS34:1 = 2.33 and 1.84, PS36:3 = 2.94 and 3.33, PS36:2 = 15.00 and 18.55, PS36:1 = 24.34 and 25.47. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

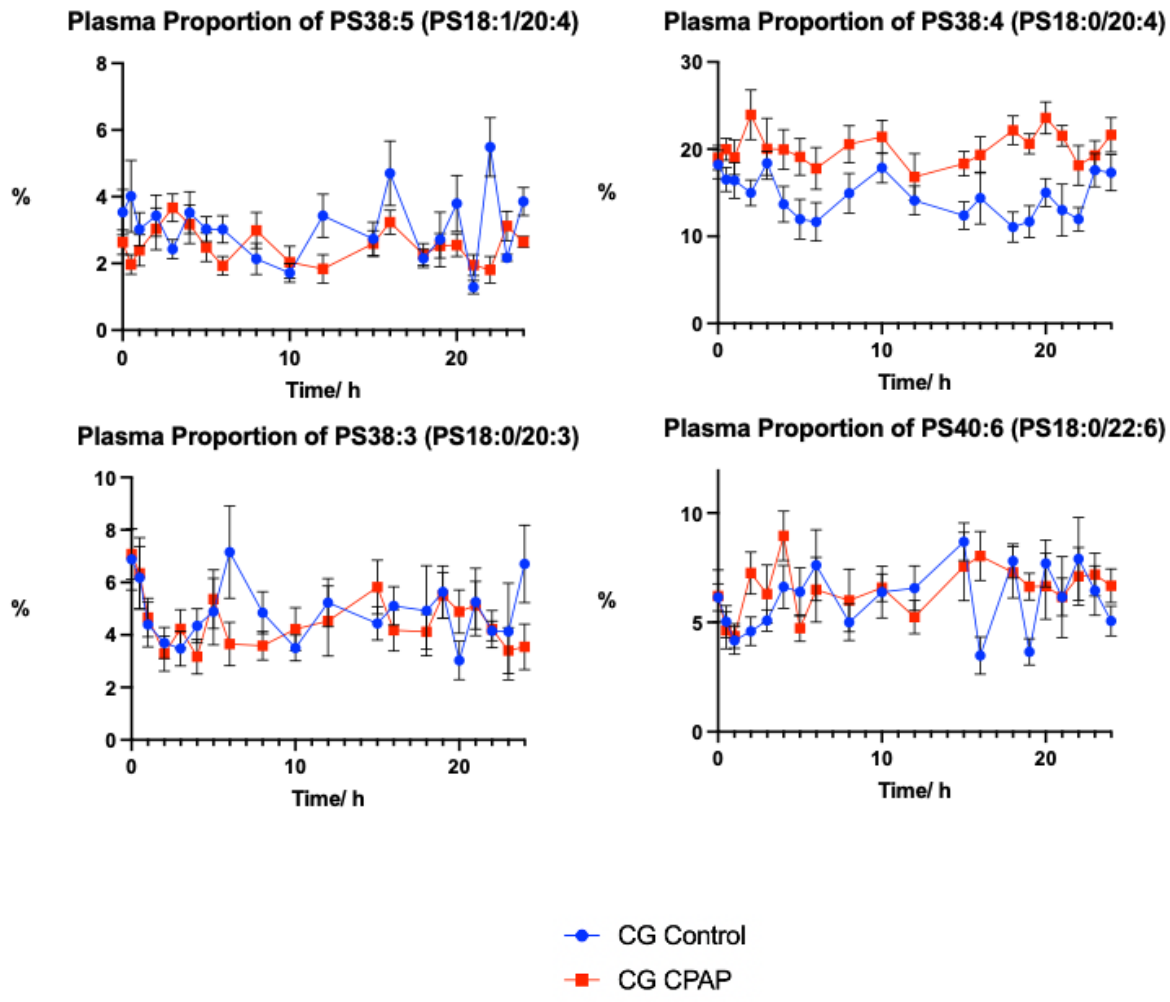

Figure 93. Plasma proportions of polyunsaturated phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly lower proportions in the CG CPAP group for PS38:5 (commonly PS18:1/20:4) ($U = 125, p = 0.0430$). In contrast, there were significantly higher proportions for PS38:4 (commonly PS18:0/20:4) ($U = 11, p < 0.0001$) in the CG CPAP group. No significant differences were observed for PS38:3 (commonly PS18:0/20:3) ($U = 163, p = 0.3273$) or PS40:6 (commonly PS18:0/22:6) ($U = 157, p = 0.2534$). Median proportions (%) for CG Control and CG CPAP were respectively: PS38:5 = 3.03 and 2.54, PS38:4 = 14.66 and 19.97, PS38:3 = 4.87 and 4.23, PS40:6 = 6.27 and 6.61. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

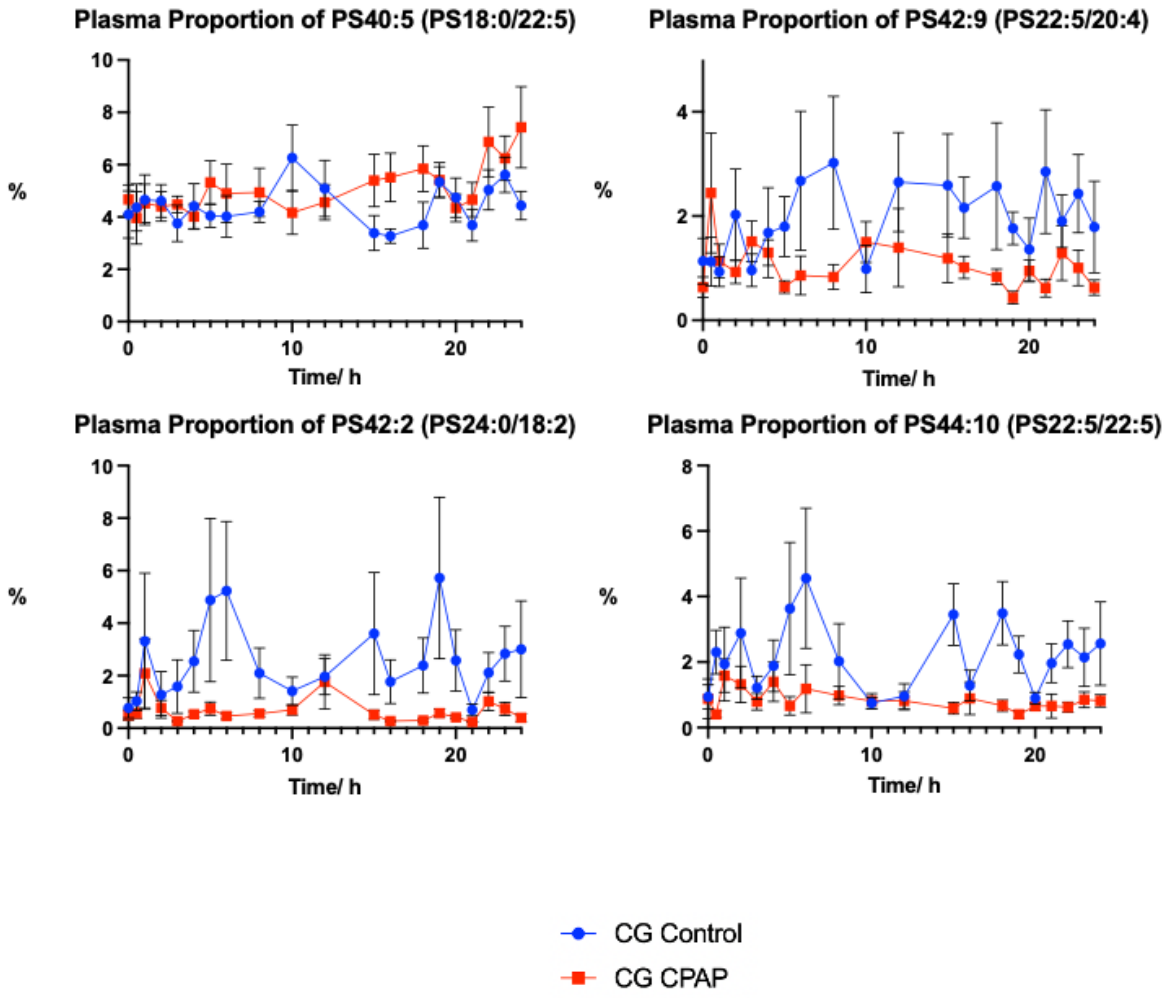

Figure 94. Plasma proportions of very long-chain phosphatidylserine (PS) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed significantly higher proportions in the CG CPAP group for PS40:5 (commonly PS18:0/22:5) ($U = 120, p = 0.0304$), and significantly lower proportions for PS42:9 (commonly PS22:5/20:4) ($U = 59, p < 0.0001$), PS42:2 (commonly PS24:0/18:2) ($U = 19, p < 0.0001$) and PS44:10 (commonly PS22:5/22:5) ($U = 33, p < 0.0001$). Median proportions (%) for CG Control and CG CPAP were respectively: PS40:5 = 4.39 and 4.79, PS42:9 = 1.85 and 0.98, PS42:2 = 2.25 and 0.54, PS44:10 = 2.08 and 0.81. There was a general reduction in the relative abundance of highly unsaturated, very long chain PS species in the CG CPAP group. Data are presented as mean ± SEM. Sample sizes were CG Control (n = 8,

decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

The overall reduction in PS in animals with PEEP applied suggests reduced cellular injury and apoptosis (38, 194), mitigating phosgene induced damage. Lower PS leakage into plasma may indicate attenuated membrane turnover and improved cell viability. Loss of shorter chain PS into plasma may simply be the result of non-specific release from damaged cells. Increased plasma PS36:2 (commonly PS18:0/18:2) in the CG CPAP group however, suggests release consistent with ongoing cellular injury despite PEEP application.

The divergence in behaviour of the arachidonyl species, PS38:5 and PS38:4 warrants exploration. It likely represents differential enzymic susceptibility, PS38:5 commonly possesses an 18:1 acyl chain conferring greater bilayer fluidity and increased susceptibility to PLA_2 hydrolysis (192). It underwent a fractional decrease with PEEP applied, possibly as a result of preserved PLA_2 function in the CG CPAP group. In contrast PS38:4 commonly has an 18:0 acyl chain, which is less reactive and less susceptible to PLA_2, and PS38:4 was higher when PEEP was applied. Relatively increased consumption of PS38:4 in the CG Control group, in the production of inflammatory mediators, could account for this (85).

The suppression of very long chain polyunsaturated PS by PEEP is likely due to reduced lipid remodelling and oxidative stress (193), in comparison to the CG Control animals. Overall, PEEP modulates PS metabolism, reducing PS leakage and preserving cell membrane components, suggestive of a mechanism that attenuates inflammatory lipid turnover and apoptosis.

5.2.7 Sphingomyelin

Overall plasma sphingomyelin (SM) concentration was modestly but significantly higher in the CG CPAP group compared with the CG Control group, consistent with reduced SM hydrolysis. There were no significant

differences observed between individual species in plasma for absolute concentrations; Figure 95 and Figure 96. Although the changes were small, PEEP appeared to slightly shift the fractional distribution toward increased unsaturated long-chain SM species. This could be due to subtle changes in sphingomyelinase activity and ceramide formation; processes which have been implicated in cell death by necrosis and apoptosis (195, 196). PEEP may be modulating sphingolipid turnover in a manner consistent with reduced cellular injury. Fractions are presented in Figure 97 and Figure 98.

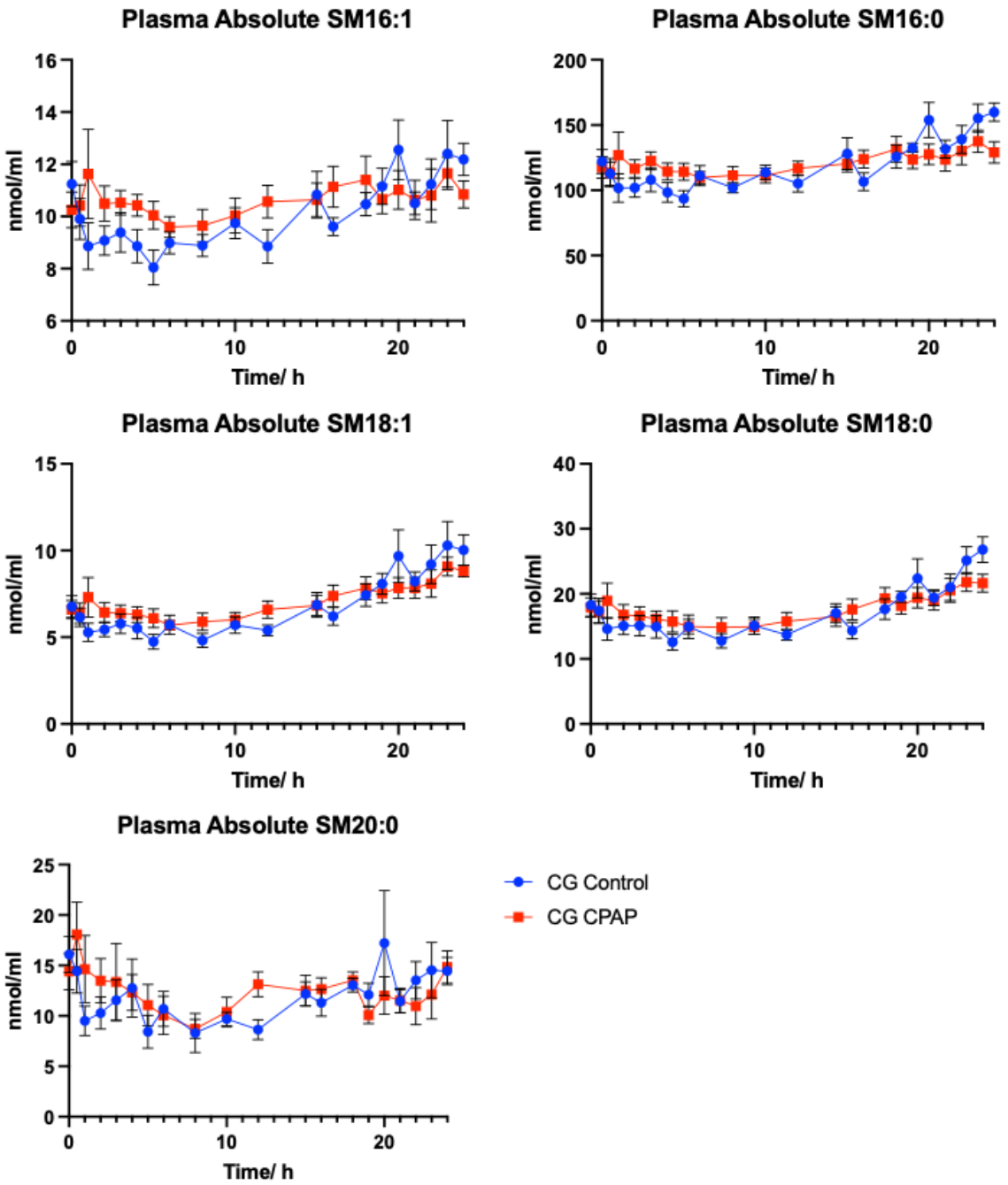

Figure 95. Plasma absolute concentrations of sphingomyelin (SM) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant group differences for any SM species analysed: SM16:1 ($U = 145, p = 0.1417$), SM16:0 ($U = 166, p = 0.3689$), SM18:1 ($U = 160, p = 0.2888$), SM18:0 ($U = 167, p = 0.3834$), and SM20:0 ($U = 174, p = 0.4945$).

Median concentrations (nmol.ml^{-1}) for CG Control and CG CPAP respectively

were: SM16:1 = 9.82 and 10.60, SM16:0 = 113.3 and 121.5, SM18:1 = 6.18 and 6.71, SM18:0 = 16.09 and 17.43, SM20:0 = 11.83 and 12.42. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

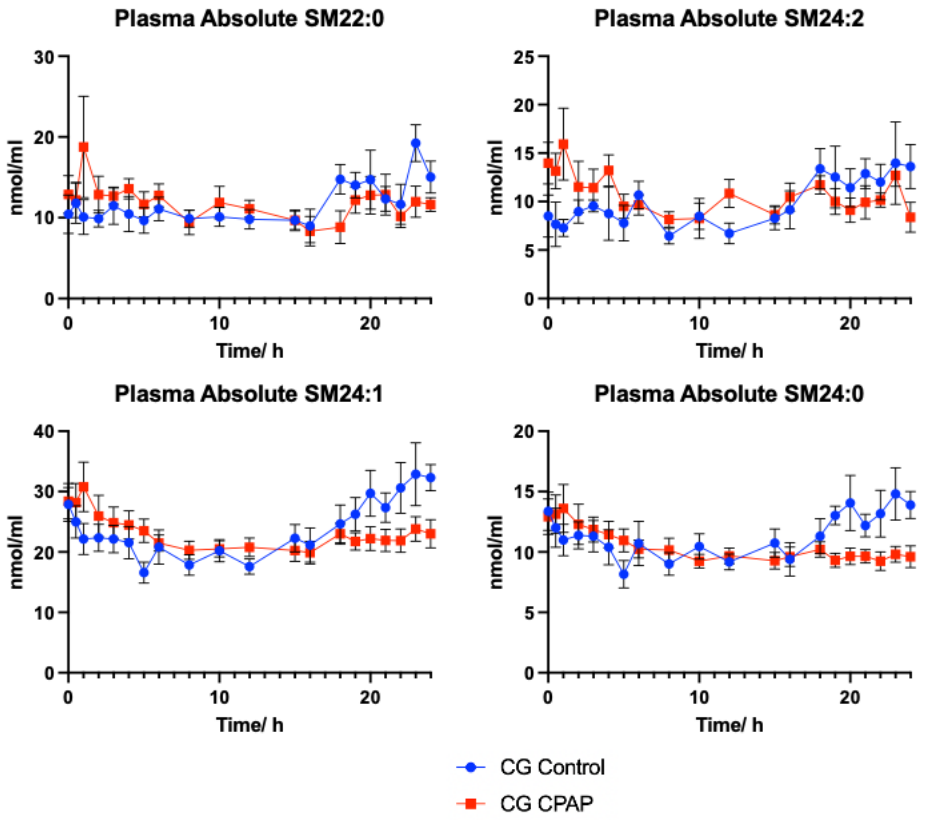

Figure 96. Plasma absolute concentrations of longer chain sphingomyelin (SM) species in CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed no significant differences for any species analysed: SM22:0 ($U = 174, p = 0.4945$), SM24:2 ($U = 151, p = 0.1918$), SM24:1 ($U = 185, p = 0.6980$) and SM24:0 ($U = 140, p = 0.1081$). Median concentrations (nmol.ml^{-1}) for CG Controls and CG CPAP were respectively: SM22:0 = 10.78 and 12.09, SM24:2 = 9.06 and 10.31, SM24:1 = 22.31 and 22.62, SM24:0 = 11.31 and 9.98. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

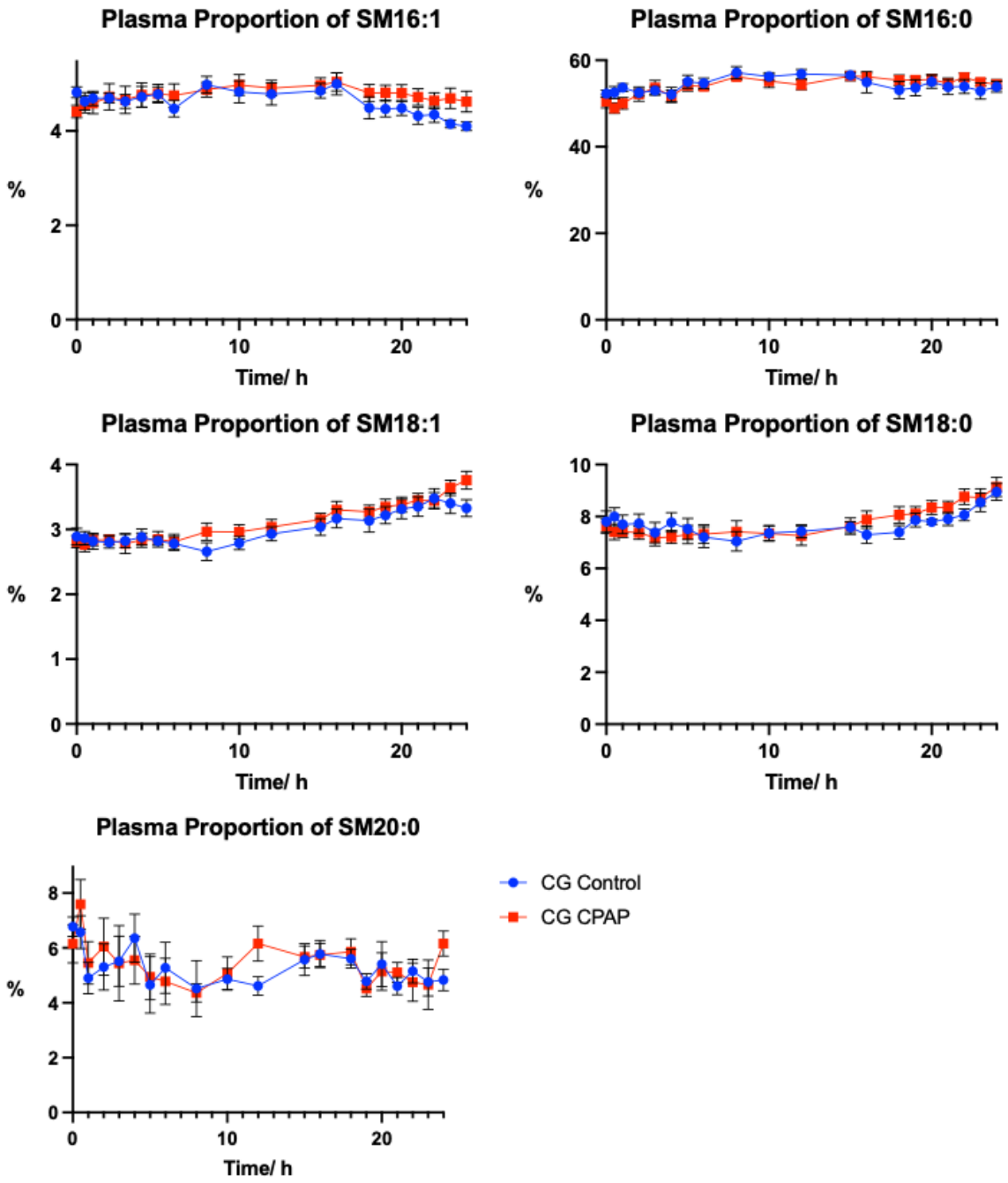

Figure 97. Fractional composition of sphingomyelin (SM) species in plasma from CG Control and CG CPAP groups. Mann–Whitney U tests revealed no significant group differences for any SM species analysed: SM16:1 ($U = 139, p = 0.1022$), SM16:0 ($U = 189, p = 0.7788$), SM18:1 ($U = 171, p = 0.4450$), SM18:0 ($U = 199, p = 0.9893$) and SM20:0 ($U = 172, p = 0.4612$). Median proportions (%) for CG Controls and CG CPAP were respectively: SM16:1 = 4.66 and 4.76, SM16:0 = 53.87 and 54.55, SM18:1 = 2.91 and

3.00, SM18:0 = 7.71 and 7.52, SM20:0 = 5.22 and 5.45. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

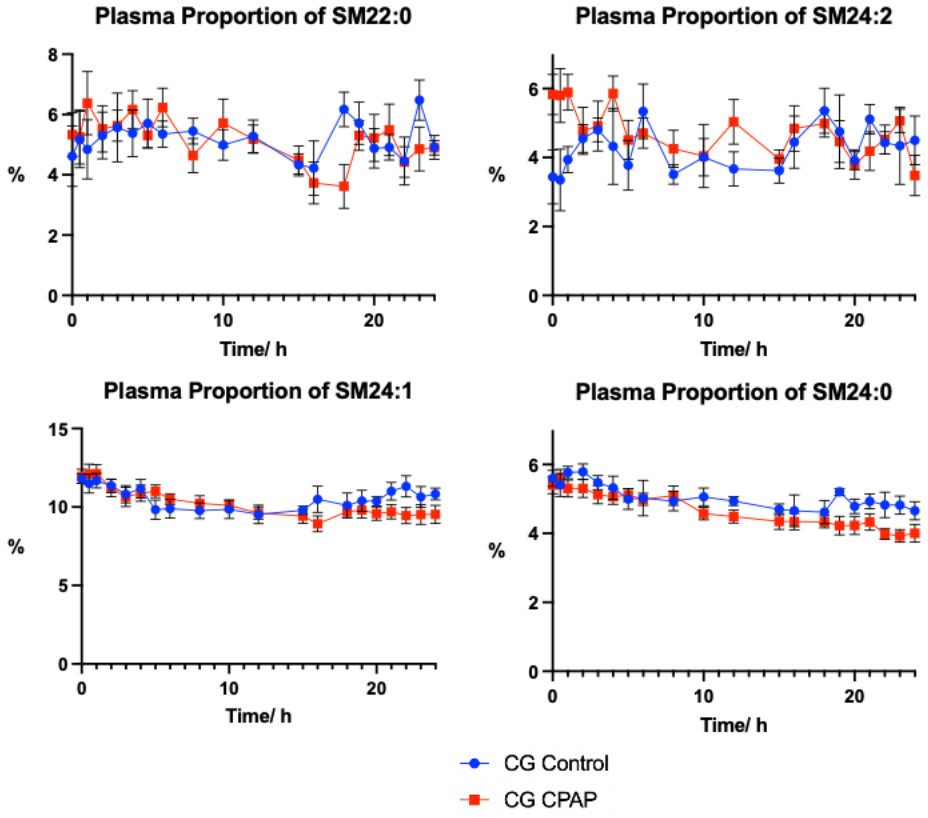

Figure 98. Fractional composition of longer chain sphingomyelin (SM) species in plasma from CG Control and CG CPAP groups for the observation period. Mann–Whitney U tests revealed small but significant differences for SM24:2 ($U = 120, p = 0.0304$) and SM24:0 ($U = 124, p = 0.0402$). SM22:0 ($U = 194, p = 0.8831$) and SM24:1 ($U = 142, p = 0.1207$) showed no significant differences. Median proportions (%) for CG Controls and CG CPAP respectively were: SM22:0 = 5.23 and 5.28, SM24:2 = 4.34 and 4.74, SM24:1 = 10.57 and 9.96, SM24:0 = 4.97 and 4.53. Data are presented as mean ± SEM. Sample sizes were CG Controls (n = 8, decreasing to 5) and CG CPAP (n = 8). CG = Phosgene, CPAP = Continuous Positive Airway Pressure.

5.2.8 Arachidonyl species

Arachidonyl containing species were detected at concentrations related to the GPL class they belong to. Preferential loss was observed in some arachidonyl PC species, namely PC38:5 (commonly PC18:1/20:4) and PC38:4 (commonly PC18:0/20:4) but less so PC36:4 (commonly PC16:0/20:4). LPC 20:4 decreased markedly during the experiment indicating a possible role in the production of inflammatory mediators (85). Beyond PC and LPC, the less abundant other classes made a smaller contribution. The 20:4 PE species, PE36:4 and PE38:5 increased in absolute amounts but fell by fraction when PEEP was applied. PS38:5 decreased by fraction and PS38:4 increased absolutely and by fraction. Together, these patterns indicate dynamic redistribution of arachidonate under the influence of PEEP.

5.3 Summary of Plasma Lipid Findings

A detailed lipidomic analysis of phosgene induced acute lung injury has not previously been reported. This analysis has not identified an easy target biomarker or change in composition ratio that could serve as an early indicator for phosgene exposure during the latent phase.

Due to limitations in sample availability, plasma from air exposed controls was not included. However, the experimental results include baseline plasma samples taken pre-exposure.

Significant differences in plasma GPL concentrations were observed in PC and PE, both of which were elevated in the CG CPAP group compared to CG Controls. These findings indicate that PEEP application influences systemic GPL regulation. In Figure 71 plasma PC concentration was significantly higher in the CG CPAP group compared to CG Controls, and in ARDS patients, plasma PC is known to be lower than in healthy individuals (88). In this context, PEEP application may mitigate phosgene induced reduction of PC suggesting a protective effect. This protective effect is likely mediated through reduction of inflammatory injury.

Nutritional factors influence plasma GPL concentration, but since both groups received identical nutrition in the form of standard pig diet, described in Section 2.1.1, this cannot account for the observed differences. Instead, PEEP likely modifies PC metabolism through mechanisms of inflammation and the hepatopulmonary axis. The preferential loss of arachidonyl species, attenuated by PEEP, to inflammatory mediators reinforces this hypothesis.

Mono- and diunsaturated PC species demonstrated stability or increases in the CG CPAP group, whereas polyunsaturated species generally declined. These findings align with observations of ARDS patients where polyunsaturated PC depletion is linked to reduced liver PEMT activity and PC consumption by inflammatory signalling pathways (80). The early spike seen in unsaturated LPC species is likely to be due to cell membrane damage. The stable overall proportions of LPC species thereafter suggest uniform remodelling by the liver rather than degradation (192).

In terminal bronchoalveolar lavage (BAL) samples, SM concentration was higher in CG Controls and highest in CG Controls that died early, illustrated in Figure 54 and Figure 55. This indicates greater cellular necrosis in the CG CPAP group, particularly in those more severely affected. By contrast plasma SM concentrations were similar in both groups. This particular observation reinforces Hill's findings from over 100y ago that the chemical effect of phosgene is confined to the lungs (19), with all other systemic changes being secondary consequences.

6 - DISCUSSION

World War 1 revealed the brutal reality of chemical warfare. It was quickly realised that phosgene is a very different chemical weapon; it accounted for a quarter of chemical weapons used but was responsible for 85% of chemical agent associated deaths (197). The pioneering work of Galwey, Hill and Barcroft showing that it has direct effects only at the alveolar level became the foundation of work exploring its pathophysiology. The discovery of surfactant followed from this work.

This thesis provides a comprehensive evaluation of the effects of phosgene exposure on pulmonary function and lipid metabolism, and examines a porcine model of phosgene induced acute lung injury. The experimental outcomes have significant implications for understanding the pathophysiology of phosgene induced acute lung injury and for developing potential therapeutic interventions for a large scale release. Notably, the application of positive end expiratory pressure (PEEP) as a surrogate for continuous positive airway pressure (CPAP) was shown to mitigate many adverse effects of phosgene exposure, including reductions in pulmonary oedema and improvements in measured gas exchange variables. Furthermore, lipidomic analyses revealed distinct changes in bronchoalveolar lavage (BAL) and plasma lipid profiles, providing novel insights into the biochemical pathways affected by phosgene toxicity.

There remains no effective pharmacological treatment of phosgene induced acute lung injury, the only effective treatments are based on ventilation and oxygenation strategies for acute respiratory distress syndrome (ARDS) (102). There is no pre-exposure prophylaxis and no biomarker for diagnosis. In combination with a sub-clinical latent phase, this has long been recognised as a significant problem (11-14); the solution of prolonged observation of potential casualties has been doctrinal following World War 1 (25). By aiming to reduce this medical logistic burden, this work has shown that the application of CPAP is effective at ameliorating the effects of phosgene.

6.1 Physiological and Cellular Responses

Mortality was reduced to zero in the CG CPAP group and every measure of oxygenation was improved by the application of PEEP. This not only delayed the onset of severe effects, but also represented an effective treatment in its own right. It may be the optimal intervention for a substantial number of casualties following a mass release of phosgene.

PEEP, as a surrogate for CPAP, was associated with reduced lung oedema and BAL protein. The disparity between derived and actual measures of lung oedema is explained in Section 3.7.3. In short, the exclusion of affected lung regions by hypoxic pulmonary vasoconstriction, causing redistribution of thermal indicator (175), and amplification of errors during the calculation are responsible. Thus, derived measurements of extravascular lung water will be underestimated in affected lung. The protective physiological effects of PEEP echo the lipidomic findings discussed in Section 6.2. Stabilisation of alveoli limits repetitive closing injury which causes atelectrauma, thus decreasing epithelial stress and dampening surfactant disturbance (198).

Phosgene exposure resulted in marked tachypnoea and hypoxaemia from increased shunt fraction. PEEP improved ventilation- perfusion mismatching lessening the observed adverse effects. The benefits of PEEP extended further than improved oxygenation and included cellular preservation. Inflammatory cell profiles within BAL were favourably altered by PEEP. Neutrophil margination into the alveolar space, a hallmark of the inflammatory component of ARDS (70, 165, 166), was increased in phosgene exposed controls, but substantially reduced in the CG CPAP group with PEEP applied. Although statistically significant differences were not detected between groups, alveolar macrophages were preserved, indicating partial preservation of these cells and their surfactant clearance capacity (62).

Correlations between BAL surfactant lipids and cellular counts provided further insight into these interactions. In the BAL of phosgene exposed animals, PEEP was associated with an inverse relationship between

neutrophil counts and both total and saturated PC. This pattern can be explained by PEEP maintaining functionally active neutrophils capable of clearing phospholipids or utilising them for mediator synthesis (62). These correlations did not meet statistical significance in either control group. A similar inverse relationship observed with PEEP, again not replicated in either control group, between BAL total PC and total LPC supports reduced PLA_2 activity and reduced hydrolysis of PC. PLA_2 enzymes play an important role in surfactant degradation and inflammatory amplification in ARDS. In ARDS, elevated secretory PLA_2-IIA activity in BAL is associated with reduced PC, increased LPC and worse disease phenotype (199). The absolute amounts of LPC16:0 and LPC20:4 increased significantly after phosgene exposure, particularly in CG Controls that died before 24h, CG Control Dead. The rise in LPC16:0 and LPC20:4 observed after phosgene exposure indicates PLA_2 mediated hydrolysis of PC and liberation of arachidonic acid (20:4) to act as a substrate for inflammatory pathways described in Section 1.3.2, Figure 19. The attenuation of LPC16:0 and LPC20:4 by PEEP therefore suggests suppression of this injurious pathway and preservation of surfactant stability. When lipid composition was examined by survival status in the CG Controls, increased alveolar PC and PG were associated with survival, while higher SM and LPC20:4 accompanied non- survivors, consistent with necrotic injury and exaggerated inflammation (200). SM is present in cell and organelle membranes; the excess in the BAL of more severely affected animals may have been derived from necrotic cell death.

6.2 Integration of Lipid Findings

This study set out to characterise the physiological and biochemical effects of phosgene exposure using a porcine model, to evaluate the effects of PEEP as a surrogate for CPAP and to explore the associated changes in lipid metabolism through detailed lipidomic analysis of terminal BAL and plasma. The findings show that phosgene induces an acute lung injury (ALI) that mirrors many aspects of human ARDS; hypoxaemia, non-cardiogenic pulmonary oedema and widespread surfactant disruption. Application of PEEP attenuated these effects through maintenance of alveolar integrity and limiting the cascades of surfactant disturbance and inflammation.

The integration of physiological and lipidomic data provides new insight into the mechanisms underlying this intervention. Animals treated with PEEP demonstrated improved oxygenation and reduced lung oedema. These physiological advantages were associated with a normalisation of surfactant lipids in BAL. In untreated animals, total surfactant lipid content increased markedly with no change in fractional composition. This is consistent with the following possibilities acting alone or in combination:

- Cellular injury and unregulated release from type II alveolar cells.
- Hyperstimulation of type II alveolar cells.
- Reduced catabolism or recycling of surfactant.

The restoration of lipid levels by PEEP to that near the amounts found in air exposed controls suggests that stabilisation of alveoli limits the mechanical and chemical stress on type II alveolar cells and preserves recycling pathways.

The use of plasma lipidomics revealed more systemic effects of phosgene induced ALI and its modulation by PEEP. There was no evidence to show that lung lipids were leaking into the circulation, as explained in Section 4.2.7, Plasma PC and PE species were elevated in the CG CPAP group compared to CG Controls, indicating that PEEP influences systemic

glycerophospholipid regulation. The liver plays a crucial role in lipid metabolism, including the synthesis, modification, and degradation of lipids. The changes in plasma lipid levels and composition may be influenced by the liver's response to lung injury. In general terms, the effects of hypoxia and inflammation, or more specifically and for example, the increase in LPC18:1 can be attributed to acyl remodelling by the liver rather than degradation of PC. These findings lend weight to the lung- liver axis hypothesis. Plasma PC has previously been shown to decrease in patients with ARDS when compared to controls as a result of impaired hepatic synthesis via the PEMT pathway (88). The relative preservation of PC in the CG CPAP group suggests that PEEP may help preserve or restore PC metabolism, potentially mitigating hepatic dysfunction associated with ARDS. LPC species tended to decrease in both groups, either due to reduced phospholipase A_2 activity or enhanced reacylation, suggesting a shift towards lipid preservation rather than breakdown. SM was increased modestly after exposure, and significantly more in the CG CPAP group, while PS was significantly higher in CG Controls. Maile *et al*. analysed plasma lipids in human ARDS, global depletion of most lipid classes was associated with a poor outcome (201). In their study PS was not examined, nor was global SM however, an increase in one plasma SM species annotated as SM43:1, was associated with a higher mortality. In a review of factors affecting the sphingomyelin- ceramide pathway in conditions associated with hypoxia, increased sphingomyelinase activity and accumulation of ceramide occurred in ARDS, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (202). While studies of plasma SM in ARDS are rare, increased hydrolysis of SM and accumulation of ceramide have been associated with a poor prognosis, implying that SM depletion is associated with more severe hypoxia, or worse lung injury. The relative preservation of plasma SM in the CG CPAP group suggests that PEEP limits activation of this pathway. Changes in plasma PS in ARDS have not been reported in the literature, however PS is externalised in conditions of cell activation and induction of cell death (38). Stressed and diseased cells presenting PS externally acts as a signal for efferocytosis and can induce thrombosis (203). Indeed, thrombosis in the form of microemboli were

observed in early phosgene studies published in 1920 (19, 21). The lower PS observed in the CG CPAP group suggests that PEEP attenuated cellular activation and mechanisms that lead to PS externalisation during stress or injury.

6.3 Comparison with Existing Literature

The findings of this thesis broadly corroborate earlier studies describing phosgene induced acute lung injury and more general acute lung injury/ ARDS. However, this work extends the understanding of phosgene toxicity by linking molecular species lipidomics to physiological outcomes. Unlike previous studies that primarily focused on histopathological changes, the lipidomic approach employed here provides molecular level insights into the disruption of surfactant homeostasis, systemic lipid metabolism and the influence of PEEP.

6.3.1 Comparison with Phosgene Literature

The acute physiological changes described here, characterised by pulmonary oedema, hypoxaemia and haemoconcentration, explain the phases of phosgene poisoning described in World War 1 (13-15), as discussed in Sections 1.1.2 and 1.1.4. The physiological signature of phosgene induced acute lung injury is replicated in this study and also fits with studies of humans accidentally exposed to phosgene (204). Studies evolved over the years to include analysis of surfactant. Frosolono and Currie reported surfactant changes observed in rats exposed to phosgene in 1985 (100). They found that all classes of measured surfactant lipids increased, echoing the findings in Section 4.2.1. Later, in 1999 Jugg *et al*. reported changes in surfactant composition in rats exposed to phosgene (101). Her findings were similar, with rises reported in all measured lipid classes.

Translational studies continued, ventilatory strategies for invasive ventilation are reviewed in Section 1.4.3, those studies are not directly applicable to this study's evaluation of CPAP as a treatment modality, however the oxygenation and ventilation principles for the general management of acute lung injury are aligned. In 2016, Holmes *et al*. emphasised surfactant dysfunction and pulmonary oedema arising from permeability changes. Their work included trialling the catalytic antioxidant AEOL 10150 in phosgene exposed mice, with limited success (205).

More recent analyses have examined the molecular mechanisms underlying alveolar damage including surfactant components as potential therapeutic targets. Lu *et al*. summarised the effects of phosgene including the direct acylation of PC, liberation of arachidonic acid, and propagation of oxidative and inflammatory cascades through reactive oxygen species (ROS) generation (102). In a re-examination of the mechanisms underlying phosgene induced acute lung injury, Pauluhn proposed that the primary insult occurs through direct modification of surfactant lipids and proteins at the alveolar lining (96). He argues that the effects of these modifications destabilise alveoli and secondary oxidative stress further damages membranes. These aspects align with the findings in this thesis, which identify specific changes in surfactant glycerolipid and sphingolipid species consistent with these mechanisms. However, physiologically Pauluhn argues that there is a cardiogenic component to phosgene induced pulmonary oedema, implying left ventricular dysfunction, and this is not supported by the preponderance of experimental evidence, including the current porcine model. Phosgene induced acute lung injury does not meet accepted diagnostic or physiological criteria for heart failure (206). The pathophysiological constellation observed; low CVP, haemoconcentration, markedly elevated alveolar protein, which is inconsistent with cardiogenic pulmonary oedema (207), contradicts a cardiogenic mechanism. Cardiogenic pulmonary oedema is a transudate whereas phosgene produces an exudate due to pathological alveolar permeability. Phosgene therefore cannot be reasonably interpreted as precipitating left sided heart failure, and any secondary right heart strain arising from the consequences of regional hypoxia cannot account for the pulmonary oedema observed.

Previous phosgene studies have not described surfactant lipids at the level of individual molecular species, nor linked those biochemical changes directly to physiological outcomes as achieved in this thesis.

6.3.2 Comparison with ARDS and Lipidomics Literature

The lipidomic and physiological patterns identified in this study share important features with those in humans with acute lung injury and ARDS. Human studies of ARDS tend to enrol patients after the diagnosis has been made, this point may well be around 72h post deterioration or longer. ARDS, systemic inflammation and multiorgan dysfunction will have become established, and undefined periods of hypoxaemia prior to stabilisation will also have an influence on lipid metabolism. In contrast, the current study captures the early molecular responses preceding respiratory failure in plasma and early changes in BAL composition post mortem.

Surfactant dysfunction is a well recognised feature of ARDS. In one of the most detailed assessments discussed in Section 1.2.5, Günther *at al*. showed that ARDS and severe pneumonia were associated with fractional reductions in BAL, of PC and PG, with reciprocal increases in fractions of SM, PI and LPC. PE and PS showed no significant change (72). No comparable changes in fraction, at class level were observed in this porcine phosgene study extending to 24h, earlier enrolment of humans in studies may eliminate this difference.

Mechanistic studies in ARDS provide further parallels with the current study. Kitsiouli *et al*. reviewed PLA_2 subclasses in ARDS (199). In particular, the activation of secretory PLA_2-IIA leads to hydrolysis of PC to LPC and the liberation of arachidonic acid with the ensuing inflammatory cascade. The observed effects of phosgene in the BAL of exposed animals aligns with this finding. Moreover, the attenuation of the increase in LPC by PEEP in this study suggests suppression of this pathway and preservation of surfactant homeostasis. Dushianthan *et al*. found reduced plasma PC associated with impaired PEMT activity in ARDS patients when compared to controls (88). Species level differences from their study are presented in

Table **2**, to summarise, many PUFA species were decreased in ARDS patients and monounsaturates were not significantly altered. Cytidine diphosphocholine (CDP): choline pathway flux is increased in ARDS while

PEMT flux is reduced, and these alterations account for the plasma changes observed. These later stage systemic alterations contrast with the present study, in which plasma PC overall was preserved under PEEP, and may indicate that the early plasma lipidomic response to phosgene exposure precedes the hepatic synthetic abnormalities observed in human ARDS. Species level plasma PC differences are presented in Section 5.2.2 and discussed in Section 5.2.3.

Maile *et al*. identified global plasma lipid depletion in the plasma of non-survivors of ARDS (201). This is examined in Section 6.2 and to summarise, the current study aligns with his findings with preservation of lipids by the application of PEEP parallels survival in patients with higher plasma lipid concentrations. Disturbances in sphingolipid metabolism are examined by Ottolenghi *et al*. (202) and are discussed in Section 6.2. Her findings of increased sphingomyelinase activity in association with reduced SM and increased ceramide accumulation in hypoxia and inflammatory lung disease align with the finding of SM preservation in phosgene exposed animals treated with PEEP.

Taken together, these comparisons demonstrate that phosgene induced acute lung injury shares common biochemical features with human ARDS, including surfactant disruption, PLA_2- mediated phospholipid hydrolysis and sphingolipid dysregulation. However, the porcine model of phosgene induced acute lung injury offers a unique perspective by characterising lipidomic changes early, before the confounding effects of established systemic inflammation occur. Moreover, the technique of tightly intubating the right median lobe for bronchoscopic sampling of BAL at post mortem allows for reproducible and accurate recovery of surfactant. As a result of this, absolute amounts of surfactant lipids can be accurately reported. Human studies are very prone to recovery errors, particularly if endotracheal aspirate is examined instead. This is why human findings are often reported as fractions because absolute amounts are not comparable. The ability of PEEP to modulate these early changes provides mechanistic support for its protective

role in preventing surfactant disruption, limiting inflammatory lipid pathways and attenuating pathophysiological deterioration.

6.4 Strengths and Limitations

6.4.1 Strengths

This porcine model replicates human physiology more accurately than other animal models. The pulmonary architecture, surfactant composition and respiratory pathophysiology more closely resemble human.

The dosing of inhaled phosgene is exceptionally precise and this was controlled by a tightly regulated and continuously monitored phosgene exposure concentration. This precision is rarely achieved in inhalational toxicology studies and, by eliminating inconsistencies in exposure, between group differences can be confidently attributed to PEEP.

The procedure for sampling BAL through tight intubation of the right median lobe allows for near complete recovery of lavage fluid and eliminates recovery errors. This enables accurate quantification of absolute surfactant lipid concentrations. Since human studies rely on variable BAL recovery, they are limited to reporting fractional changes, this is a major strength.

This work presents a comprehensive analysis of combined physiological, biochemical and molecular species level lipidomics data and offers a multidimensional understanding of phosgene toxicity. Mechanistic linkage between surfactant disruption, inflammatory activation, liver metabolism, physiological deterioration and the protective effects of PEEP as a surrogate for CPAP is characterised.

Animals were enrolled prior to respiratory failure, allowing the capture of early molecular events that precede deterioration. This timing distinguishes the work from most human ARDS studies and provides novel insights into the initial dysregulation of lipid metabolism in acute lung injury.

6.4.2 Limitations

The cost and logistical complexity of the model limits the sample size and may have extended effects on generalisability. Smaller numbers reduced statistical power for some comparisons.

The individual experiments could not be blinded for practical and safety reasons, and the potential for observer bias could have been introduced. Efforts were made to minimise this through predefined protocols and objective physiological endpoints. Lipidomic samples were labelled clearly with the study number without reference to which group they fell into.

The post mortem BAL recovery method, remains a regional sampling technique, and findings may not capture heterogeneity across the whole lung. However, this would compound lung weight data if implemented. The model was insufficiently robust for antemortem bronchoscopy, and the sampling was from a single timepoint. This limits temporal interpretation of some pathways. Endotracheal aspirates could have been made, but these would only allow for fractional comparisons of surfactant composition.

6.5 Future

The findings of this thesis yield several strands for future investigation in the detection and treatment of phosgene induced acute lung injury. Identifying those likely to develop the effects of phosgene after a release, and discharging the *worried well* have obvious logistic benefits. Early diagnostic biomarkers capable of identifying exposed individuals before inaugural symptoms and signs remain a priority. In World War 1 asymptomatic individuals could often predict impending deterioration by a change in the flavour of their cigarettes to one reminiscent of rotten eggs (11, 12). This approach is not acceptable or reliable but may draw upon perturbations in the ‘reactive species interactome’ (208). Perhaps measurements could be made and used as markers for any pulmonary agent influencing redox homeostasis. An alternative biomarker worthy of further investigation is surfactant protein D (209, 210). Another method could involve the inhalation

of a non-toxic compound that is permeable only to injured lungs and is measured in a capillary blood sample.

While there is no merit in pursuing the effects of steroids in this context (211, 212), several antioxidants have been evaluated with variable success in chemical agent induced ALI, indicating that our understanding of pulmonary redox is lacking. Since the completion of the experimental work on CPAP, N-acetylcysteine has been trialled and found to be ineffective as a post exposure treatment for phosgene induced acute lung injury in pigs (104). Catalytic antioxidants such as AEOL10150 have shown potential benefits in conditions of oxidative stress (205, 213, 214). The explosive rapidity of phosgene's reaction with biological molecules makes complete reversal impossible within the constraints of our current understanding. Further understanding and exploitation of biomarkers for diagnosis and the use of catalytic antioxidants within treatment, and possibly pre-exposure, could transform the care of phosgene casualties.

6.6 Conclusion

This thesis set out to evaluate the physiological and biochemical consequences of phosgene inhalation and to determine whether the early application of PEEP, as a surrogate for CPAP, could mitigate the onset and severity of phosgene induced acute lung injury. The findings demonstrate that PEEP substantially reduces the adverse effects of phosgene exposure, improving oxygenation, delaying physiological deterioration, reducing pulmonary oedema and preventing mortality in this model. The results support the use of CPAP as an early scalable intervention capable of extending the timeline for safe triage, treatment and transport of patients in a mass casualty scenario.

A second objective was to characterise the lipid disturbances at the level of individual molecular species. This aim was also achieved. Detailed lipidomic analysis of BAL and plasma identified specific alterations in glycerophospholipid and sphingolipid metabolism, hydrolysis of PC, arachidonic acid liberation, and changes in SM and PS associated with injury severity. These results provide the first molecular species profile of surfactant and plasma lipid disruption in phosgene induced acute lung injury.

Although the study showed clear lipidomic signatures of the injury, no single lipid species or combination, emerged as a unique biomarker of phosgene exposure. Instead, the results call attention to early, pathway specific disturbances, particularly related to PLA_2 and sphingomyelinase pathways, that may inform future biomarker development rather than identifying a standalone diagnostic marker at present.

The survival benefits of PEEP as a surrogate for CPAP corroborate previous research on ventilation strategies for ARDS. The novel contribution of this thesis lies in demonstrating CPAP's specific efficacy in a chemical injury model, suggesting its utility as a field deployable intervention during mass casualty scenarios. The findings have direct implications for civil and military preparedness, advance the mechanistic understanding of phosgene toxicity and provide a strong foundation for future work.

APPENDIX- PUBLICATIONS

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Continuous positive airway pressure: An early intervention to prevent phosgene-induced acute lung injury

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ABSTRACT

Exposure to toxic industrial chemicals such as phosgene may occur through accidental or deliberate release. Inhalation may result in an acute lung injury which manifests as hypoxaemia with insufficient oxygen being delivered to the tissues resulting in hypoxia, respiratory failure and death. No effective pharmacological therapy currently exists and treatment remains supportive, often requiring intensive care facilities. In a mass casualty scenario the logistical burden of managing exposed individuals would rapidly overwhelm healthcare systems. This highlights the need to develop post exposure therapeutic strategies to minimise injury severity and increase survival in individuals exposed to toxic chemicals.

Our research objective was to investigate a commercial off the shelf (COTS) therapy; ambient air continuous positive airway pressure (CPAP) support, initiated 1 h post exposure to explore the concept that early intervention with positive airway pressure would reduce or ameliorate lung injury following exposure to phosgene.

This study has demonstrated that CPAP, initiated before overt signs of exposure become manifest, significantly improved survival as well as improving some clinically relevant physiological measures of phosgene-induced acute lung injury over 24 h.

1. Introduction

Phosgene ($COCl_2$) is an important, high volume, globally produced toxic industrial chemical (TIC) used as a reactive intermediate in the production of plastics, dyes and agricultural products (Sciuto and Hurt, 2004). As such, despite restrictions on its storage and supply, accidental exposures e.g. from transportation and storage facilities, from thermal decomposition of chlorinated hydrocarbons, or through deliberate releases, remain a potential problem. Exposure of unprotected individuals to phosgene can result in an acute lung injury (ALI), which may further develop into acute respiratory distress syndrome (ARDS), for which there are no proven pharmacological interventions currently available. Treatment remains supportive, requires intensive care facilities and can be ineffective in cases of severe poisoning. Despite decades of research into the use of novel ventilation and medical management strategies, mortality rates from ARDS remain high. The potential for exposure to lung damaging chemicals such as phosgene to cause mass casualties, either in a military or civilian context, highlights the need to develop post-exposure therapeutic strategies to minimise lung injury and increase survival in exposed individuals.

The clinical effects of exposure to high concentrations of phosgene are well characterised (Diller, 1985). Following acute exposure there is an asymptomatic period with a variable duration (6–12 h) which is inversely proportional to the exposure dose; however, while the clinical effects may not be apparent during this "clinical latent phase" the biochemical effects are ongoing. This latent period can complicate diagnosis and triage especially since initial symptoms may not predict outcome (Borak and Diller, 2001; Diller, 1985; Grainge and Rice, 2010). Additionally, there is currently no way to discriminate between unexposed and exposed individuals early on. Damage to the respiratory

Abbreviations: COTS, commercial off the shelf; CPAP, continuous positive airway pressure; TIC, toxic industrial chemical; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; IPPV, intermittent positive pressure ventilation; PEEP, positive end expiratory pressure; COPD, chronic obstructive pulmonary disease; SF(Qs:Qt), shunt fraction; ET, endotracheal tube; ECG, Electrocardiogram; IV, intravenous; BAL, bronchoalveolar lavage; WBC, white blood cell; LWW:BW, lung wet weight to body weight ratio; LWW:DW, lung wet weight to dry weight ratio; ANOVA, analysis of variance; SD, standard deviation; PaO_2, partial pressure of oxygen (arterial); $PaCO_2$, partial pressure of carbon dioxide (arterial); O_2Hb, oxyhaemoglobin; SaO_2, oxygen saturation (arterial)

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epithelium may occur within minutes and cause disruption to the integrity of the alveolar epithelial blood–air barrier, which can result in the movement of tissue fluid and inflammatory cells (mainly neutrophils) into the alveolar space. Accumulation of activated neutrophils in the lung can cause injury through the release of reactive oxygen species. Phosgene causes upregulation of oxidative response enzymes such as glutathione reductase and superoxide dismutase activity (Jaskot et al., 1991; Sciuto and Moran, 2001). The formation of a non-cardiogenic pulmonary oedema and resulting impaired gas exchange culminate in adverse physiological changes including decreased lung compliance, arterial pH and arterial oxygen concentration which may lead to respiratory failure and death (Brown et al., 2002; Jugg, 2016; Marrs et al., 1996). Where fatalities ensue, 80% may be expected to die within the first 24–48 h after exposure.

Previous studies have demonstrated a significant improvement in survival following phosgene- induced ALI using intermittent positive pressure ventilation (IPPV) with high positive end expiratory pressure (PEEP) and low tidal volume, initiated 6 h post-exposure (Jugg, 2016; Parkhouse et al., 2007), and with supplemental oxygen (Grainge et al., 2010a). However, these interventions are resource intensive and impractical following a mass casualty event. A way in which aspects of this strategy, such as PEEP, could be implemented early might be of benefit. Therefore, there remains a need for the development and evaluation of effective, simple and less resource intensive interventions. CPAP is a treatment that uses air pressures between 4 cm H_2O and 20 cm H_2O (in adults (Burns et al., 2001)) to keep the airways open. It is used by health care providers world-wide to treat conditions such as sleep apnoea and chronic obstructive pulmonary disease (COPD) and it is also effective in patients who are hypoxic or in respiratory failure due to cardiogenic pulmonary oedema. In these circumstances supplemental oxygen would also be administered. A CPAP machine can be purchased commercially and has three main parts; a face/nose mask, a tube that connects the mask to the machine's motor, and a motor that blows air into the tube. It facilitates and augments spontaneous breathing activity in patients with ALI, and also recruits collapsed or consolidated lung (Putensen and Wrigge, 2004). These effects may provide benefit in casualties who have been exposed to toxic chemicals.

There are a number of similarities which exist between phosgene-induced ALI and that seen in patients with non-toxic ALI or ARDS, including hypoxaemia, alveolar capillary damage characterized by inflammation, increased alveolar-capillary permeability, leading to the development of alveolar oedema (Brown et al., 2002; Parkhouse et al., 2007; Ware and Matthay, 2000). It is therefore reasonable to assume that treatments developed for ALI of non-chemical origin could be used to treat chemically-induced ALI, albeit without the use of supplemental oxygen. The hypothesis for use of ambient air CPAP is that by non-invasively increasing the positive pressure in the lungs at the very early stages of injury development (i.e. while changes to the blood-air barrier are occurring) and before full clinical assessment, it might be possible to reduce or delay the progression of the injury by preventing fluid influx into the alveoli. Any collapsed or consolidated lung due to phosgene injury may also potentially be recruited (opened), thus keeping the airways and alveoli open and increasing the functional residual capacity of the lung. Alveolar recruitment, which has for many years been a primary goal in respiratory care for ALI, should result in early improvement in oxygenation and gas exchange (Mols et al., 2006). Redistribution of lung water from the alveoli to the perivascular space, as a result of increased mean airway pressure, will also result in improvement of shunt fraction (SF) resulting from ventilation-perfusion mismatch.

Though an established treatment modality within the hospital setting, ambient air CPAP has not been studied either in the context of ALI due to phosgene exposure or as a "buddie-buddie" first aid or first responder delivered treatment. CPAP may provide a means by which large numbers of casualties could be treated by non-specialist medical personnel as well as reducing the number of casualties requiring intubation at the scene of an incident and the need for intensive care management with its associated logistical burden (mechanical ventilators, medical oxygen and skilled manpower). CPAP may delay the progression of ALI in order for definitive care to be delivered in more appropriate, safer surroundings. The use of ambient air CPAP as an early non-invasive intervention to reduce/eliminate ALI from chemical exposure is a novel and untested therapy.

2. Materials and methods

2.1. Animals

Large white juvenile female pigs (47–55 kg, n = 22) were obtained from a commercial source approved as a supplier by Dstl's Animal Welfare Ethical Review body. Animals were housed in pairs and allowed access to food and water *ad libitum* as previously described (Brown et al., 2002). All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.2. Surgical procedures

Experimental procedures have been previously described in detail (Grainge et al., 2009, 2010a). Briefly, following pre-medication, animals were anaesthetised with isoflurane in oxygen and nitrous oxide. Once anaesthetised, the animals were intubated with a size 8 cuffed endotracheal tube (ET) (Portex, Smiths Medical Int. Ltd, UK). A carotid artery and both internal jugular veins were surgically exposed and cannulated and connected to a pressure transducer and the blood pressure and heart rate monitored. Electrocardiogram (ECG), pulse oximetry, exhaled carbon dioxide, and central venous pressure were measured using a Propaq 246 monitor (Protocol systems Inc., Beaverton, USA). A Bonanno bladder catheter was introduced into the bladder via an open cystotomy. To replace insensible fluid loses, animals were maintained on an infusion of Vetivex 11 (Hartmann's solution, 2.0 ml kg^{-1} h^{-1}).

Following surgery, anaesthesia was maintained intravenously with propofol 2% (~10–12 mg kg^{-1} h^{-1}) ("Propofol" Diprivan®, Astra Zeneca, UK; 20 mg ml^{-1}) and alfentanil hydrochloride (~0.5–2.5 µg kg^{-1} h^{-1}) (Rapifen, Janssen Pharmaceuticals Ltd, Ireland; 500 µg ml^{-1}).

2.3. Study protocol

Baseline physiological and biochemical measurements were taken for 1 h following the completion of surgery. The exposure system consisted of a delivery apparatus, an exposure tube and effluent and analysis equipment and has been previously described (Brown et al., 2002). The delivery system was a 10 L Tedlar® bag mounted within a rigid Perspex box and filled with phosgene (BDH, UK) diluted with nitrogen. Delivery of phosgene was achieved by admitting nitrogen to the outer Perspex chamber, thereby subjecting the Tedlar® bag to a positive pressure and directing the outflow to the exposure tube inlet to produce the required concentration.

The animal was connected to the exposure apparatus, via its ET tube, allowed to acclimatise for 10 min and then exposed to air or phosgene. Carrier air was drawn through the exposure tube at a flow rate of 78–85 L min^{-1}. For phosgene exposures the concentration of phosgene within the exposure tube was monitored continuously using a Miran infrared gas analyser (Foxboro Instruments, UK). Phosgene concentrations were recorded every 15 s and were integrated over time to calculate the dosage (concentration (mg m^{-3}) x time (min); Ct) delivered to the animal. The Inhaled dose was determined from the mean phosgene concentration (mg m^{-3}) x total inhaled volume (m^3, L/1000) divided by body weight (kg). Following exposure the animal remained attached to the exposure apparatus for 5 min and physiological monitoring was continued.

The animals were randomly assigned to the following groups:

- Air-exposed controls (n = 4)
- Phosgene-exposed controls (n = 10)
- Phosgene + CPAP (n = 8)

The air-exposed control animals remained breathing air, whilst the other groups were exposed to phosgene for approximately 10 min to deliver a target inhaled dose of 0.24 mg kg^{-1}.

2.4. CPAP

On returning to the experimental room and for the first 1 h post exposure, animals assigned to the 'phosgene + CPAP' group were attached to a ventilator (Evita XL, Dräger Medical) without CPAP being initiated. At 1 h post exposure CPAP was initiated using medical air at 5 cm H_2O and every 5 min thereafter increased by increments of 1 cm H_2O to a maximum of 10 cm H_2O (limit of tolerability). Once CPAP values were set, they remained unchanged for the remainder of the study. Air-exposed and phosgene-exposed control animals were not attached to the ventilator and remained breathing room air spontaneously to the end of the study.

2.5. Physiological measurements

Physiological measurements were made every 20 min during the 1 h baseline period after which measurements were recorded every 30 min until the end of the experimental period (24 h or death).

Arterial and central venous blood gas samples were taken at hourly intervals and immediately analysed using a GEM Premier 3500 blood-gas analyser and co-oximetry analyser (Instrumentation Laboratories UK Ltd, UK). Temperature adjusted values are reported. Haematological analysis was performed on EDTA blood samples using an Advia 120 Hematology system (Siemens, UK).

Shunt fraction (Qs:Qt; the ratio of shunted blood (Qs) to total cardiac output (Qt)) is a calculated measure of the amount of blood passing through the lungs that remains unoxygenated and was calculated using standard formulae (Edwards et al., 1993).

2.6. Post mortem and histopathology

At the end of the 24 h monitoring period, or when the animal became moribund (defined as periods of asystole and a central venous oxygenation < 15%) it was killed by an overdose of sodium pentabarbitone (200 mg ml^{-1}; IV) ("Euthatal" Rhone Merieux Ltd., UK) and a full *post mortem* examination carried out. Following clamping of the trachea distal to the end of the ET tube to prevent loss of fluid from the respiratory tract and potential contamination of the airway during *post mortem*, the thorax was opened and the lungs and heart removed. Bronchoalveolar lavage (BAL) of the right medial lung lobe was performed as previously described (Grainge et al., 2009). Lavage fluid was analysed for total white blood cell (WBC) counts (Advia 120 Hematology system) and for differential cell counts. Protein content of BAL supernatant was determined using Coomassie blue (Bradford, 1976).

Following lavage, the lungs were weighed for lung wet weight to body weight (LWW:BW) ratio determinations (the weight of the remaining lavage saline being taken into account) and lung wet weight to dry weight (LWW:DW) ratios. Samples from each lung lobe and all major organs were taken, fixed in neutral buffered formalin and processed for histopathological examination using standard processing techniques. Histology slides were assessed by an independent veterinary pathologist blinded to the study design, using a previously developed scoring system (Grainge et al., 2010a; Jugg et al., 2013).

2.7. Statistical analysis

Data from the 24 h monitoring period were analysed using a linear mixed-effects model (http://CRAN.R-project.org/package=lme4) that describes the relationship between a response variable (e.g. blood analysis result) and one or more covariates recorded with it (treatment, time and animal). The three treatments were compared against each other at all time points throughout the study. The fixed effects are compared using Tukey's multiple comparisons test within the lsmeans package within R (https://cran.r-project.org/web/packages/lsmeans/index.html) and the results were determined based on a 95% confidence level so found to be significant at $p < 0.05$.

All other data distributions were subjected to a preliminary statistical screen to ensure acceptable fit for a one-way analysis of variance (ANOVA). Where data was shown to be non-normally distributed, a non-parametric ANOVA (Kruskal Wallis) was applied. Where the non-parametric ANOVA (Kruskal Wallis) was applied, and the Z value found to be significant ($p < 0.05$) a Dunn's Test was used to examine planned comparisons of treatment medians.

3. Results

All data are expressed as mean ± SD.

3.1. Phosgene exposures

All animals exposed to phosgene received the intended target inhaled dose of 0.24 mg·kg^{-1} (mean inhaled dose 0·241 ± 0·008 mg·kg^{-1}). The mean Ct required to provide this inhaled dose was 1846 ± 432 mg min m^{-3} (C = 185.4 ± 38.5 mg m^{-3} (~ 45 ppm); t = 9.94 ± 0.53 min).

3.2. Survival

All air-exposed controls (without CPAP) survived to the end of the 24 h monitoring period (Fig. 1). Five of 10 phosgene-exposed control animals survived to 24 h. There was a significant improvement in survival between the phosgene + CPAP group when compared to the phosgene-exposed control group (100% vs 50%, $p < 0.05$) with all CPAP treated animals surviving to 24 h.

3.3. Oxygenation

It was noted that all groups had similar blood gas values (PaO_2, SaO_2, bicarbonate and $PaCO_2$), shunt fraction and respiratory rates during the postsurgical baseline period and for 1 h post exposure. After

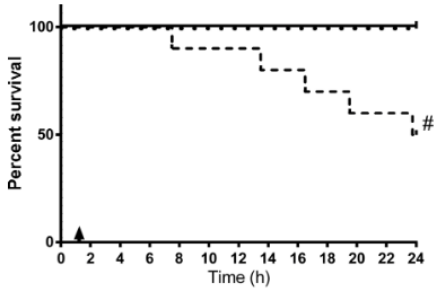

Fig. 1. Kaplan-Meier percentage survival graph. Air-exposed controls (—, n = 4); phosgene-exposed controls (– – –, n = 10); phosgene + CPAP treated (■■■, n = 8, CPAP initiated from 1 h post exposure). "▲" represents CPAP initiation. # Significant difference between phosgene-exposed control and phosgene + CPAP groups ($p < 0.05$).

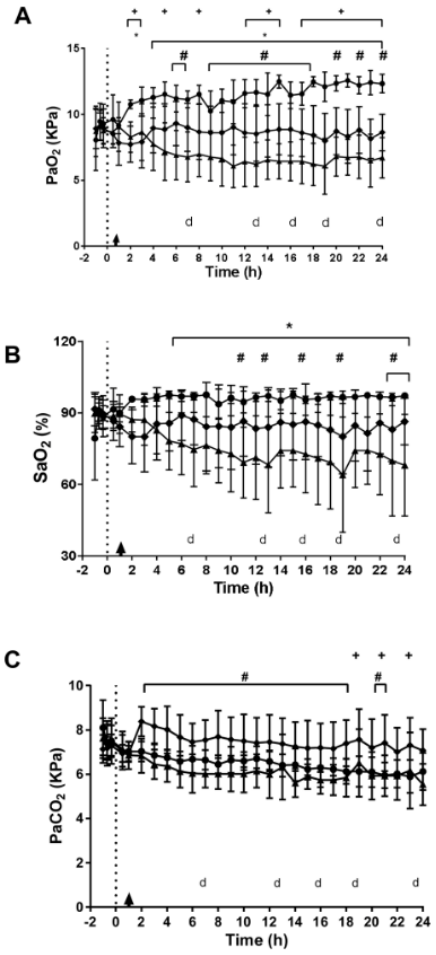

Fig. 2. Changes in temperature adjusted (T) arterial blood PaO_2 (A) SaO_2 (B) and $PaCO_2$ (C). Air-exposed controls (●, n = 4), Phosgene-exposed controls (▲, n = 10), Phosgene + CPAP treated (♦, n = 8, CPAP from +1 h post exposure). "▲ " represents CPAP initiation. Each "d" represents the death of an animal. + Significant difference between air-exposed controls and phosgene + CPAP group. * Significant difference between air-exposed controls and phosgene-exposed control group. # Significant difference between phosgene-exposed controls and phosgene + CPAP group. All differences significant at $p < 0.05$.

this time the air-exposed control animals stabilise at levels consistent with previous studies (Jugg et al., 2013, 2016). These early changes reflect the fact that the animals are anaesthetised, spontaneously breathing, and laying supine.

Phosgene caused a significant reduction in PaO_2 (Fig. 2A), SaO_2 (Fig. 2B) and O_2Hb (data not shown), when compared to air-exposed controls from 2 to 24 h post exposure ($p < 0.05$). Following CPAP the fall in PaO_2 was diminished, being significantly less than that seen in phosgene-exposed controls from 6 through to 24 h ($p < 0.05$). PaO_2 did not return to levels seen in the air-exposed controls and there remained a small but significant reduction in PaO_2 in the phosgene + CPAP animals when compared to air-exposed controls from 2 h post exposure onwards ($p < 0.05$).

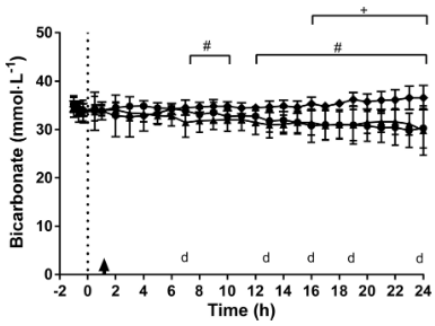

Fig. 3. Changes in arterial blood bicarbonate. Air-exposed controls (●, n = 4), Phosgene-exposed controls (▲, n = 10), Phosgene + CPAP treated (♦, n = 8, CPAP from +1 h post exposure). "▲ " represents CPAP initiation. Each "d" represents the death of an animal. + Significant difference between air-exposed controls and phosgene + CPAP group. # Significant difference between phosgene-exposed controls and phosgene + CPAP group. All differences significant at $p < 0.05$.

While there was no significant difference in $PaCO_2$ between the phosgene-exposed controls and the air-exposed controls, the CPAP treated animals had a small but significant increase in $PaCO_2$ compared to both air and phosgene-exposed controls (Fig. 2C)($p < 0.05$).

3.4. Acid base analysis

The base excess of air and phosgene-exposed controls remained stable throughout the study (data not shown). CPAP treatment resulted in higher base excess levels compared to the air and phosgene-exposed controls, becoming significant from 17 to 24 h post exposure ($p < 0.05$).

The arterial blood bicarbonate levels of the air and phosgene-exposed controls remain stable throughout (Fig. 3). CPAP treatment resulted in small but significant increase in bicarbonate levels compared to both air and phosgene-exposed controls ($p < 0.05$).

The arterial blood pH of air and phosgene exposed animals remained constant throughout the study (data not shown). There was an early, small (0.1 unit) but significant decrease in pH following CPAP treatment (2 and 6 h).

3.5. Shunt fraction

The shunt fraction of air-exposed controls was maintained at stable levels from 2 h post exposure (Fig. 4). Exposure to phosgene resulted in a significant increase in shunt fraction, reaching levels of approximately 50%, from 4 to 24 h compared to the air-exposed controls ($p < 0.05$). CPAP treated animals had a smaller increase in shunt fraction, reaching average levels of 30%, when compared to the air-exposed controls ($p < 0.05$). There was no significant difference in shunt fraction between the CPAP treated animals and the phosgene-exposed controls over the course of the study.

3.6. Respiratory rate

The respiratory rate of air-exposed controls remained stable (Fig. 5). The phosgene-exposed controls were observed to exhibit rapid, shallow breathing, with a sustained and significant increase in respiratory rate compared to the air-exposed controls from 2.5 to 24 h ($p < 0.05$), and when compared to the phosgene + CPAP animals from 1.5 to 24 h ($p < 0.05$). The CPAP treated animals' maintained normal stable

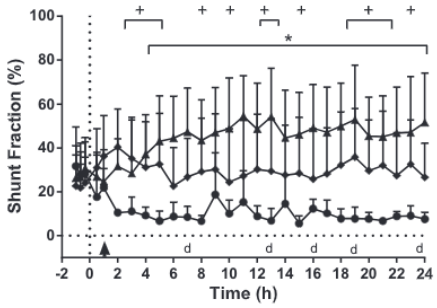

Fig. 4. Changes in shunt fraction. Air-exposed controls (●, n = 4), Phosgene-exposed controls (▲, n = 10), Phosgene + CPAP treated (♦, n = 8, CPAP from +1 h post exposure). "▲" represents CPAP initiation. Each "d" represents the death of an animal. + Significant difference between air-exposed controls and phosgene + CPAP group. * Significant difference between air-exposed controls and phosgene-exposed control group. All differences significant at $p < 0.05$.

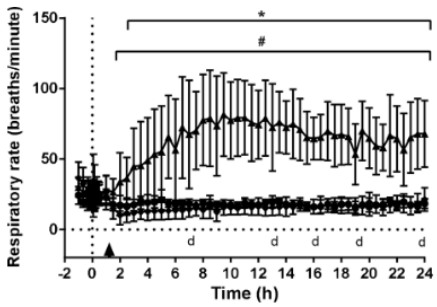

Fig. 5. Changes in respiratory rate. Air-exposed controls (●, n = 4), Phosgene-exposed controls (▲, n = 10), Phosgene + CPAP treated (♦, n = 8, CPAP from +1 h post exposure). "▲" represents CPAP initiation. Each "d" represents the death of an animal. * Significant difference between air-exposed controls and phosgene-exposed control group. # Significant difference between phosgene-exposed controls and phosgene + CPAP group. All differences significant at $p < 0.05$.

respiratory rate throughout the study.

3.7. Measures of pulmonary oedema

For all terminal variables, data was pooled irrespective of the time of death of each animal in each group.

The lung wet weight to body weight (LWW:BW) ratio (data not shown) and lung wet weight to dry weight (LWW:DW) ratio (data not shown) are measures of extravascular lung water and indicate the degree of alveolar permeability. Exposure to phosgene resulted in a significant increase in LWW:BW ratio compared to air-exposed controls ($p < 0.05$). The LWW:BW of CPAP treated animals was not significantly different from the air or phosgene-exposed controls.

Exposure to phosgene resulted in a significant increase in LWW:DW ratio in the left apical ($p < 0.05$) and right apical ($p < 0.05$) lobes. Following CPAP the LWW:DW ratio was significantly decreased in the left apical lobe as compared to phosgene-exposed controls ($p < 0.05$) but remained significantly elevated in the right apical lobe compared to the air-exposed controls ($p < 0.05$).

There were no other significant differences in LWW:DW ratios in the remaining lung lobes between the three groups.

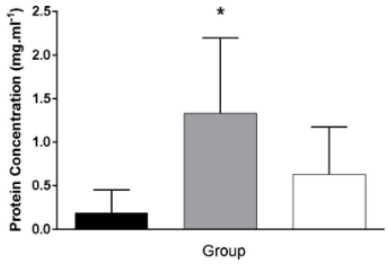

Fig. 6. Protein concentration in terminal lavage fluid. Air-exposed controls (black bar, n = 4). Phosgene-exposed controls (grey bar, n = 10) Phosgene + CPAP treated (white bar, n = 8, CPAP from 1 h post exposure). * Significant difference between air-exposed controls and phosgene-exposed control group. Significant at $p < 0.05$.

3.7.1. Protein

There was a significant increase in the amount of protein in the terminal BAL fluid of the phosgene-exposed controls when compared to the air-exposed controls ($p < 0.05$) (Fig. 6). Following CPAP the protein concentration was reduced compared to phosgene-exposed controls and was not significantly different from the air control animals.

3.7.2. Differential WBC count

Differential WBC count analysis of terminal BAL fluid demonstrates a normal cellular distribution in the lungs of the air-exposed control animals with the majority of cells being the resident alveolar macrophage (Fig. 7).

There was a significant increase in the percentage (Fig. 7) and absolute number (0.18 ± 0.14 vs $0.014 \pm 0.015 \times 10^6$ mL^{-1} respectively) of neutrophils recruited into the lungs of the phosgene-exposed controls when compared to the air-exposed controls ($p < 0.05$). In addition, there was a concomitant significant decrease in the percent of alveolar macrophages ($p < 0.05$). There were no other significant differences in WBC populations in the BAL fluid.

3.8. Pathology

There was a significant increase in total aggregate lung pathology score (determined from bronchiolar epithelial degeneration and necrosis, cellular debris in bronchiolar lumen, perivascular oedema, fibrin and cell infiltrates, interstitial oedema, alveolar oedema and fibrin and

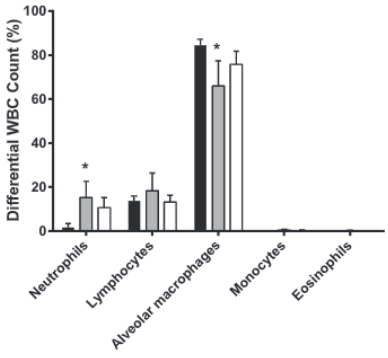

Fig. 7. Differential WBC count analysis of terminal BAL fluid. Air-exposed controls (black bar, n = 4). Phosgene-exposed controls (grey bar, n = 10) Phosgene + CPAP treated (white bar, n = 8, CPAP from 1 h post exposure). * Significant difference between phosgene-exposed controls and air-exposed controls ($p < 0.05$).

alveolar cell infiltrate) from all lung lobes in the phosgene-exposed control and the phosgene + CPAP animals when compared to the air-exposed controls (data not shown; $p < 0.05$) (this analysis excluded the right medial lobe which had previously been lavaged). Early use of CPAP did not reduce the total aggregate lung pathology score compared with phosgene-exposed control animals.

4. Discussion

This study has demonstrated that CPAP initiated 1 h post-phosgene exposure i.e. before overt signs of exposure become manifest, significantly improved survival at 24 h after poisoning as well as improving some clinically relevant physiological measures of phosgene-induced ALI (e.g. arterial blood oxygenation and respiratory rate). Although the physical injury, as assessed by histopathology, to the lungs was not improved, improved oxygenation up to and beyond 24 h would potentially reduce the immediate medical logistical burden following a mass casualty event. In the context of a large number of casualties, such an effect could reduce mortality as well as the sophistication of medical intervention required on reaching hospital.

Although the clinical effects of exposure to high concentrations of phosgene are well characterised, there remain gaps in our understanding of the mechanisms by which phosgene causes its injury to the lungs (Diller, 1985; Jugg, 2016). Following exposure there is an asymptomatic period that varies in duration; however, it is during this latent period that the immediate biochemical effects of phosgene occur (Jugg, 2016). As a highly reactive hydrophobic gas, phosgene penetrates the lower respiratory tract having a direct effect on the respiratory epithelium. Damage by acylation reactions with primary and secondary amines as well as hydroxyl and sulfhydryl groups causes the downstream release of arachidonic acid-derived mediators such as leukotrienes and the denaturation of both proteins and lipids which results in irreversible changes in the cell membrane and intracellular structures. These changes result in increasing fluid transfer across the alveolar membrane, and clinically evident pulmonary oedema (Diller, 1985; Grainge and Rice, 2010). A dysregulated and continued overstimulation of J-receptors (also known as pulmonary C-fibres) has been proposed as an essential contributing factor for the lethal acute lung oedema to occur following phosgene exposure (Li et al., 2013, 2011). Therefore, a means by which the movement of fluid into the lungs can be prevented may result in a better clinical outcome for casualties.

Inhalation of 0.24 mg kg^{-1} phosgene caused 50% mortality at 24 h, with early deaths due to fulminating proteinaceous pulmonary oedema and consequent hypoxia as demonstrated by the increased terminal BAL fluid protein, neutrophil number and concomitant increase in the LWW:BW ratio. This occurs following breakdown of the alveolar-endothelial barrier. Indeed, the histopathology demonstrated a significant increase in alveolar oedema and fibrin as well as bronchiolar epithelial degeneration and necrosis, perivascular oedema, fibrin and cell infiltrates which was consistent with those reported by Li et al., in the dog (Li et al., 2015). It should be noted that the variability in terminal assessment of histopathology, protein and LWW:BW ratio probably reflect the different time points at which the animals died; pooling data from these different time points is a limitation of the study and may reduce the power of the statistical analysis of these variables.

Phosgene caused a significant decrease in arterial blood PaO_2 from as early as 2 h post-exposure while the $PaCO_2$, BE and bicarbonate levels remained unchanged. Hypoxaemic respiratory failure is characterised by a PaO_2 of < 8 kPa (60 mm Hg) with normal or low arterial $PaCO_2$. The hypoxaemia is likely due, at least in part, to impaired gas exchange resulting from oedema formation, firstly within the perivascular space and septal interstitium and subsequently within the alveolar space (Brown et al., 2002). This may contribute to shunting of blood, resulting from ventilation-perfusion mismatch as demonstrated by the increase in shunt fraction seen from 4 h. The results are in agreement with other studies using a similar model following exposure to phosgene (Brown et al., 2002; Grainge et al., 2010a; Parkhouse et al., 2007).

Phosgene caused a significant and sustained increase in respiratory rate, possibly due to the hypoxaemia stimulating peripheral chemoreceptors to increase both the rate and depth of respiration. However, although tidal volume was not measured in this study, it was noted that respiration became rapid and shallow (tachypnoea) indicating another mechanism may be involved. The increased respiratory rate was also noted to occur before the hypoxaemia became evident i.e. before PaO_2 fell to < 8 kPa and may therefore result from stimulation of J-receptors previously described as being involved in phosgene-induced lung injury and known to induce a reflex rapid shallow breathing pattern (Anand et al., 1986; Li et al., 2013; Paintal, 1969). Paintal (1969) postulated that stimulation of J-receptors occurs as a result of a rise in pulmonary interstitial pressure or volume produced by a rise in pulmonary capillary pressure and that it is almost certain they would be stimulated during phosgene inhalation (Paintal, 1969).

Initiation of CPAP from 1 h post exposure resulted in 100% survival at 24 h proving the hypothesis that early intervention with positive airway pressure would reduce or ameliorate the lung injury, or its effects, following chemical exposure. Treatment with CPAP provided functional benefit to the animals as demonstrated by the improved hypoxaemia. The phosgene-induced increase in respiratory rate was prevented; this may be due, not only to improved and stable arterial blood oxygen levels but also due to reduced pulmonary oedema fluid (BALF protein and LWW:BW ratios not different from air-exposed controls) seen in the CPAP treated animals. CPAP can cause changes in alveolar/hydrostatic pressure dynamics (Mattera, 2010). An increase in alveolar pressures can counterbalance interstitial or capillary hydrostatic pressures and slow or stop movement of fluid into the alveoli. Positive airway pressure can push fluid out of the alveoli in pulmonary oedema and stop further influx. It may be that the phosgene-induced stimulation of J-receptors was prevented by the positive airway pressure keeping fluid out of the alveolar spaces.

The role of CPAP is to deliver a constant positive pressure to the airways which allows recruitment of alveoli not involved in gas exchange due to the presence of pulmonary oedema fluid. As more alveoli stay open, the surface area for diffusion or gas-exchange increases resulting in better alveolar ventilation, improved gas exchange and increased arterial blood PaO_2 (Mattera, 2010), as demonstrated in our study. Improvement in ventilation/perfusion mismatch occurs. The shunt fraction was improved following the application of CPAP.

Previous work undertaken by this group has used terminally anaesthetised, conventionally ventilated pigs to test the efficacy of single treatments e.g. intravenous and inhaled steroids (Smith et al., 2009), inhaled salbutamol (Grainge et al., 2009) and inhaled furosemide (Grainge et al., 2010b). This model has also been used to assess strategies available in the field hospital and ICU, and demonstrated the beneficial effects of protective ventilation (Parkhouse et al., 2007) and supplemental oxygen therapy (Grainge et al., 2010a) to phosgene exposure. However these methods require mechanical ventilation, are resource intensive and would be unsuitable in a mass casualty scenario. Ambient air CPAP is a non-invasive therapy commonly employed in spontaneously breathing patients with obstructive sleep apnoea (Giles, 2015) and in the treatment of acute respiratory failure due to cardiogenic pulmonary oedema (Baudouin et al., 2002). CPAP delivers a constant positive pressure above atmospheric pressure throughout the respiratory cycle (Duncan et al., 1986) and limits collapse of bronchioles and alveoli by acting as a counter-pressure to fluid movement. Entire regions of the lung that would otherwise collapse are forced or held open (recruitment). The functional residual capacity is greater such that the chest and lungs are more expanded with improved compliance. From this more expanded resting position the mechanical work of breathing is improved (Wild and Alagesan, 2001). It has previously been shown in patients with a 'moderate' ALI, that applying CPAP of 10 and 15 cm H_2O provided the greatest beneficial effect in oxygenation

regardless of tidal volume (Burns et al., 2001). The CPAP value of 10 cm H_2O administered in this study was the maximum tolerated by the animals. The current study was designed to use air to drive CPAP instead of medical oxygen as this would be in very limited supply or not available in remote locations or in mass casualty events. In light of the known benefits of increasing the inspired concentration of oxygen following phosgene exposure (Grainge et al., 2010a) the use of supplemental oxygen with CPAP may further improve outcomes and should be investigated.

In studying the effects of ALI induced by phosgene in any animal there are numerous limitations. The use of anaesthesia is essential in performing studies *in vivo*, but is also known to affect physiology including oxygenation and respiration in a dose dependent manner (Hockel et al., 2012). However, by having the appropriate negative (air-exposed) and positive (phosgene-exposed) control groups the study was designed to allow assessment of the role of CPAP against phosgene-induced ALI. The current study only examined physiology up to 24 h after phosgene exposure; therefore a delayed decline or improvement in the treatment group after this time could not be assessed though would be of significant clinical interest. It is also unknown what benefit CPAP would deliver if initiated at a later time point or when the animals were exhibiting signs of poisoning.

5. Conclusions

This study has demonstrated that early use of CPAP following phosgene exposure significantly improved survival and arterial oxygenation, while also reducing markers of blood-air barrier dysfunction. Initiation at 1 h post exposure proved the concept that early intervention with positive airway pressure could reduce the effects of lung injury. Ambient air CPAP is a therapy which can be implemented using simple COTS equipment which might quickly be transitioned into use in a mass casualty event. As such it could provide a means by which large numbers of casualties could be more quickly and easily treated, thereby reducing the medical logistical burden. This suggests that CPAP might be an effective means of delaying respiratory deterioration and improving survival, thereby enabling evacuation to more definitive care.

Disclaimer

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Conflict of interest

None.

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References

Anand, A., Paintal, A.S., Whitteridge, D., 1986. Phosgene stimulates J-Receptors and produces increased respiratory drive in cats. J. Physiol.-London 371, P109.
Baudouin, S., Blumenthal, S., Cooper, B., Davison, A., Elliott, M., Kinnear, W., Paton, R., Sawicka, E., Turner, L., 2002. Non-invasive ventilaton in acute respiratory failure. Thorax 57, 192–211.
Borak, J., Diller, W.F., 2001. Phosgene exposure: mechanisms of injury and treatment strategies. J. Occup. Environ. Med. 43, 110–119.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brown, R.F., Jugg, B.J., Harban, F.M., Ashley, Z., Kenward, C.E., Platt, J., Hill, A., Rice, P., Watkins, P.E., 2002. Pathophysiological responses following phosgene exposure in the anaesthetized pig. J. Appl. Toxicol. 22, 263–269.
Burns, D., West, T.A., Hawkins, K., O'Keefe, G.E., 2001. Immediate effects of positive end-expiratory pressure and low and high tidal volume ventilation upon gas exchange and compliance in patients with acute lung injury. J. Trauma 51, 1177–1181.
Diller, W.F., 1985. Pathogenesis of phosgene poisoning. Toxicol. Ind. Health 1, 7–15.
Duncan, A.W., Oh, T.E., Hillman, D.R., 1986. PEEP and CPAP. Anaesth. Intensive Care 14, 236–250.
Edwards, J.D., Shoemaker, W.C., Vincent, J.L., 1993. Oxygen Transport: Principles and Practice. W B Saunders, London.
Giles, K., 2015. Adherence to CPAP in obstructive sleep apnoea. Nurs. Times 111, 23.
Grainge, C., Rice, P., 2010. Management of phosgene-induced acute lung injury. Clin. Toxicol. 48, 497–508.
Grainge, C., Brown, R., Jugg, B.J., Smith, A.J., Mann, T.M., Jenner, J., Rice, P., Parkhouse, D.A., 2009. Early treatment with nebulised salbutamol worsens physiological measures and does not improve survival following phosgene induced acute lung injury. J. R. Army Med. Corps 155, 105–109.
Grainge, C., Jugg, B.J., Smith, A.J., Brown, R.F., Jenner, J., Parkhouse, D.A., Rice, P., 2010a. Delayed low-dose supplemental oxygen improves survival following phosgene-induced acute lung injury. Inhal. Toxicol. 22, 552–560.
Grainge, C., Smith, A.J., Jugg, B.J., Fairhall, S.J., Mann, T., Perrott, R., Jenner, J., Millar, T., Rice, P., 2010b. Furosemide in the treatment of phosgene induced acute lung injury. J. R. Army Med. Corps 156, 245–250.
Hockel, K., Trabold, R., Scholler, K., Torok, E., Plesnila, N., 2012. Impact of anesthesia on pathophysiology and mortality following subarachnoid hemorrhage in rats. Exp. Transl. Stroke Med. 5 (4), 1–9.
Jaskot, R.H., Grose, E.C., Richards, J.H., Doerfler, D.L., 1991. Effects of inhaled phosgene on rat lung antioxidant systems. Fundam. Appl. Toxicol. 17, 666–674.
Jugg, B., Fairhall, S., Smith, A., Rutter, S., Mann, T., Perrott, R., Jenner, J., Salguero, J., Shute, J., Sciuto, A.M., 2013. N-acetyl-L-cysteine protects against inhaled sulfur mustard poisoning in the large swine. Clin. Toxicol. 51, 216–224.
Jugg, B., Hoard-Fruchey, H.M., Rothwell, C.C., Dillman, J., David, J., Jenner, J., Sciuto, A.M., 2016. Acute gene expression profile of lung tissue following sulfur mustard inhalation exposure in large anaesthetized swine. Chem. Res. Toxicol. 29, 1602–1610.
Jugg, B., 2016. Toxicology and treatment of phosgene induced lung injury. In: In: Worek, F., Jenner, J., Thiermann, H. (Eds.), Chemical Warfare Toxicology, vol. 1. Royal Society of Chemistry, London, pp. 117–153 (Fundamental aspects).
Li, W.L., Hai, C.X., Pauluhn, J., 2011. Inhaled nitric oxide aggravates phosgene model of acute lung injury. Inhal. Toxicol. 23, 842–852.
Li, W., Liu, F., Wang, C., Truebel, H., Pauluhn, J., 2013. Novel insights into phosgene-induced acute lung injury in rats: role of dysregulated cardiopulmonary reflexes and nitric oxide in lung edema pathogenesis. Toxicol. Sci. 131, 612–628.
Li, W.L., Rosenbruch, M., Pauluhn, J., 2015. Effect of PEEP on phosgene-induced lung edema: pilot study on dogs using protective ventilation strategies. Exp. Toxicol. Pathol. 67, 109–116.
Marrs, T.C., Maynard, R.J., Sidell, F.R., 1996. Phosgene. Chemical Warfare Agents: Toxicology and Treatment. John Wiley (p. 185).
Mattera, C.J., 2010. Physiology explains CPAP's effectiveness. J. Emerg. Med. Serv.
Mols, G., Priebe, H.-J., Guttmann, J., 2006. Alveolar recruitment in acute lung injury. Br. J. Anaesth. 96, 156–166.
Paintal, A.S., 1969. Mechanism of stimulation of type: j pulmonary receptors. J. Physiol. 203, 511–532.
Parkhouse, D.A., Brown, R.F., Jugg, B.J., Harban, F.M., Platt, J., Kenward, C.E., Jenner, J., Rice, P., Smith, A.J., 2007. Protective ventilation strategies in the management of phosgene-induced acute lung injury. Mil. Med. 172, 295–300.
Putensen, C., Wrigge, H., 2004. Clinical review: biphasic positive airway pressure and airway pressure release ventilation. Crit. Care 8, 492–497.
Sciuto, A.M., Hurt, H.H., 2004. Therapeutic treatments of phosgene-induced lung injury. Inhal. Toxicol. 16, 565–580.
Sciuto, A.M., Moran, T.S., 2001. Effect of dietary treatment with n-propyl gallate or vitamin E on the survival of mice exposed to phosgene. J. Appl. Toxicol. 21, 33–39.
Smith, A., Brown, R., Jugg, B., Platt, J., Mann, T., Masey, C., Jenner, J., Rice, P., 2009. The effect of steroid treatment with inhaled budesonide or intravenous methylprednisolone on phosgene-Induced acute lung injury in a porcine model. Mil. Med. 174, 1287–1294.
Ware, L.B., Matthay, M.A., 2000. The acute respiratory distress syndrome. N. Engl. J. Med. 342, 1334–1349.
Wild, M.R., Alagesan, K., 2001. PEEP and CPAP. Br. J. Anaesth. 1, 89–92.

Phosgene use in World War 1 and early evaluations of pathophysiology

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ABSTRACT
World War 1 ended 100 years ago. The aftermath included the consolidation of significant advances in medical care of casualties. Some of these advances were made in the care of chemical casualties, in particular the mechanisms of toxicity and treatment of phosgene exposure. Phosgene, or carbonyl chloride, is an extremely poisonous vapour that was used to devastating effect during World War 1. Observations made of acutely poisoned casualties formed the basis of much research in the early post-World War 1 era. Some extremely elegant experiments, some at the nascent Porton Down research facility, further evaluated the toxin and defences against it. Researchers drew on knowledge that was later forgotten and has since been relearnt later in the 20th century and made many correct assumptions. Their work is the bedrock of our understanding of phosgene toxicity that survives to this day. The horrors of chemical warfare prompted the Geneva Protocol of 1925, prohibiting the use of chemical agents in warfare, and chemical warfare on this scale has not been repeated. The ease with which phosgene can be synthesised requires healthcare providers to be familiar with its effects.

Key messages

- Phosgene is an extremely toxic vapour that is easily manufactured. Healthcare workers should be aware of its effects.
- Phosgene was used as a chemical weapon in World War 1 where much of the clinical effects including delayed death, were described.
- In the aftermath of World War 1, tremendous work was done in evaluating thepathophysiology of phosgene with some extremely elegant experiments.

PHOSGENE DISCOVERY AND USES

Phosgene was first synthesised by John Davy[1] by exposing equal volumes of carbon monoxide and chlorine to sunshine for 15 min. He noted that the mixture contracted to half the original volume and the chlorine colour disappeared to form a colourless gas. He named it phosgene, from the Greek φως, phos meaning light, and γνομαι, gene meaning to produce,[1] born of light. Its odour was recklessly described thus:

> Thrown into the atmosphere, it did not fume. Its odour was different from that of chlorine, something like that which one might imagine would result from the smell of chlorine combined with that of ammonia, yet more intolerable and suffocating than chlorine itself, and affecting the eyes in a peculiar manner, producing a rapid flow of tears and occasioning painful sensations.

The odour has been variously described as of green corn or musty hay,[2] or as new mown hay.

Phosgene is a highly reactive compound with the formula $COCl_2$. It is 3.5 times denser than air, with a boiling point of 7.4°C and a critical temperature of 182°C.[3] It exists as a vapour at normal temperature and pressure.

Kirby Jackson[4] described four chief methods for producing phosgene:

1. The photochemical combination of carbon monoxide and chlorine.
2. The oxidation of chlorinated hydrocarbons with chromic acid.
3. The interaction of sulfur trioxide or oleum with chlorinated hydrocarbons.
4. The combination of carbon monoxide and chlorine in the presence of a solid catalyst.

It is also produced in the thermal decomposition of certain chlorinated hydrocarbons.[5 6]

Phosgene is widely used commercially in the production of many chemical compounds. It reacts with a multitude of nitrogen, oxygen, sulfur and carbon centres, as well as with a variety of other inorganic compounds, by acylation, chlorination, decarboxylation and dehydration. It is an important chemical intermediate in many manufacturing processes.[7] The majority of phosgene is used in the production of isocyanates for the synthesis of polyurethanes; other uses are in the production of polycarbonates, and chloroformates used to make pharmaceuticals and pesticides.[8]

PHOSGENE IN WORLD WAR 1

Gas attacks in World War 1 began on 22 April 1915 with the release of chlorine from vast arrays of cylinders, such as those in Figure 1, by the Germans. Phosgene was used to devastating effect during World War 1. The first recorded use was at Ypres on 19 December 1915 in combination with chlorine. Allied intelligence was such that respirators had been developed containing cotton impregnated with sodium phenolate, and later sodium phenolate in combination with hexamethylenetetramine.[2] However the troops at Ypres on that occasion were inadequately protected. The Allies had adopted gas warfare in response to repeated German gas attacks by the end of September 1915, and this included the development of personal protective equipment (PPE) and tactics, techniques and procedures. Thus the gas attacks became less effective as time went on.[2 9] Because of its relatively high boiling point of 7.4°C, phosgene could not be

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Figure 1 A typical cylinder release gas attack. It was possible to use 3000 cylinders across a front of 3000 yards.[9] https://commons.wikimedia.org/wiki/File:Poison_gas_attack.jpg.

used alone in cylinders and was usually combined with chlorine in a ratio of 1:3 or 1:1. It could be used as a sole agent in large projected shells, sometimes adsorbed into pumice.

The Germans had an established industrial use for phosgene in the manufacture of dyes.[2] Chlorine had established peacetime uses but carbon monoxide had not. It became necessary for large quantities of carbon monoxide to be produced in order to manufacture phosgene. The Germans and Americans favoured reacting carbon dioxide with carbon, usually charcoal, but the French and the British used incomplete combustion of coke. All sides combined chlorine and carbon monoxide passing over a carbon catalyst and then dried before storage.[2]

Accurate meteorology was vital for the deployment of phosgene. Aside from the obvious wind direction and chance of change, the presence of water in the atmosphere would greatly diminish the effectiveness of phosgene. Phosgene reacts with water to produce carbon dioxide and hydrochloric acid:

$$COCl_2 + H_2O \rightarrow CO_2 + 2HCl$$

This reaction was exploited in early PPE by keeping it moist, in addition to the countermeasures discussed above. The last gas cloud attack occurred on 8 August 1916 and attention was focused on firing shells, including ones containing phosgene.[9] In April 1917 the British introduced the projector which could fire a drum of pressurised liquid into enemy territory where it would rupture and leak large quantities of vapour.[9]

Vedder[10] describes the symptoms of phosgene poisoning as quite different from other agents. Even at high concentrations the inhalation does not cause irritation of the upper airways. Immediately following exposure, the victim is largely asymptomatic. Some soldiers reported a change in the taste of cigarettes to an unpleasant flavour, often reminiscent of rotten eggs.[11 12] Later, and often after exertion, victims develop dyspnoea, which is often followed by cyanosis and death.[10] Many authors subdivide cases of phosgene-induced pulmonary oedema into two groups[10 13 14]:

1. Venous engorgement with cyanosis. Congested plum to blue face with tachypnoea and increased respiratory excursion. These patients may be coughing up large volumes of pulmonary oedema. Pulse is typically 100 beats per minute.
2. Grey pallor. These patients are collapsed with ashen, grey lips. Their breathing is rapid and shallow, and pulse may be up to 140 beats per minute. Cough is often absent.

Most phosgene casualties are in group 2, and those in group 1 can progress to group 2. Severe cases in either group exhibit extreme anxiety, reduced level of consciousness or delirium.[13] Survivors often have no recollection of their illness, even those who held normal conversations at the Casualty Clearing Station.[14]

Physiologist John Scott Haldane witnessed first hand the effects of phosgene and delivered a lecture on the subject at the Royal Army Medical College on 8 October 1919[14]. He described multiple stupefied casualties in respiratory distress, with deeply cyanosed, plum-coloured lips and distended neck veins. One of the casualties died while he was visiting a clearing station, and a postmortem was immediately carried out by a Dr McNee. *'The lungs were voluminous and much congested. Albuminous liquid could be squeezed from them in abundance. The bronchi and alveoli were inflamed, and a great deal of emphysema was present'*. He concluded that the cyanosis was due to anoxaemia and the distended neck veins secondary to an increase in pulmonary vascular resistance and distension of the right side of the heart. He noticed the characteristic dose response to phosgene, whereby a large inhalation causes immediate severe signs and symptoms, often resulting in death. At lower doses, exposure to a low concentration for a prolonged period or a relatively high concentration for a short duration, the signs and symptoms are delayed by hours and often precipitated by exertion. He goes on to describe how the administration air with supplemental oxygen titrated to effect can reverse the cyanosis. It did not, however, reverse the tachypnoea, which he surmised was driven by arterial carbon dioxide content. Oxygen was recommended in a dose of 2 L/min, and up to 5 L/min in severe cases. Both Haldane and Galwey[15] recognised the toxicity of oxygen in higher concentrations, and Haldane's[16] oxygen apparatus could deliver oxygen with entrained air up to 10 L/min,[16] although the logistic burden must have been considerable. Venesection was another treatment that proved effective in cases where venous congestion was prominent. Galwey[15] states that the intervention is to reduce right heart strain and is achieved by the removal of 650 mL blood over 20 min. This removal reduces capillary pressure, causing uptake of interstitial fluid from the peripheries, possibly including the lungs and bringing the haematocrit closer to normal. He thought that the rise in haematocrit phosgene causes could be ameliorated by the intravenous injection of saline.

Vedder recognised that it was difficult to determine the pathology of phosgene in human because when weaponised it was commonly mixed with chlorine. However he reported that lung weights were increased, with the right lung typically weighing 1000 g and the left 875 g, and often cover the heart when the chest is opened.[10] The lungs were grossly abnormal with dark discolouration and exuding frothy serous fluid when sectioned, and although full of this frothy serous fluid, the upper airways exhibited little or no inflammatory change. Fluid from brown discoloured lung was acidic when tested with litmus paper, and the right heart was generally dilated and often with petechial haemorrhages beneath the endocardium.

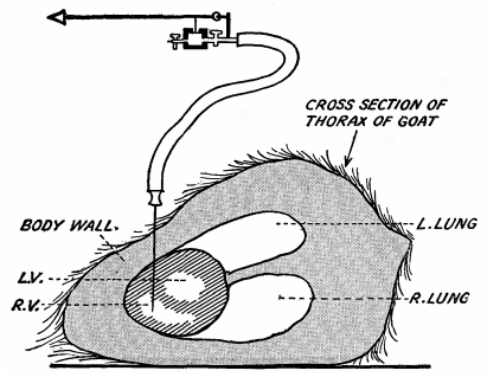

Figure 2 Schematic produced by Barcroft illustrating the method of recording intracardiac pressure by direct puncture developed in conjunction with Barcroft.[20]

Many of the organs show venous congestion. On microscopic examination of the alveoli, he noticed that they are full of fluid with desquamated alveolar epithelium, white cells and red cells. Fibrin was observed crossing the alveoli and the capillary beds. He concluded that this was the basis for obstruction of the pulmonary circulation and right heart strain, and therefore a mechanism for generalised venous engorgement. *'Thus the important acute changes caused by phosgene poisoning are practically limited to the lungs'*.[10]

Further inflammatory changes are noted with disease progression, with patches of bronchopneumonia. The majority of cases recover if they survive the first 48 hours.[10]

By the end of World War 1, there were 180 983 British *'gas casualties'*, including 6062 deaths. These official figures are a gross underestimate; records begin in 1916 and they do not include the missing or captured.[17] These are victims of chlorine, phosgene and mustard. Of the three, total phosgene production was the smallest, yet it was responsible for more devastation, completely out of proportion to the other agents.

EARLY EVALUATIONS OF THE PATHOPHYSIOLOGY OF PHOSGENE

It has long been recognised that phosgene acts primarily at the alveolar level. Hill[18] showed in a series of elegant experiments that the poisoned lung, homogenised, extracted with Ringer's solution and injected into a healthy animal, caused no adverse effect. Likewise the oedema fluid of poisoned animals injected into healthy subjects and plasma through which phosgene has been bubbled produced no adverse effects. He went a stage further and showed that by isolating a cat lung in vivo using a bronchial blocker, and then allowing the anaesthetised animal to breathe phosgene, only the exposed lung suffered the effects. Cyanosis and cardiovascular variables returned to baseline once the animal breathed air on removal of the blocker. Thus Hill[18] demonstrated that phosgene acts only locally with no *direct* systemic effects. It was well known that the loss of oedema fluid was significant to cause a rise in haematocrit, and Hill[18] suggested that this could be corrected by the intravenous infusion of gum saline. Gum saline is a solution of gum acacia of 6%–7% in 0.9% saline and acts as a colloid.[19] This was rejected as a therapy on the grounds that it may worsen pulmonary oedema. Hill[18] was unable to explain the selective alveolar damage and wrongly assumed that this damage was caused by hydrochloric acid formed by the reaction of phosgene with water.

Barcroft[20] summarised his work on the pathophysiology of phosgene poisoning in a lecture given at the Royal Army Medical College in 1919. His work was based on animal experiments conducted at the Royal Engineers' Experimental Ground at Porton. His chamber experiments were the first large-scale exposures to known concentrations of phosgene vapour. Histological examination showed that increased lung damage occurs with increasing doses, as expected. Macroscopic changes were not uniform, and the microscopic changes included damage to the alveolar epithelium, causing oedema formation and capillary changes including thrombosis. Although hypoxic pulmonary vasoconstriction was first recognised in 1894,[21] it was not eponymised as the Euler-Liljestrand mechanism until 1946.[22 23] Barcroft[20] observed normoxic goats with lungs four times the normal weight. Rather than using hypoxic pulmonary vasoconstriction as the explanation, Barcroft[20] first assumed that that oedematous alveoli were not perfused as a result of capillary thrombi. After developing an in vitro model to test the effects of raised pulmonary vascular resistance, he went on to test his hypothesis in vivo. By introducing a needle into the right ventricle of a goat, it was possible to measure and trace the pressure transmitted via the needle (Figure 2).

These goats were then exposed to phosgene, and he observed the typical findings illustrated in Figure 3. Soon after the animals were exposed to phosgene, respiratory rate and right ventricular pressure increase, in line with the anticipated increase in pulmonary vascular resistance. Later in the experiments the right ventricular systolic pressure normalises; however, the diastolic pressures are much lower, reflecting hypovolaemia. Given that the pulse pressures remain similar, postexposure, the increased pulmonary vascular resistance is likely to persist. Vedder[10] noted that a man's lungs could contain up to 2 kg of oedema, yet Barcroft paid little attention to the effects of hypovolaemia in his analysis and made no measurement of hypoxaemia to correlate with minute volume, linking minute volume with right ventricular systolic pressure. He went on to show that in experiments of oil or starch embolism, there is a transient rise in right ventricular pressure, but minute volume would either increase or decrease. To further evaluate the relationship between minute volume and right ventricular pressure, he showed that right ventricular pressure falls during an increase in minute volume in the exercising goat.

While he recognised that there is a vasomotor component to phosgene lung injury, Barcroft summarised that the pulmonary capillaries are compressed by oedema fluid in the alveoli. On exertion these capillaries dilate under vasomotor influences, creating a large physiological shunt that worsens hypoxaemia. He demonstrated this in a series of experiments using oximetry of blood in phosgene-exposed goats at rest and during exercise, unfortunately not linking the data to right ventricular pressure.

ANALYSIS OF EARLY CONCLUSIONS

The classical descriptions of early and late effects were made during World War 1. Their importance to the management of a large number of casualties persists to this day. Barcroft's correct observation of increased pulmonary vascular resistance was not underpinned by the hypoxic pulmonary vasoconstriction

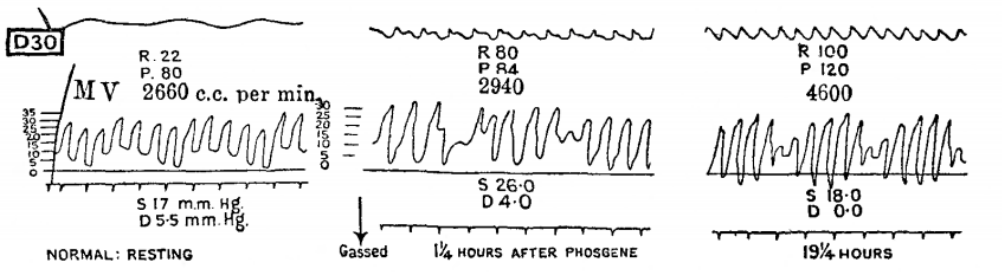

Figure 3 Tracings of respiratory rhythm above the right ventricular pressure pre-exposure and postexposure to phosgene (1.25 and 19.25 hours postexposure). MV, minute volume; P, pulse rate; R, respiratory rate. The y-axis is the right ventricular pressure measured in cmH_2O. D, mean diastolic pressure expressed in mm Hg; S, mean systolic pressure expressed in mm Hg. The bottom scale is time, measured in seconds.

mechanism to minimise shunt. However the sudden collapse of patients yet to experience severe intoxication is reasonably explained by his hypothesis that it is pulmonary vasodilation known to occur on exertion that is the causative factor.

Many have classified the casualties into groups 1 (venous engorgement with cyanosis) and 2 (grey pallor), and this warrants further discussion. It was known that hypoxia and increased arterial carbon dioxide content will increase respiratory rate, and there was considerable debate over the relative contributions made by hypoxia and carbon dioxide, including the thought that elevated carbon dioxide contributed to the venous engorgement by contributing to vasodilation. Arterial carbon dioxide was not measured, and elevated carbon dioxide seems unlikely when considering the alveolar air equation:

$$PA_{O2} = PI_{O2} - (PA_{CO2} / R)$$

Alveolar oxygen is calculated by the partial pressure of inspired oxygen; note that it is less than atmospheric after humidification, with the partial pressure of alveolar carbon dioxide subtracted and divided by the respiratory quotient (R). Thus, alveolar oxygen can be increased by reducing the alveolar carbon dioxide, most simply by hyperventilation. In fact the explanation may be even simpler. Group 1 casualties are yet to experience hypovolaemia secondary to fluid losses from extreme pulmonary oedema; their veins are engorged by increased pulmonary vascular resistance and they appear cyanosed from hypoxaemia. Group 2 casualties have significant fluid losses as evidenced by their rapid HR and pallor from vasoconstriction and appear grey because they are cyanosed. It has been stated that a patient's disease may progress from group 1 to 2, and this is likely due to circulatory fluid loss. For all his astonishing work, Barcroft neglected to note the reduced and often transiently negative diastolic pressures measured in severely intoxicated goats.

Upper airway sparing and Hill's work elegantly demonstrated that phosgene is decomposed by lungs, limiting its effect to that organ system. The cardiac effects follow from that.

CONCLUSIONS

In this article we have seen the maleficent adoption of chemical warfare by German forces in World War 1 and the Allied response. PPE and other protective techniques employed have not been dwelt on in detail; however, these were quickly developed by the Allies. The Allied combination of superior PPE and the rapid development of their own chemical arsenal and delivery methods meant that towards the end, the Germans had been hoisted by their own petard by launching a chemical war. Many of the senior commanders in World War 2 had at least indirect experience of chemical warfare; its hideous nature discouraged them and politicians from bringing it to bear a second time.

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REFERENCES

1. Davy J. On a gaseous compound of carbonic oxide and chlorine. *Philos Trans R Soc Lond* 1812;102:144–51.
2. Fries AA, West CJ. *Chemical warfare*. 1st edn. 370 Seventh Avenue, NEW YORK and 6 & 8 Bouverie Street, LONDON EC4: McGraw-Hill Book Company, 1921: 445.
3. BOC. *Safety data sheet: phosgene MSDS Nr: 331-00-0002BOC(U)*. BOC, Priestley Road, Worsley, Manchester M28 2UT: British Oxygen Company, 2000.
4. Jackson KE. Phosgene. *Journal of Chemical Education* 1933;10:622–6.
5. Brown JE, Birky MM. Phosgene in the thermal decomposition products of poly(vinyl chloride): generation, detection and measurement. *J Anal Toxicol* 1980;4:166–74.
6. Wyatt JP, Allister CA. Occupational phosgene poisoning: a case report and review. *J Accid Emerg Med* 1995;12:212–3.
7. Babad H, Zeiler AG. Chemistry of phosgene. *Chem Rev* 1973;73:75–91.
8. Pyo S-H, Persson P, Mollaahmad MA, *et al*. Cyclic carbonates as monomers for phosgene- and isocyanate-free polyurethanes and polycarbonates. *Pure and Applied Chemistry* 2011;84:637–61.
9. Galwey WR. Gas Warfare: Adoption: Methods of Use: Protection of Troops. *J R Army Med Corps* 1922;38:62–72.
10. Vedder EB. *The medical aspects of chemical warfare*. Waverly Press, Baltimore MD: Williams & Wilkins Company, 1925: 327.
11. Ward C. *Living on the western front: annals and stories, 1914-1919*. Bloomsbury Academic, 2013.
12. Barnes D, Cane B. *it made you think of home: the haunting journal of deward barnes, canadian expeditionary force, 1916-1919*. Dundurn, 2004.
13. Galwey WR. Gas Warfare: Lecture II: Effects of poisonous gases- early and late. *J R Army Med Corps* 1922;38:144–54.
14. Haldane JS. Lung-irritant gas poisoning and its sequelæ. *Journal of the Royal Army Medical Corps* 1919;33:494–507.
15. Galwey WR. Gas warfare: lecture iii: the treatment of gas casualties- early and late. *J R Army Med Corps* 1922;38:223–34.
16. Haldane JS. The therapeutic administration of oxygen. *Br Med J* 1917;1:181–3.
17. Harris R. *Paxman J. A higher form of killing*. New York: Random House, 2002.

18. Hill L. Lecture on the pathology of war poison gases. *J R Army Med Corps* 1920;35:334–42.
19. Bayliss WM. The action of gum acacia on the circulation. *Journal of Pharmacology and Experimental Therapeutics* 1920;15:29–74.
20. Barcroft J. Some problems of the circulation during gas poisoning. *J R Army Med Corps* 1920;34:155–73.
21 Bradford JR, Dean HP. The pulmonary circulation. *J Physiol* 1894; 16:34–158.
22. USv E, Liljestrand G. Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiologica Scandinavica* 1946;12:301–20.
23. Dunham-Snary KJ, Wu D, Sykes EA, *et al*. Hypoxic pulmonary vasoconstriction: from molecular mechanisms to medicine. *Chest* 2017;151:181–92.

REFERENCES

1. BOC. Safety Data Sheet: Phosgene MSDS Nr: 331-00-0002BOC(U). BOC, Priestley Road, Worsley, MANCHESTER M28 2UT: British Oxygen Company; 2000.

2. Davy J. On a gaseous compound of carbonic oxide and chlorine. Phil Trans R Soc Lond. 1812;102:144-51.

3. Fries AA, West CJ. Chemical Warfare. First Edition ed. 370 Seventh Avenue, NEW YORK and 6 & 8 Bouverie Street, LONDON EC4: McGraw-Hill Book Company Inc; 1921. 445 p.

4. Cowell E. CHEMICAL WARFARE AND THE DOCTOR—I. British Medical Journal. 1939;2(4109):736.

5. Jackson KE. Phosgene. Journal of Chemical Education. 1933;10(10):622- 6.

6. Brown JE, Birky MM. Phosgene in the Thermal Decomposition Products of Poly (Vinyl Chloride): Generation, Detection and Measurement. Journal of Analytical Toxicology. 1980;4:166- 74.

7. Wyatt JP, Allister CA. Occupational phosgene poisoning: a case report and review. Emergency Medicine Journal. 1995;12(3):212-3.

8. Babad H, Zeiler A. The Chemistry of Phosgene. Chemical Reviews. 1973;73(1):75- 91.

9. Pyo S-H, Persson P, Mollaahmad MA, Sörensen K, Lundmark S, Hatti-Kaul R. Cyclic carbonates as monomers for phosgene- and isocyanate-free polyurethanes and polycarbonates. Pure and Applied Chemistry. 2011;84(3).

10. Galwey WR. Gas Warfare: Adoption: Methods of Use: Protection of Troops. Journal of the Royal Army Medical Corps. 1922;38(1):62-72.

11. Ward C. Living on the Western Front: Annals and Stories, 1914-1919: Bloomsbury Academic; 2013.

12. Barnes D, Cane B. It Made You Think of Home: The Haunting Journal of Deward Barnes, Canadian Expeditionary Force, 1916-1919: Dundurn; 2004.

13. Vedder EB. The Medical Aspects of Chemical Warfare. Waverly Press, Baltimore MD: Williams & Wilkins Company; 1925. 327 p.

14. Galwey WR. Gas Warfare: Lecture II: Effects of poisonous gases- early and late. Journal of the Royal Army Medical Corps. 1922;38(2):144-54.

15. Haldane JS. Lung-Irritant Gas Poisoning and Its Sequelæ. Journal of the Royal Army Medical Corps. 1919;33(6):494-507.

16. Galwey WR. Gas Warfare: Lecture III: The treatment of gas casualties- early and late. Journal of the Royal Army Medical Corps. 1922;38(3):223-34.

17. Haldane JS. The therapeutic administration of oxygen. BMJ. 1917;i:181-3.

18. Harris R, Paxman J. A Higher Form of Killing. New York: Random House; 2002.

19. Hill L. Lecture on the Pathology of War Poison Gases. Journal of the Royal Army Medical Corps. 1920;35(4):334-42.

20. Bayliss WM. The Action of Gum Acacia on the Circulation. Journal of Pharmacology and Experimental Therapeutics. 1920;15(1):29-74.

21. Barcroft J. Some Problems of the Circulation during Gas Poisoning. Journal of the Royal Army Medical Corps. 1920;34(2):155-73.

22. Bradford JR, Dean HP. The Pulmonary Circulation. The Journal of Physiology. 1894;16(1-2):34-158.

23. Euler USv, Liljestrand G. Observations on the Pulmonary Arterial Blood Pressure in the Cat. Acta Physiologica Scandinavica. 1946;12(4):301-20.

24. Dunham-Snary KJ, Wu D, Sykes EA, Thakrar A, Parlow LR, Mewburn JD, et al. Hypoxic Pulmonary Vasoconstriction: From Molecular Mechanisms to Medicine. Chest. 2017;151(1):181-92.

25. Medical manual of chemical warfare, 1939: His Majesty's Stationery Office; 1939 0, 1940. 104+15 p.

26. West JB. Respiratory physiology: the essentials. 6 ed: Lippincott Williams & Wilkins; 2000.

27. von Neergaard K. Neue Auffassungen uber einen Grundbegriff der Atemmehanik: die Retraktionskraft der Lunge, abhagig von der Oberflachenspannung in den Alveolen. Z Gesamte Exp Med. 1929;66:373-94.

28. Obladen M. History of surfactant up to 1980. Biol Neonate. 2005;87(4):308-16.

29. Pattle RE. Properties, Function and Origin of the Alveolar Lining Layer. Nature. 1955;175(4469):1125-6.

30. Pattle R. Surface lining of lung alveoli. Physiological reviews. 1965;45(1):48-79.

31. Avery M, Mead J. Surface properties in relation to atelectasis and hyaline membrane disease. AMA Journal of Diseases of Children. 1959;97(5_PART_I):517-23.

32. Pattle R. lung surfactant: an introduction. J Reprod Fertil, Suppl. 1975.

33. P Haagsman H, V Diemel R. Surfactant-associated proteins: Functions and structural variation2001. 91-108 p.

34. Quintero OA, Wright JR. Metabolism of phosphatidylglycerol by alveolar macrophages in vitro. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2000;279(2):L399-L407.

35. Hagen FS, Grant FJ, Kuijper JL, Slaughter CA, Moomaw CR, Orth K, et al. Expression and characterization of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides. Biochemistry. 1991;30(34):8415-23.

36. W. Glasser S. Surfactant Protein-C in the Maintenance of Lung Integrity and Function. Journal of Allergy & Therapy. 2012;01(S7).

37. Harvey R, Ferrier D. Biochemistry (Lippincott's Illustrated Reviews Series). 5th ed: Lippincott Williams & Wilkins, Baltimore, MD and Philadelphia, PA, USA; 2011.

38. Calianese DC, Birge RB. Biology of phosphatidylserine (PS): basic physiology and implications in immunology, infectious disease, and cancer. Cell Commun Signal. 2020;18(1):41.

39. Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Murphy RC, et al. A comprehensive classification system for lipids. Journal of Lipid Research. 2005;46(5):839-62.

40. Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CR, Shimizu T, et al. Update of the LIPID MAPS comprehensive classification system for lipids. J Lipid Res. 2009;50 Suppl:S9-14.

41. Postle A, L Heeley E, C Wilton D. A comparison of the molecular species compositions of mammalian lung surfactant phospholipids2001. 65-73 p.

42. Body DR. The phospholipid composition of pig lung surfactant. Lipids. 1971;6(9):625.

43. Andreeva AV, Kutuzov MA, Voyno-Yasenetskaya TA. Regulation of surfactant secretion in alveolar type II cells. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2007;293(2):L259-L71.

44. Alcorn JL. Pulmonary surfactant trafficking and homeostasis. Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease: Elsevier; 2017. p. 59-75.

45. Moessinger C, Klizaite K, Steinhagen A, Philippou-Massier J, Shevchenko A, Hoch M, et al. Two different pathways of phosphatidylcholine synthesis, the Kennedy Pathway and the Lands Cycle, differentially regulate cellular triacylglycerol storage. BMC Cell Biol. 2014;15:43.

46. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res. 2008;49(6):1187-94.

47. Walkey CJ, Donohue LR, Bronson R, Agellon LB, Vance DE. Disruption of the murine gene encoding phosphatidylethanolamine N-methyltransferase. Proc Natl Acad Sci U S A. 1997;94(24):12880-5.

48. Vance DE, Vance JE. Phospholipid biosynthesis in eukaryotes. Biochemistry of lipids, lipoproteins and membranes: Elsevier; 2008. p. 213-44.

49. Gibellini F, Smith TK. The Kennedy pathway--De novo synthesis of phosphatidylethanolamine and phosphatidylcholine. IUBMB Life. 2010;62(6):414-28.

50. Kennedy EP, Weiss SB. The Function of Cytidine Coenzymes in the Biosynthesis of Phospholipides. Journal of Biological Chemistry. 1956;222(1):193-214.

51. Ecker J, Liebisch G. Application of stable isotopes to investigate the metabolism of fatty acids, glycerophospholipid and sphingolipid species. Prog Lipid Res. 2014;54:14-31.

52. Hishikawa D, Shindou H, Kobayashi S, Nakanishi H, Taguchi R, Shimizu T. Discovery of a lysophospholipid acyltransferase family essential for membrane asymmetry and diversity. Proc Natl Acad Sci U S A. 2008;105(8):2830-5.

53. Chevalier G, Collet AJ. In vivo incorporation of choline-3H, leucine-3H and galactose-3H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. The Anatomical Record. 1972;174(3):289-310.

54. Osanai K, Mason RJ, Voelker DR. Pulmonary surfactant phosphatidylcholine transport bypasses the brefeldin A sensitive compartment of alveolar type II cells. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2001;1531(3):222-9.

55. Osanai K, Tsuchihara C, Hatta R, Oikawa T, Tsuchihara K, Iguchi M, et al. Pulmonary surfactant transport in alveolar type II cells. Respirology. 2006;11 Suppl:S70-3.

56. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell 4th edn (New York: Garland Science). 2002:629- 31.

57. Nogee LM, Demello DE, Dehner LP, Colten HR. Deficiency of Pulmonary Surfactant Protein B in Congenital Alveolar Proteinosis. New England Journal of Medicine. 1993;328(6):406-10.

58. Stahlman MT, Gray MP, Falconieri MW, Whitsett JA, Weaver TE. Lamellar Body Formation in Normal and Surfactant Protein B-Deficient Fetal Mice. Laboratory Investigation. 2000;80(3):395-403.

59. Veldhuizen RAW, Inchley K, Hearn SA, Lewis JF, Possmayer F. Degradation of surfactant-associated protein B (SP-B) during in vitro conversion of large to small surfactant aggregates. Biochemical Journal. 1993;295(1):141-7.

60. Günther A, Schmidt R, Feustel A, Meier U, Pucker C, Ermert M, et al. Surfactant Subtype Conversion Is Related to Loss of Surfactant Apoprotein B and Surface Activity in Large Surfactant Aggregates. American Journal of Respiratory and Critical Care Medicine. 1999;159(1):244-51.

61. Brackenbury AM, Malloy JL, McCaig LA, Yao LJ, Veldhuizen RAW, Lewis JF. Evaluation of alveolar surfactant aggregatesin vitroandin vivo. European Respiratory Journal. 2002;19(1):41-6.

62. Olmeda B, Martínez-Calle M, Pérez-Gil J. Pulmonary surfactant metabolism in the alveolar airspace: Biogenesis, extracellular conversions, recycling. Annals of Anatomy - Anatomischer Anzeiger. 2017;209:78-92.

63. Jacobs H, Jobe A, Ikegami M, Conaway D. The significance of reutilization of surfactant phosphatidylcholine. Journal of Biological Chemistry. 1983;258(7):4159-65.

64. Bates S. P63 (CKAP4) as an SP-A receptor: Implications for surfactant turnover. Cellular Physiology and Biochemistry. 2010;25(1):41-54.

65. Postle AD, Henderson NG, Koster G, Clark HW, Hunt AN. Analysis of lung surfactant phosphatidylcholine metabolism in transgenic mice using stable isotopes. Chem Phys Lipids. 2011;164(6):549-55.

66. Goss V, Hunt AN, Postle AD. Regulation of lung surfactant phospholipid synthesis and metabolism. Biochim Biophys Acta. 2013;1831(2):448-58.

67. Bernhard W, editor 82@ Regulation of Surfactant-Associated Phospholipid Synthesis and Secretion2017.

68. Agassandian M, Mallampalli RK. Surfactant phospholipid metabolism. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2013;1831(3):612-25.

69. Ashbaugh D, Boyd Bigelow D, Petty T, Levine B. ACUTE RESPIRATORY DISTRESS IN ADULTS. The Lancet. 1967;290(7511):319-23.

70. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, et al. Acute respiratory distress syndrome. Nat Rev Dis Primers. 2019;5(1):18.

71. Network ARDS, Brower R, Matthay M, Morris A, Schoenfeld D, Thompson B, et al. Ventilation with lower tidal volumes as compared with

traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. N Engl J Med. 2000;342(18):1301-8.

72. Gunther A, Siebert C, Schmidt R, Ziegler S, Grimminger F, Yabut M, et al. Surfactant alterations in severe pneumonia, acute respiratory distress syndrome, and cardiogenic lung edema. Am J Respir Crit Care Med. 1996;153(1):176-84.

73. Lange Y, Swaisgood M, Ramos B, Steck T. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. Journal of Biological Chemistry. 1989;264(7):3786-93.

74. Kobayashi T. Mapping trasmembrane distribution of sphingomyelin. Emerg Top Life Sci. 2023;7(1):31-45.

75. Nakos G, Kitsiouli EI, Lekka ME. Bronchoalveolar lavage alterations in pulmonary embolism. Am J Respir Crit Care Med. 1998;158(5 Pt 1):1504-10.

76. Calkovska A, Mokra D, Calkovsky V. Lung surfactant alterations in pulmonary thromboembolism. Eur J Med Res. 2009;14 Suppl 4:38-41.

77. Rooney SA, Young SL, Mendelson CR. Molecular and cellular processing of lung surfactant 1. The FASEB journal. 1994;8(12):957-67.

78. R. W. Wirtz H, G Dobbs L. Wirtz, H. R. W. & Dobbs, L. G. Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. Science 250, 1266-12691990. 1266-9 p.

79. Carroll Jr JL, McCoy DM, McGowan SE, Salome RG, Ryan AJ, Mallampalli RK. Pulmonary-specific expression of tumor necrosis factor-α alters surfactant lipid metabolism. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2002;282(4):L735-L42.

80. Dushianthan A, Goss V, Cusack R, Grocott MP, Postle AD. Altered molecular specificity of surfactant phosphatidycholine synthesis in patients with acute respiratory distress syndrome. Respir Res. 2014;15:128.

81. Uphoff A, Hermansson M, Haimi P, Somerharju P. Analysis of complex lipidomes. Medical Applications of Mass Spectrometry: Elsevier; 2008. p. 223-49.

82. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J Lipid Res. 2010;51(11):3299-305.

83. Quehenberger O, Dennis EA. The human plasma lipidome. N Engl J Med. 2011;365(19):1812-23.

84. Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. J Lipid Res. 2009;50(6):1015-38.

85. Wang B, Wu L, Chen J, Dong L, Chen C, Wen Z, et al. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. Signal Transduct Target Ther. 2021;6(1):94.

86. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest. 1955;34(9):1345-53.

87. Snider AJ, Orr Gandy KA, Obeid LM. Sphingosine kinase: Role in regulation of bioactive sphingolipid mediators in inflammation. Biochimie. 2010;92(6):707-15.

88. Dushianthan A, Cusack R, Grocott MPW, Postle AD. Abnormal liver phosphatidylcholine synthesis revealed in patients with acute respiratory distress syndrome. J Lipid Res. 2018;59(6):1034-45.

89. Triggiani M, De Marino V, de Crescenzo G, Marone G. Arachidonic acid remodeling in human inflammatory cells migrating to the lung in vivo. Int Arch Allergy Immunol. 1997;113(1-3):190-2.

90. Hanley SP. Prostaglandins and the lung. Lung. 1986;164(2):65-77.

91. Masclans JR, Bermejo B, Picó M, de Latorre FJ, Rodríguez-Roisin R, Planas M. [The prognostic value of eicosanoids in the acute respiratory distress syndrome]. Med Clin (Barc). 1999;112(3):81-4.

92. Murphy RC, Folco G. Lysophospholipid acyltransferases and leukotriene biosynthesis: intersection of the Lands cycle and the arachidonate PI cycle. J Lipid Res. 2019;60(2):219-26.

93. Potts A, Simon F, Gerard R. The mechanism of action of phosgene and diphosgene. Archives of biochemistry. 1949;24(2):329.

94. Nash T, Pattle RE. The absorption of phosgene by aqueous solutions and its relation to toxicity. Ann Occup Hyg. 1971;14(3):227-33.

95. Ghio AJ, Kennedy TP, Hatch GE, Tepper JS. Reduction of neutrophil influx diminishes lung injury and mortality following phosgene inhalation. Journal of Applied Physiology. 1991;71(2):657-65.

96. Pauluhn J. Phosgene inhalation toxicity: Update on mechanisms and mechanism-based treatment strategies. Toxicology. 2021;450:152682.

97. Lee LY. Respiratory sensations evoked by activation of bronchopulmonary C-fibers. Respir Physiol Neurobiol. 2009;167(1):26-35.

98. Li W, Liu F, Wang C, Truebel H, Pauluhn J. Novel insights into phosgene-induced acute lung injury in rats: role of dysregulated cardiopulmonary reflexes and nitric oxide in lung edema pathogenesis. Toxicol Sci. 2013;131(2):612-28.

99. Bruner H, Boche R, Gibbon M, McCarthy M. VI. Electrocardiographic Study of Heart and Effect of Vagotomy in Phosgene Poisoning. Proceedings of the society for experimental biology and medicine. 1948;68(2):279-81.

100. Frosolono MF, Currie WD. Response of the pulmonary surfactant system to phosgene. Toxicol Ind Health. 1985;1(2):29-35.

101. Jugg B, Jenner J, Rice P. The effect of perfluoroisobutene and phosgene on rat lavage fluid surfactant phospholipids. Human & experimental toxicology. 1999;18(11):659-68.

102. Lu Q, Huang S, Meng X, Zhang J, Yu S, Li J, et al. Mechanism of Phosgene-Induced Acute Lung Injury and Treatment Strategy. Int J Mol Sci. 2021;22(20).

103. Sciuto AM, Clapp DL, Hess ZA, Moran TS. The temporal profile of cytokines in the bronchoalveolar lavage fluid in mice exposed to the industrial gas phosgene. Inhal Toxicol. 2003;15(7):687-700.

104. Rendell R, Fairhall S, Graham S, Rutter S, Auton P, Smith A, et al. Assessment of N-acetylcysteine as a therapy for phosgene-induced acute lung injury. Toxicology letters. 2018;290:145-52.

105. McElroy CS, Day BJ. Antioxidants as potential medical countermeasures for chemical warfare agents and toxic industrial chemicals. Biochem Pharmacol. 2016;100:1-11.

106. Wijte D, Alblas MJ, Noort D, Langenberg JP, van Helden HP. Toxic effects following phosgene exposure of human epithelial lung cells in vitro using a CULTEX(R) system. Toxicol In Vitro. 2011;25(8):2080-7.

107. Grainge C, Rice P. Management of phosgene-induced acute lung injury. Clin Toxicol (Phila). 2010;48(6):497-508.

108. Diller WF. Early diagnosis of phosgene overexposure. Toxicol Ind Health. 1985;1(2):73-80.

109. Black RM. An overview of biological markers of exposure to chemical warfare agents. Journal of analytical toxicology. 2008;32(1):2-9.

110. Grainge C, Brown R, Jugg B, Smith A, Mann T, Jenner J, et al. Early treatment with nebulised salbutamol worsens physiological measures and does not improve survival following phosgene induced acute lung injury. Journal of the Royal Army Medical Corps. 2009;155(2):105-9.

111. Haldane J. A Lecture on the Symptoms, Causes, and Prevention of Anoxaemia (Insufficient Supply of Oxygen to the Tissues), and the Value of Oxygen in its Treatment. British medical journal. 1919;2(3055):65.

112. Bruner H, Boche RD, Chapple CC, Gibbon MH, McCARTHY MD. Studies on experimental phosgene poisoning. III. Oxygen therapy in phosgene-poisoned dogs and rats. The Journal of clinical investigation. 1947;26(5):936-44.

113. Grainge C, Jugg BJ, Smith AJ, Brown RF, Jenner J, Parkhouse DA, et al. Delayed low-dose supplemental oxygen improves survival following phosgene-induced acute lung injury. Inhal Toxicol. 2010;22(7):552-60.

114. Brown RFR, Jugg BJA, Harban FMJ, Ashley Z, Kenward C, Platt J, et al. Pathophysiological responses following phosgene exposure in the

anaesthetized pig. Journal of Applied Toxicology: An International Journal. 2002;22(4):263-9.

115. Parkhouse DA, Brown RF, Jugg BJ, Harban FM, Platt J, Kenward CE, et al. Protective ventilation strategies in the management of phosgene-induced acute lung injury. Military medicine. 2007;172(3):295-300.

116. Toxbase. Phosgene [website]. [Available from: https://www.toxbase.org/Poisons-Index-A-Z/P-Products/Phosgene/.

117. Weibel ER. Geometric and Dimensional Airway Models of Conductive, Transitory and Respiratory Zones of the Human Lung. Morphometry of the Human Lung. Berlin, Heidelberg: Springer Berlin Heidelberg; 1963. p. 136-42.

118. Maina JN, van Gils P. Morphometric characterization of the airway and vascular systems of the lung of the domestic pig, Sus scrofa: comparison of the airway, arterial and venous systems. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2001;130(4):781-98.

119. Nakakuki S. Bronchial tree, lobular division and blood vessels of the pig lung. J Vet Med Sci. 1994;56(4):685-9.

120. Judge EP, Hughes JM, Egan JJ, Maguire M, Molloy EL, O'Dea S. Anatomy and bronchoscopy of the porcine lung. A model for translational respiratory medicine. Am J Respir Cell Mol Biol. 2014;51(3):334-43.

121. Chiari H. Ueber einen neuen Typus von Missbildung an der Trachea des Menschen. Ziegler's Beiträge z path Anat u allgem Pathol. 1889;5.

122. Doolittle AM, Mair EA. Tracheal bronchus: classification, endoscopic analysis, and airway management. Otolaryngol Head Neck Surg. 2002;126(3):240-3.

123. Terry PB, Traystman RJ. The Clinical Significance of Collateral Ventilation. Annals of the American Thoracic Society. 2016;13(12):2251-7.

124. Takeda J, MacKenzie CF, Watson R, Roberts HGJ, Moorman R, Hoff BH, et al. Collateral ventilation of obstructed lung during high-frequency oscillation in dogs and pigs. Critical Care Medicine. 1987;15(9):858-62.

125. Borg T, Alvfors A, Gerdin B, Modig J. A porcine model of early adult respiratory distress syndrome induced by endotoxaemia. Acta anaesthesiologica scandinavica. 1985;29(8):814-30.

126. Wang H, Bodenstein M, Markstaller K. Overview of the pathology of three widely used animal models of acute lung injury. European surgical research. 2008;40(4):305-16.

127. Pauluhn J, Carson A, Costa D, Gordon T, Kodavanti U, Last J, et al. Workshop summary: phosgene-induced pulmonary toxicity revisited: appraisal of early and late markers of pulmonary injury from animal models with emphasis on human significance. Inhalation toxicology. 2007;19(10):789-810.

128. Union EPaCotE. Directive 2010/63/EU of the European parliament and of the council of 22 September 2010. Off J Eur Union. 2010;276:33-74.

129. Dräger. Suitable products for Phosgene COCl2 Germany: Dräger; 2025 [Available from: https://www.draeger.com/en_uk/Substances/349.

130. Protocol G. Protocol for the prohibition of the use in war of asphyxiating, poisonous or other gases, and of bacteriological methods of warfare. Signed at Geneva, June. 1925;17.

131. United States. War Dept. Enemy Tactics in Chemical Warfare. Washington, D.C.: War Department; 1944. p. 82.

132. Central Intelligence Agency. Impact and Implications of Chemical Weapons Use in the Iran-Iraq War. 1988. Report No.: NI IIM 88-10004C.

133. Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction. Paris: United Nations; 1993. p. 1974 UNTS 45.

134. The Project Anthracite Team. Hungnam Fertiliser Complex: DPRK's Chemical Facilities. Royal United Services Institute, London; 2025. Report No.: Site Profile 4.

135. Stock T, Lohs K. The challenge of old chemical munitions and toxic armament wastes: Oxford University Press; 1997.

136. Ryan TA, Seddon E, Seddon K, Ryan C. Phosgene: And Related Carbonyl Halides: Elsevier; 1996.

137. Cotarca L, Lange C, Meurer K, Pauluhn J. Phosgene. Ley C, editor. Weinheim, Germany: Wiley-VCH; 2019.

138. U.S. Chemical Safety and Hazard Investigation Board. Investigation Report: DuPont Belle Toxic Chemical Releases. Washington (DC); 2011. Report No.: 2010-6-I-WV.

139. IMPEL tENftIaEoEL. Accidental release of phosgene on a chemical platform. France; 2012. Report No.: 42163.

140. TNN. Phosgene gas leak in Gujarat plant kills 4. The Times of India. 2016 4 November 2016.

141. Occupational Safety and Health Administration. Accident Search Results. Keyword: "Phosgene". [Webpage]. Washington, DC: U.S. Department of Labor; 2025 [Available from:

https://www.osha.gov/ords/imis/AccidentSearch.search?acc_keyword=%22Phosgene%22&keyword_list=on.

142. Bell DG. Severe lung injury following exposure to chlorine gas: a case series. Chest. 2007;132(4):566A.

143. Cornish P. The CBRN system: assessing the threat of terrorist use of chemical, biological, radiological and nuclear weapons in the United Kingdom. Chatham House, 10 St James's Square, London. SW1Y 4LE: Royal Institute of International Affairs; 2007.

144. Federal Bureau of Investigation. Domestic Terrorists Intent and Capability to Use Chemical, Biological, Radiological, and Nuclear Weapons. Washington, D.C.: Federal Bureau of Investigation, Weapons of Mass Destruction Directorate CD, DHS, Office of Intelligence & Analysis 2008.

145. Uncle Fester. Silent Death. 2nd ed. ed. Port Townsend, Washington.: Loopmanics Unlimited; 1997.

146. NAS/COT Subcommittee for AEGLs. Interim Acute Exposure Guideline Levels (AEGLs) for Sulfuric Acid, Sulfur Trioxide, and Oleum. Washington, D.C.; 2009. Report No.: Interim 1: 12/2008.

147. UK Health Security Agency. Carbon tetrachloride: general information. UK: UK Health Security Agency; 2022 [updated 17 November 2022. Available from: https://www.gov.uk/government/publications/carbon-tetrachloride-properties-incident-management-and-toxicology/carbon-tetrachloride-general-information.

148. The Control of Explosives Precursors and Poisons Regulations 2023, Pub. L. No. SI 2023/63 Stat. SI 2023/63 (2023).

149. Animals (Scientific Procedures) Act, Stat. C.14 (1986).

150. Graham S, Fairhall S, Rutter S, Auton P, Rendell R, Smith A, et al. Continuous positive airway pressure: An early intervention to prevent phosgene-induced acute lung injury. Toxicol Lett. 2018;293:120-6.

151. Arifin WN, Zahiruddin WM. Sample Size Calculation in Animal Studies Using Resource Equation Approach. Malays J Med Sci. 2017;24(5):101-5.

152. Charan J, Kantharia ND. How to calculate sample size in animal studies? J Pharmacol Pharmacother. 2013;4(4):303-6.

153. Grainge C, Smith A, Jugg B, Fairhall S, Mann T, Perrott R, et al. Furosemide in the Treatment of Phosgene Induced Acute Lung Injury. Journal of the Royal Army Medical Corps. 2010;156(4):245-.

154. Odenstedt H, Lindgren S, Olegard C, Erlandsson K, Lethvall S, Aneman A, et al. Slow moderate pressure recruitment maneuver minimizes negative circulatory and lung mechanic side effects: evaluation of recruitment maneuvers using electric impedance tomography. Intensive Care Med. 2005;31(12):1706-14.

155. NATO Standardization Office. Allied Medical Publication AMedP-7.1: Medical management of CBRN casualties. Brussels; 2018.

156. Chapman CR, Foley KM. Oxygen Transport. Principles and Practice. JD Edwards, WC Shoemaker, J.-L. Vincent (Eds.). WB Saunders Company Ltd., London, England. 1993. 367 pages. ISBN 0-7020-1576-8 Springer.

157. Shanmukhappa C, Lokeshwaran S. Venous oxygen saturation. 2020.

158. Reinhart K, Rudolph T, Bredle DL, Hannemann L, Cain SM. Comparison of central-venous to mixed-venous oxygen saturation during changes in oxygen supply/demand. Chest. 1989;95(6):1216-21.

159. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.

160. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37(8):911-7.

161. de Hoffmann E. Tandem mass spectrometry: a primer. Journal of mass spectrometry. 1996;31(2):129-37.

162. Miller WC, Rice DL, Unger KM, Bradley BL. Effect of PEEP on lung water content in experimental noncardiogenic pulmonary edema. Crit Care Med. 1981;9(1):7-9.

163. Maybauer DM, Talke PO, Westphal M, Maybauer MO, Traber LD, Enkhbaatar P, et al. Positive end-expiratory pressure ventilation increases extravascular lung water due to a decrease in lung lymph flow. Anaesth Intensive Care. 2006;34(3):329-33.

164. Colmenero-Ruiz M, Fernández-Mondéjar E, Fernández-Sacristán MA, Rivera-Fernández R, Vazquez-Mata G. PEEP and low tidal volume ventilation reduce lung water in porcine pulmonary edema. Am J Respir Crit Care Med. 1997;155(3):964-70.

165. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med. 2000;342(18):1334-49.

166. Zemans RL, Matthay MA. What drives neutrophils to the alveoli in ARDS? Thorax. 2017;72(1):1-3.

167. Steinberg KP, Milberg JA, Martin TR, Maunder RJ, Cockrill BA, Hudson LD. Evolution of bronchoalveolar cell populations in the adult

respiratory distress syndrome. American Journal of Respiratory and Critical Care Medicine. 1994;150(1):113-22.

168. Abraham E. Neutrophils and acute lung injury. Crit Care Med. 2003;31(4 Suppl):S195-9.

169. Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. Mol Med. 2011;17(3-4):293-307.

170. Yang SC, Tsai YF, Pan YL, Hwang TL. Understanding the role of neutrophils in acute respiratory distress syndrome. Biomed J. 2021;44(4):439-46.

171. Hall JE, Hall ME. Guyton and Hall Textbook of Medical Physiology E-Book: Guyton and Hall Textbook of Medical Physiology E-Book: Elsevier Health Sciences; 2020.

172. Rudloff E, Hopper K. Crystalloid and Colloid Compositions and Their Impact. Front Vet Sci. 2021;8:639848.

173. Monkman SL, Andersen CC, Nahmias C, Ghaffer H, Bourgeois JM, Roberts RS, et al. Positive end-expiratory pressure above lower inflection point minimizes influx of activated neutrophils into lung. Crit Care Med. 2004;32(12):2471-5.

174. Buonacera A, Stancanelli B, Colaci M, Malatino L. Neutrophil to Lymphocyte Ratio: An Emerging Marker of the Relationships between the Immune System and Diseases. International Journal of Molecular Sciences. 2022;23(7):3636.

175. Jozwiak M, Teboul JL, Monnet X. Extravascular lung water in critical care: recent advances and clinical applications. Ann Intensive Care. 2015;5(1):38.

176. Malo J, Ali J, Wood L. How does positive end-expiratory pressure reduce intrapulmonary shunt in canine pulmonary edema? Journal of applied physiology. 1984;57(4):1002-10.

177. Simmons DH, Linde LM, Miller JH, O'Reilly RJ. Relation Between Lung Volume and Pulmonary Vascular Resistance. Circulation Research. 1961;9(2):465-71.

178. Howell J, Permutt S, Proctor D, Riley R. Effect of inflation of the lung on different parts of pulmonary vascular bed. Journal of Applied Physiology. 1961;16(1):71-6.

179. Frostell C, Blomqvist H, Hedenstierna G, Halbig I, Pieper R. Thoracic and abdominal lymph drainage in relation to mechanical ventilation and PEEP. Acta anaesthesiologica scandinavica. 1987;31(5):405-12.

180. Reed HO, Wang L, Sonett J, Chen M, Yang J, Li L, et al. Lymphatic impairment leads to pulmonary tertiary lymphoid organ formation and alveolar damage. J Clin Invest. 2019;129(6):2514-26.

181. Wright JR, Dobbs LG. Regulation of pulmonary surfactant secretion and clearance. Annual review of physiology. 1991;53(1):395-414.

182. Simonato M, Baritussio A, Ori C, Vedovelli L, Rossi S, Dalla Massara L, et al. Disaturated-phosphatidylcholine and surfactant protein-B turnover in human acute lung injury and in control patients. Respir Res. 2011;12:36.

183. Heeley EL, Hohlfeld JM, Krug N, Postle AD. Phospholipid molecular species of bronchoalveolar lavage fluid after local allergen challenge in asthma. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2000;278(2):L305-L11.

184. Hunt AN, Postle AD, Thomassen MJ. The Lipidomics Of Alveolar Proteinosis. D43 PULMONARY ALVEOLAR PROTEINOSIS: American Thoracic Society; 2012. p. A5804-A.

185. Testi R. Sphingomyelin breakdown and cell fate. Trends in biochemical sciences. 1996;21(12):468-71.

186. Oda Y, Kai H, Isohama Y, Takahama K, Miyata T. Stimulation of pulmonary surfactant secretion by activating neutrophils in rat type II pneumocytes culture. Life sciences. 1991;49(11):803-11.

187. Ryan SF, Ghassibi Y, Liau DF. Effects of activated polymorphonuclear leukocytes upon pulmonary surfactant in vitro. Am J Respir Cell Mol Biol. 1991;4(1):33-41.

188. Quintero OA, Wright JR. Clearance of surfactant lipids by neutrophils and macrophages isolated from the acutely inflamed lung. Am J Physiol Lung Cell Mol Physiol. 2002;282(2):L330-9.

189. Wright J. Clearance and recycling of pulmonary surfactant. American Journal of Physiology-Lung Cellular and Molecular Physiology. 1990;259(2):L1-L12.

190. Dushianthan A, Cusack R, Goss V, Koster G, Grocott MPW, Postle AD. In Vivo Cellular Phosphatidylcholine Kinetics of CD15+ Leucocytes and CD3+ T-Lymphocytes in Adults with Acute Respiratory Distress Syndrome. Cells. 2024;13(4).

191. Hunt AN, Malur A, Monfort T, Lagoudakis P, Mahajan S, Postle AD, et al. Hepatic Steatosis Accompanies Pulmonary Alveolar Proteinosis. Am J Respir Cell Mol Biol. 2017;57(4):448-58.

192. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chem Rev. 2011;111(10):6130-85.

193. Virk R, Cook K, Cavazos A, Wassall SR, Gowdy KM, Shaikh SR. How Membrane Phospholipids Containing Long-Chain Polyunsaturated Fatty Acids and Their Oxidation Products Orchestrate Lipid Raft Dynamics to Control Inflammation. J Nutr. 2024;154(9):2862-70.

194. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death & Differentiation. 1998;5(7):551-62.

195. Taniguchi M, Okazaki T. Ceramide/Sphingomyelin Rheostat Regulated by Sphingomyelin Synthases and Chronic Diseases in Murine Models. J Lipid Atheroscler. 2020;9(3):380-405.

196. Taniguchi M, Okazaki T. Role of ceramide/sphingomyelin (SM) balance regulated through “SM cycle” in cancer. Cellular signalling. 2021;87:110119.

197. Chauhan S, D’cruz R, Faruqi S, Singh K, Varma S, Singh M, et al. Chemical warfare agents. Environmental Toxicology and Pharmacology. 2008;26(2):113-22.

198. Slutsky AS, Ranieri VM. Ventilator-induced lung injury. N Engl J Med. 2013;369(22):2126-36.

199. Kitsiouli E, Nakos G, Lekka ME. Phospholipase A2 subclasses in acute respiratory distress syndrome. Biochim Biophys Acta. 2009;1792(10):941-53.

200. Ghidoni R, Caretti A, Signorelli P. Role of Sphingolipids in the Pathobiology of Lung Inflammation. Mediators Inflamm. 2015;2015:487508.
201. Maile MD, Standiford TJ, Engoren MC, Stringer KA, Jewell ES, Rajendiran TM, et al. Associations of the plasma lipidome with mortality in the acute respiratory distress syndrome: a longitudinal cohort study. Respir Res. 2018;19(1):60.
202. Ottolenghi S, Zulueta A, Caretti A. Iron and Sphingolipids as Common Players of (Mal)Adaptation to Hypoxia in Pulmonary Diseases. Int J Mol Sci. 2020;21(1).
203. Pulica R, Aquib A, Varsanyi C, Gadiyar V, Wang Z, Frederick T, et al. Dys-regulated phosphatidylserine externalization as a cell intrinsic immune escape mechanism in cancer. Cell Commun Signal. 2025;23(1):131.
204. Vaish AK, Consul S, Agrawal A, Chaudhary SC, Gutch M, Jain N, et al. Accidental phosgene gas exposure: A review with background study of 10 cases. J Emerg Trauma Shock. 2013;6(4):271-5.
205. Holmes WW, Keyser BM, Paradiso DC, Ray R, Andres DK, Benton BJ, et al. Conceptual approaches for treatment of phosgene inhalation-induced lung injury. Toxicol Lett. 2016;244:8-20.
206. McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Bohm M, et al. 2023 Focused Update of the 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Eur Heart J. 2023;44(37):3627-39.
207. Sprung CL, Rackow EC, Fein IA, Jacob AI, Isikoff SK. The spectrum of pulmonary edema: differentiation of cardiogenic, intermediate, and

noncardiogenic forms of pulmonary edema. American Review of Respiratory Disease. 1981;124(6):718-22.

208. Cortese-Krott MM, Koning A, Kuhnle GGC, Nagy P, Bianco CL, Pasch A, et al. The Reactive Species Interactome: Evolutionary Emergence, Biological Significance, and Opportunities for Redox Metabolomics and Personalized Medicine. Antioxid Redox Signal. 2017;27(10):684-712.

209. Eisner MD. Plasma surfactant protein levels and clinical outcomes in patients with acute lung injury. Thorax. 2003;58(11):983-8.

210. Mokra D, Kosutova P. Biomarkers in acute lung injury. Respiratory Physiology & Neurobiology. 2015;209:52-8.

211. Smith A, Brown R, Jugg B, Platt J, Mann T, Masey C, et al. The effect of steroid treatment with inhaled budesonide or intravenous methylprednisolone on phosgene-induced acute lung injury in a porcine model. Military medicine. 2009;174(12):1287-94.

212. Liu F, Pauluhn J, Trubel H, Wang C. Single high-dose dexamethasone and sodium salicylate failed to attenuate phosgene-induced acute lung injury in rats. Toxicology. 2014;315:17-23.

213. McGovern T, Day BJ, White CW, Powell WS, Martin JG. AEOL10150: a novel therapeutic for rescue treatment after toxic gas lung injury. Free Radic Biol Med. 2011;50(5):602-8.

214. McElroy CS, Min E, Huang J, Loader JE, Hendry-Hofer TB, Garlick RB, et al. From the cover: catalytic antioxidant rescue of inhaled sulfur mustard toxicity. Toxicological Sciences. 2016;154(2):341-53.