

**The impact of acute lung injury, ECMO and transfusion on oxidative stress and plasma selenium levels in an ovine model**

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1 The impact of Acute Lung Injury, ECMO and transfusion on oxidative stress and  
2 plasma selenium levels in an ovine model.

3

4 **Short title:** Oxidative stress and selenium levels during ECMO

5

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2

### 3 **Competing Interests**

4 The authors declare that they have no competing interests.

5

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10

1 **Abstract:**

2 The purpose of this study was to determine the effects of smoke induced acute lung  
3 injury (S-ALI), extracorporeal membrane oxygenation (ECMO) and transfusion on  
4 oxidative stress and plasma selenium levels. Forty ewes were divided into (i) healthy  
5 control (n=4), (ii) S-ALI control (n=7), (iii) ECMO control (n=7) (iv) S-ALI + ECMO  
6 (n=8) and (v) S-ALI + ECMO + packed red blood cell (PRBC) transfusion (n=14).  
7 Plasma thiobarbituric acid reactive substances (TBARS), selenium and glutathione  
8 peroxidase (GPx) activity were analysed at baseline, after smoke injury (or sham) and  
9 0.25, 1, 2, 6, 7, 12 and 24 hours after initiation of ECMO. Peak TBARS levels were  
10 similar across all groups. Plasma selenium decreased by 54% in S-ALI sheep  
11 ( $1.36\pm 0.20$  to  $0.63\pm 0.27$   $\mu\text{mol/L}$ ,  $p < 0.0001$ ), and 72% in sheep with S-ALI + ECMO  
12 at 24hr ( $1.36\pm 0.20$  to  $0.38\pm 0.19$ ,  $p < 0.0001$ ). PRBC transfusion had no effect on  
13 TBARS, selenium levels or glutathione peroxidase activity in plasma. While ECMO  
14 independently increased TBARS in healthy sheep to levels which were similar to the  
15 S-ALI control, the addition of ECMO after S-ALI caused a negligible increase in  
16 TBARS. This suggests that the initial lung injury was the predominant feature in the  
17 TBARS response. In contrast, the addition of ECMO in S-ALI sheep exacerbated  
18 reductions in plasma selenium beyond that of S-ALI or ECMO alone. Clinical studies  
19 are needed to confirm the extent and duration of selenium loss associated with  
20 ECMO.

21

22 **Key words:** Extracorporeal membrane oxygenation, acute lung injury, selenium,  
23 oxidative stress, antioxidants.

24

25 **Abbreviations**

1 ACT, Activated clotting time; ALT, Alanine transaminase; ARDS, Acute respiratory  
2 distress syndrome; CoHb, Carboxyhemoglobin; ECMO, Extracorporeal membrane  
3 oxygenation; GPx, Glutathione peroxidase; ICE, Intracardiac echocardiography; ICU,  
4 Intensive care unit; MDA, Malondialdehyde; MOF, Multi-organ failure; MV,  
5 Mechanical ventilation; PRBC, Packed red blood cell; ROS, Reactive oxygen species;  
6 TBARS, Thiobarbituric acid reactive substances.

7

8

9

1 **Introduction**

2 Patients with acute respiratory distress syndrome (ARDS) are critically ill and exhibit  
3 increased oxidative stress and reduced selenium levels compared to other hospital in-  
4 patients [1, 2]. Regardless of the cause of lung injury, oxidative stress is thought to be  
5 a major contributor to the pathogenesis and progression of ARDS [1, 3]. The mortality  
6 among patients with acute lung injury (ALI) and ARDS may be as high as 41% and in  
7 severe cases of ARDS, veno-venous extracorporeal membrane oxygenation (VV  
8 ECMO) provides a rescue therapy for temporary respiratory support [4, 5].

9

10 VV ECMO involves the insertion of large cannulas into the central venous circulation,  
11 redirecting the blood through an oxygenator (artificial lung), where carbon dioxide is  
12 removed and oxygen is added. (Fig. 1) Oxygenated blood is then returned through a  
13 cannula to the right side of the heart before passing through the lungs. ECMO circuits  
14 have a large surface area to enable efficient gaseous exchange, however exposure of  
15 the patient's blood to these foreign surfaces initiates an inflammatory response [6] as  
16 well as absorbing trace elements and drugs [7, 8].

17

18 *[insert Figure 1]*

19

20 Oxidative stress occurs when excessive production of reactive species of oxygen  
21 (ROS) overwhelms the antioxidant system [9]. Excessive ROS production during  
22 sepsis, systemic inflammation and/or ischemia-reperfusion injury has been associated  
23 with cellular, tissue and ultimately organ injury [9]. Additionally, extracorporeal  
24 circuits (ECMO, dialysis, cardiac bypass) involve extensive blood contact with a  
25 foreign surface and this coupled with hyperoxia and massive blood transfusions have

1 the potential to augment oxidative stress [10, 11]. Whether oxidative stress in this  
2 setting contributes to the morbidity/mortality risk of patients on ECMO is unclear.  
3  
4 Selenium is a trace element incorporated into a variety of selenoproteins involved in  
5 antioxidant, thyroid, immunity and inflammatory regulation [12]. While some studies  
6 have demonstrated a negative correlation between selenium and oxidative stress [13,  
7 14], reduced plasma selenium levels have also been independently associated with  
8 worse outcomes in some critically ill patients [15]. Glutathione peroxidase (GPx) a  
9 primary antioxidant enzyme, requires selenium for normal functioning [16]. The  
10 reduction of serum selenium levels during critical illness and inflammatory  
11 syndromes [17] may compromise GPx activity. Despite its importance to antioxidant  
12 function (as well as thyroid and immune function), the impact of ECMO on selenium  
13 levels has not been previously investigated. We hypothesised that the addition of an  
14 ECMO circuit to a critically ill host would result in selenium reductions and  
15 exacerbate oxidative stress levels. We sought to investigate the impact of acute lung  
16 injury, (i.e. critical injury), ECMO and transfusion, individually and in combination  
17 on oxidant status and plasma selenium levels using an ovine model of smoke induced  
18 acute lung injury (S-ALI).

19

## 20 **Materials and Methods**

21 The animal ethics committees of The University Of Queensland (QUT/194/12) and  
22 The Queensland University of Technology (1100000053) approved this study that  
23 adhered to the National Health and Medical Research Council (NHMRC) Code of  
24 Practice for the Care and Use of Animals for Scientific Purposes [18]. Forty  
25 Australian Sann Border Leicester Cross ewes (1-3 yr old) were divided into the

1 following groups: (i) healthy control (n=4), (ii) ECMO control (healthy sheep +  
2 ECMO, n=7), (iii) S-ALI control (n=7), (iv) S-ALI + ECMO (n=8) and (v) S-ALI +  
3 ECMO + PRBC transfusion (n=14). Half the transfused group received fresh PRBC  
4 (< 5 days old) and the other half received aged PRBC (>35 days old).

5

### 6 ***Animal Preparation:***

7 Sheep were allocated to five different groups. Each sheep was anaesthetised and  
8 instrumented as previously detailed [19]. Briefly, standard monitoring was utilised,  
9 including- arterial blood pressure; three-lead electrocardiograph (ECG); oxygen  
10 saturation monitoring; continuous hemodynamic monitoring; regular blood gas  
11 analysis (ABL825 blood gas analyser, Radiometer, Copenhagen, Denmark); and  
12 continuous cardiac output and SvO<sub>2</sub> monitoring (Swan-Ganz CCOmbo, Edwards  
13 Lifesciences, California, USA). Sheep were ventilated through a 10Fr tracheostomy  
14 tube. Normothermia (39°C) was maintained with a warming blanket.

15

### 16 ***Experimental Protocol***

17 Acute lung injury was delivered as previously described [20]. Cooled cotton smoke  
18 was administered through a custom designed hand ventilator. Animals received a total  
19 of 14 breaths of cotton smoke (or sham for controls) to achieve a carboxyHb (COHb)  
20 level of 35-45%. A two hour lung injury development period was allowed to prior to  
21 initiating ECMO.

22

### 23 ***ECMO Circuits***

24 The ECMO circuits comprised a Quadrox PLS oxygenator, Bioline tubing and  
25 Rotaflow pump head (Maquet Cardiopulmonary AG, Hechinger Strabe Germany).

1 Circuits were primed with Plasmalyte P-148 (Baxter Healthcare Pty Ltd. Toongabbie,  
2 NSW, Australia), 4% human albumin (CSL Behring. Broadmeadows, Vic, Australia),  
3 1000IU porcine heparin (Pfizer Australia Pty Ltd. West Ryde, NSW, Australia) and  
4 warmed to 38°C. Target ECMO flows were calculated at two thirds of the pre-ECMO  
5 cardiac output. Sodium chloride (0.9%) (Baxter Healthcare Pty Ltd. Toongabbie,  
6 NSW, Australia) were administered intravenously as needed to maintain these target  
7 flows. FiO<sub>2</sub> was set at 100% and ECMO gas flows set to a ratio 0.8:1 of blood flow.  
8 Anticoagulation was achieved with 3000IU of heparin given to the sheep prior to  
9 ECMO cannulation and the activated clotting time (ACT) was targeted between 200-  
10 300sec. Cannulas were a 19 Fr (return cannula) and a 21Fr (access cannula)  
11 Carmeda™ bonded femoral venous cannula (Medtronic Pty Ltd, Minneapolis, MN)  
12 positioned via the right external jugular. Final cannula position was confirmed using a  
13 technique of intra-cannula echo using an intra-cardiac echocardiography probe [21].  
14  
15 Bilateral 20Fr intercostal catheters were inserted two hours after commencement of  
16 ECMO to collect pleural effusion fluid. After 24 hours sheep were euthanized using  
17 sodium pentobarbitone (100mg/kg).

18

### 19 ***Ovine PRBC preparation***

20 Sheep in the transfusion arm received two units of leukofiltered cross-match  
21 compatible ovine PRBC that was (i) less than 5 days (fresh) or (ii) 35-42 days (aged)  
22 using a previously described protocol [22]. Transfusion of the PRBC commenced  
23 after the 6 hr samples had been collected.

24

### 25 ***Blood Sample Collection***

1 Arterial blood samples were collected at anaesthetic induction (baseline), after smoke  
2 injury (PS), 15 minutes after ECMO start (0.25 hr) then at 1, 2, 6, 7, 12, and 24 hours.  
3 Blood for selenium analysis was collected into trace free element tubes (Greiner Bio-  
4 One, Monroe, North Carolina, United States), and the separated plasma was stored at  
5 4°C until analysis. EDTA blood samples were immediately centrifuged 3000g x  
6 10min @ 4°C and plasma stored at -80°C until analysis of thiobarbituric acid reactive  
7 substances (TBARS) and GPx.

8

### 9 ***Measurement of Selenium, GPx and TBARS***

10 Plasma selenium levels were analysed by graphite furnace atomic absorption  
11 spectrometry with Zeeman background correction (GF AAS) using a Varian AA280Z  
12 analyzer (Agilent Technologies Inc, Santa Clara CA, United States) as previously  
13 described.[23] Inter-assay imprecision was 5.5%. Plasma TBARS and GPx were  
14 analysed using commercially available assay kits (Cayman Chemical Company, Ann  
15 Arbor, MI).

16 Baseline data depicted in figure 3 (A, B and C) include baseline data from all groups  
17 (i.e. n=40)

18

### 19 ***Statistical Analysis***

20 Summary data are displayed as mean  $\pm$  standard deviation with associated 95% C.I.  
21 (difference of the mean) unless otherwise stated. A two-way ANOVA with  
22 unmatched pairs and a Tukey *post hoc* correction was used to compare means within  
23 and between groups (Graphpad Prism 6.0a). Statistical significance was assumed  
24 when  $p < 0.05$ . A linear regression was performed to determine the relationship  
25 between selenium and GPx or selenium and TBARS (Graphpad Prism 6.0a).

1

## 2 **Results**

3 The sheep in this study weighed an average of  $48.6 \pm 6.0$  kg. Table 1 summarizes  
4 PaO<sub>2</sub>, and COHb levels, heart rate, mean arterial pressure (MAP), inotrope use  
5 (noradrenaline, dopamine and vasopressin) and fluid requirements across the various  
6 study groups. COHb levels ranged from 25.3-53.6% after smoke injury while PaO<sub>2</sub>  
7 levels progressively declined in the S-ALI control sheep over the 24 hours period.  
8 (Table 1). Heart rate was similar across all the groups, however the average MAP  
9 over the study period was lower in the S-ALI control, S-ALI + ECMO and S-ALI +  
10 ECMO + Tf sheep compared to healthy control and ECMO controls. Inotropes  
11 (noradrenaline and dopamine) were required in the S-ALI control, S-ALI + ECMO  
12 and S-ALI + ECMO + Tf sheep to maintain cardiac function and blood pressure, but  
13 not in the healthy control or ECMO control sheep. Vasopressin was required in 5/8 of  
14 the S-ALI + ECMO and 4/14 of the S-ALI + ECMO + Tf sheep as they were mildly  
15 vasoplegic and non-responsive to noradrenaline. (Table 1). Saline was administered  
16 intravenously as required to maintain ECMO flows at two thirds cardiac output. As a  
17 result sheep in the S-ALI control, S-ALI + ECMO and S-ALI + ECMO + Tf groups  
18 received significantly more fluids and thus had a positive fluid balance at 24hr.  
19 Despite a higher positive fluid balance sheep in these groups, there was no significant  
20 difference in Hb compared to the ECMO control sheep. (Figure 2).

21

22 Serial changes to relevant biochemical parameters are detailed in Figure 2. The pH  
23 levels remained consistent throughout the study (Figure 2B). Albumin levels were  
24 reduced significantly ( $p < 0.05$ ) in the S-ALI control and S-ALI + ECMO groups after  
25 6 hours (Figure 2C). Bilirubin levels rose significantly in the healthy control and

1 ECMO control sheep ( $p < 0.05$ ) after 12 and 18 hours respectively but remained  
2 unchanged in the S-ALI control and S-ALI + ECMO groups (Figure 2F). Samples in  
3 S-ALI + ECMO +Tf group were unavailable for albumin, creatinine, ALT and  
4 bilirubin analysis.

5

6 As no significant differences were detected between fresh and aged PRBC transfused  
7 sheep for plasma TBARS, selenium and GPx, the data was combined into a single  
8 transfusion group ( $n=14$ ) and henceforth labelled S-ALI + ECMO + Tf.

9

10 *[insert table 1]*

11 *[insert figure 2]*

12

13

#### 14 **Plasma Thiobarbituric Acid Reactive Substances (TBARS)**

15 Plasma TBARS levels remained unchanged in the healthy control sheep (Figure 3A).  
16 Compared to healthy control sheep, plasma TBARS were significantly higher at  
17 corresponding time points between 0.25h and 24h for all groups ( $p < 0.001$ ). Plasma  
18 TBARS were significantly elevated in S-ALI control sheep, peaking at 2h ( $1.32 \pm 0.40$   
19 to  $2.90 \pm 0.35 \mu\text{M}$ , 95% CI -2.78 to -1.16;  $p < 0.001$ ). Plasma TBARS rose rapidly with  
20 the initiation of ECMO in ECMO control sheep, peaking at 6h ( $1.32 \pm 0.40$  to  
21  $3.11 \pm 0.70 \mu\text{M}$ , 95% CI -2.60 to -0.98;  $p < 0.0001$ ). In S-ALI + ECMO sheep plasma  
22 TBARS were 155% above baseline ( $1.32 \pm 0.40$  vs  $3.37 \pm 0.35 \mu\text{M}$ , 95% CI -2.82 to -  
23 1.29;  $p < 0.0001$ ) and 200% above baseline in S-ALI + ECMO + Tf sheep at 24h,  
24 ( $1.32 \pm 0.40$  vs  $3.97 \pm 0.44 \mu\text{M}$ , 95% CI -3.27 to -2.04;  $p < 0.0001$ ), but this was not  
25 significantly higher than S-ALI control or ECMO control. No differences were

1 detected due to PRBC transfusion between 7h and 24h in the S-ALI + ECMO + Tf  
2 group.

3

4 *[insert figure 3]*

5

## 6 **Plasma Selenium levels**

7 Baseline plasma selenium levels were similar amongst all sheep. In ECMO control  
8 sheep, plasma selenium levels declined at 2h to 46% ( $1.36 \pm 0.20$  to  $0.73 \pm 0.21$   $\mu\text{mol/L}$ ,  
9 95% CI 0.29 to 0.97;  $p < 0.0001$ ), they rose after 6h but were still below baseline at  
10 24h ( $0.96 \pm 0.17$   $\mu\text{mol/L}$ , 95% CI 0.06 to 0.74;  $p < 0.01$ ). In S-ALI control sheep  
11 plasma selenium began to decline at 6h and were significantly below baseline levels at  
12 24h (54% reduction;  $1.36 \pm 0.20$  to  $0.63 \pm 0.27$   $\mu\text{mol/L}$  at 24h, 95% CI, 0.39 to 1.07;  $p <$   
13  $0.0001$ ) (Figure 3B). The double insult of S-ALI followed by ECMO produced the  
14 greatest decline in plasma selenium of 72% at 24h ( $1.36 \pm 0.20$  to  $0.38 \pm 0.19$ , 95% CI  
15 0.66 to 1.3;  $p < 0.0001$ ). Between group comparisons of plasma selenium results  
16 detected a significant difference between S-ALI + ECMO vs ECMO control at 24h  
17 ( $0.38 \pm 0.19$  vs  $0.96 \pm 0.17$   $\mu\text{mol/L}$ , 95% CI 0.15 to 1.01;  $p < 0.001$ ). PRBC transfusions  
18 did not cause any change.

19

20 Pleural effusion fluid collected from S-ALI+ ECMO sheep had selenium levels of  
21  $0.5 \pm 0.1$   $\mu\text{mol/L}$  (n=16). Linear regression revealed that decreased plasma selenium  
22 was associated with increased plasma TBARS for S-ALI + ECMO sheep ( $r^2 = 0.50$ ,  $p$   
23  $< 0.0001$ ). This association was reduced when data from all sheep on ECMO was  
24 combined (healthy and S-ALI;  $r^2 = 0.39$ ,  $p < 0.001$ ).

25

1 **Plasma Glutathione Peroxidase activity**

2 No changes in plasma GPx activity occurred in healthy control sheep (Figure 3C).

3 Though not statistically significant, the following trends were observed: (i) Smoke

4 injury (S-ALI control) induced a rapid but transient increase in plasma GPx,

5 ( $1.93\pm 0.55$  to  $2.67\pm 0.32$  nmol/min/ml), returning to baseline levels at 24h ( $1.94\pm 0.41$

6 nmol/min/ml). (ii) ECMO-control experienced a similar increase in plasma GPx

7 ( $1.93\pm 0.55$  to  $2.63\pm 0.47$  nmol/min/ml) after ECMO initiation, trending back to

8 baseline levels at 24h. (iii) S-ALI + ECMO produced peak plasma GPx responses

9 similar to ECMO control ( $2.86\pm 0.6$  nmol/min/ml), which then declined 1h after

10 ECMO began, being below baseline at 24h ( $1.43\pm 0.24$  vs  $1.93\pm 0.55$  nmol/min/ml).

11 (iv) plasma GPx activity was elevated after smoke injury and during the initial stages

12 of ECMO in ECMO + S-ALI + Tf group. No effect of PRBC transfusion was

13 detected.

14

15

16

17 **Discussion**

18 This ovine model has generated two significant findings. First, we found that both S-

19 ALI and ECMO independently increased oxidative stress, but when conducted

20 sequentially (i.e. S-ALI followed by ECMO) the latter caused no additional impact on

21 oxidative stress. Secondly, S-ALI caused significant reductions in plasma selenium

22 and subsequent ECMO support exacerbated this loss.

23

24 ARDS is associated with a dramatic and uncontrolled increase in ROS production that

25 contributes to morbidity and mortality [3, 24]. When conventional medicine fails in

1 these patients, ECMO is one of the few remaining life-saving options [4]. Previous  
2 ECMO studies in pediatrics, [25] rabbits, [26] lambs [27] sheep [28] and pigs [29]  
3 have shown significant elevations in lipid peroxides. However it is difficult to  
4 translate these findings to critically ill adult patients as (i) pediatric patients have  
5 significantly different bodyweight to ECMO surface area ratios and (ii) (with the  
6 exception of the sheep study [28]) in all the other animal models ECMO was initiated  
7 in a healthy host. Thus the impact of acute illness on overall oxidative stress could not  
8 be determined.

9

10 We demonstrated that both S-ALI and ECMO independently increased lipid  
11 peroxidation in plasma. Over the 24h study period, we found that while ECMO use in  
12 sheep with S-ALI resulted in an increase in lipid peroxides, the levels were not  
13 significantly greater than for S-ALI or ECMO alone. Our results contradict the  
14 findings of an earlier similar study, [28] which measured lipid peroxides in a model of  
15 S-ALI and veno-arterial ECMO, and demonstrated significant increases in lipid  
16 peroxides after ECMO was initiated in a S-ALI animal. As that study was conducted  
17 20 years ago their ECMO circuit was significantly different, which may account for  
18 the disparity in results to our study. Their circuit included a silicon membrane  
19 oxygenator with a large surface area ( $4.5 \text{ m}^2$ ), a servo controlled roller pump and  
20 venous blood drainage reservoir in a venous arterial ECMO configuration. In  
21 comparison, our study utilised a low volume closed circuit, polymethylpentene fibre  
22 oxygenator, (surface area  $1.8 \text{ m}^2$ ), with centrifugal pump and coated tubing in a VV  
23 ECMO configuration. We speculate that the improvements in ECMO technology and  
24 circuit biocompatibility have reduced the trauma associated with continual blood

1 exposure to the foreign circuit and consequently dampened any increase in lipid  
2 peroxidation.

3  
4 Independent of oxidative stress, reductions in serum or plasma selenium during sepsis  
5 and ARDS are associated with multi-organ failure, inflammatory syndromes,  
6 increased infections and increased mortality [3, 15, 24, 30, 31]. Some studies have  
7 shown that selenium replacement in critically ill patients is beneficial in reducing  
8 infections (such as ventilator acquired pneumonia) and mortality [32, 33]. This led us  
9 to investigate the impact of ECMO on plasma selenium levels. We demonstrated that  
10 S-ALI and ECMO independently led to significant reductions in plasma selenium (54  
11 and 46% respectively). Notably the “two-hit” combination of S-ALI + ECMO  
12 resulted in a greater loss (72%). We postulate that the observed plasma selenium  
13 reductions may be attributed to an acute phase response, circuit absorption and  
14 hemodilution (due to the ECMO priming solution and ongoing fluid replacement).  
15 The acute phase response seen during inflammatory syndromes increases the  
16 permeability of cellular membranes to proteins and micronutrients, that are  
17 redistributed to tissues [34]. Hypoalbuminaemia, ascites, high volume requirement  
18 and significant pleural effusions confirm this response in the sheep of this current  
19 study. Measurement of the selenium concentration in the pleural effusion fluid also  
20 confirms this fluid to be one avenue of significant selenium loss. This may have some  
21 clinical relevance as pleural effusions are not uncommon in VV ECMO patients.

22  
23 We have previously demonstrated that cardiopulmonary bypass circuits absorb trace  
24 elements [7]. Given the similarities between cardiopulmonary bypass and ECMO  
25 circuits this presents another mechanism for the loss of trace elements such as

1 selenium. In addition because patients on ECMO are critically ill, the associated  
2 altered gut motility and absorption may compromise dietary intake of trace elements  
3 [35]. Since ECMO support often lasts for over a week, the combination of acute phase  
4 redistribution, decreased dietary intake and loss through circuits absorption could lead  
5 to a true selenium deficiency .

6  
7 While many studies cite plasma or serum selenium reductions in the critically ill as an  
8 independent predictor of poor outcome, it is important to recognise that plasma  
9 selenium levels provide limited information regarding functional selenium status.

10 Selenium in plasma exists in several forms, 40-70% is in the functional form  
11 selenoprotein P, GPx-3 accounts for another 20-40% and 6-10% is bound to albumin  
12 [36]. Unfortunately in this present study we did not have the capacity to measure  
13 selenoprotein P. Plasma GPx (GPx-3) is an important extracellular antioxidant and  
14 can be used at times as a surrogate marker of selenium status. GPx is produced  
15 primarily in the kidneys and secondarily in the liver [37] and while the association  
16 between selenium and GPx activity is well established, how rapidly GPx synthesis in  
17 the kidneys and liver is altered by acute reductions in selenium is largely unknown.

18 We attempted to measure kidney (creatinine) and liver function (bilirubin and ALT).  
19 Creatinine levels remained stable in all groups, however there were increased  
20 bilirubin levels in the healthy control and ECMO control sheep but these were stable  
21 in the S-ALI control and S-ALI + ECMO sheep (Figure 2 E). In contrast ALT levels  
22 were similar across the study groups (Figure 2 F). The inconsistency between  
23 bilirubin and ALT levels suggests that the high positive fluid balance in the S-ALI  
24 control and S-ALI + ECMO sheep has possibly diluted both bilirubin and ALT levels.  
25 For GPx activity our data suggests a trend towards GPx reductions after 6 hours in S-

1 ALI + ECMO sheep, which we may be indicative of a delayed response to the  
2 reductions in plasma selenium.

3

4 ECMO patients often required multiple transfusions [38, 39], which may convey an  
5 increased risk of transfusion related complications. Previous studies in neonates and  
6 animals have demonstrated increases in lipid peroxidation after PRBC transfusions  
7 [40-43]. We and others have shown that PRBC contain low selenium levels [44] and  
8 reduced antioxidant capacity [45]. Despite these observations, in this model PRBC  
9 transfusions had no significant impact on oxidative stress, selenium or GPx. One  
10 reason for the lack of response may be because of the small volume of PRBC  
11 transfused and the period of observation too short. In addition, the magnitude of the S-  
12 ALI induced oxidative stress and selenium alterations could have masked any  
13 transfusion related effect. Thus the effect of massive PRBC transfusion during a  
14 prolonged ECMO course remains to be investigated.

15

16 Investigations into the *in vivo* impact of ECMO using modern adult ECMO cannulas  
17 and circuits can only be performed with a large animal model. Sheep models have the  
18 advantage of weight ranges, pulmonary and cardiac physiology, coagulation and  
19 inflammatory systems which are similar to humans [19]. However, large animal  
20 models are expensive and this restricted the sample size of each group in our study.

21 The incremental design of this *in vivo* study enabled the differentiation of the effect of  
22 ECMO from that of other variables such as S-ALI and transfusion but had several  
23 limitations. The complexity of maintaining a critically ill animal restricted the  
24 duration of the experiment to 24 hours, thus preventing the study of longer-term  
25 effects on oxidative stress. While this study utilised a common measure of lipid

1 peroxidation (TBARS), it may have benefited from analysing F<sub>2</sub>-isoprostanes or  
2 protein carbonyl measurements. The analysis of tissue samples from specific organs  
3 may have also revealed localized decreases in selenium as well as consequences of  
4 oxidative stress that were not apparent using systemic blood samples, however in an  
5 ECMO model where sheep are anticoagulated, serial tissue samples are not practical  
6 due to the risk of catastrophic bleeding.

7

8 During VV ECMO, blood flow is a key determinant of oxygenation as significant  
9 shunting can occur in injured lungs, and suboptimal ECMO flows may result in  
10 systemic hypoxia. Clinically, patients with a high cardiac output who are dependent  
11 on higher ECMO flows for oxygenation require a degree of fluid resuscitation.  
12 Alternatives to optimise oxygenation and minimize fluid resuscitation in these  
13 patients include pharmacological interventions to reduce cardiac output, deep  
14 sedation, paralysis or induced hypothermia. To avoid additional confounders in this  
15 study we chose to maintain ECMO flows to 2/3 of cardiac output across all sheep and  
16 to only use fluids and vasoactive agents to achieve this. Despite the high dose  
17 vasopressor and inotrope drugs in the S-ALI sheep (Table 1) the excessive capillary  
18 leak necessitated significant fluid resuscitation to maintain target ECMO blood flows.  
19 Notwithstanding this effort, we acknowledge that the resulting higher fluid balance in  
20 S-ALI + ECMO ± Tf sheep (Table 1) may have contributed to lower selenium levels  
21 and a blunted TBARS response.

22

### 23 ***Conclusions***

24 We have demonstrated that 24 hours of ECMO induced increased plasma TBARS in a  
25 healthy host. However in a S-ALI animal, ECMO did not cause any additional

1 TBARS increase. Nevertheless the combination of S-ALI and ECMO lead to  
2 profound reductions in plasma selenium. It is unclear if this represents a true  
3 deficiency or is a result of fluid shifts associated with the acute phase response. In  
4 addition to influencing antioxidant function, reductions in plasma selenium may also  
5 compromise the function of thyroid and immune function. Clinical studies are now  
6 needed to confirm if these effects on selenium, and oxidative stress are augmented or  
7 diminished with longer periods of ECMO support, and to assess if normalisation of  
8 selenium through supplementation is beneficial.

9  
10

11 **Competing Interests**

12 The authors declare that they have no competing interests.

13

14 **Author Contributions**

15 CIM, YLF, KS and JFF participated in the study design, manuscript drafting and  
16 critically editing the manuscript. CIM, SDD, KRD, MRP, SRF, GS and DP and JFF  
17 and KS were involved with data acquisition and analysis as well as manuscript  
18 drafting. CIM performed the statistical analysis. All authors read and approved the  
19 final manuscript and agree to be accountable for all aspects of the work.

20

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3

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1 **Header: Table 1. Comparison of basic physiological variables.**

2 **Footer:** COHb- carboxyhemoglobin; Tf- transfusion; MAP- mean arterial pressure.

3 COHb values are after smoke breath cycles completed. Heart rate, MAP and Inotrope  
4 values are mean±S.D. for the period between smoke injury/SHAM (2 hrs prior to  
5 ECMO) and 24 hrs ECMO. Asterisk denotes comparison between S-ALI control and  
6 S-ALI+ECMO / S-ALI+ECMO+transfusion ( $p < 0.05$ ).

7

8 **Figure 1. Schematic diagram of sheep on VV ECMO.** ECMO cannulas are inserted  
9 via right internal jugular vein (RIJV). Reproduced with permission [19]

10

11 **Figure 2. Effect of ECMO and/or S-ALI on parameters of Hemoglobin (A), pH**  
12 **(B), Albumin (C), Creatinine (D), Alanine Transaminase (E) and Bilirubin (F).** B,  
13 baseline; PS, post-smoke injury, numbers represent hours of ECMO support. a and b  
14 (Fig C.) indicate significant difference ( $p < 0.05$ ) with respect to healthy control and  
15 ECMO control. Asterisks indicate significant difference ( $p < 0.05$ ) with respect to  
16 baseline.

17

18

19 **Figure 3. Effects of smoke injury (S-ALI) ± ECMO ± transfusion (Tf) on (A)**  
20 **plasma thiobarbituric acid reactive substance (TBARS), (B) selenium and (C)**  
21 **glutathione peroxidase (GPx) levels.** The mean baseline data is represented by a full  
22 horizontal line. Dashed horizontal line is ± 1SD. PS-2h post smoke/sham injury.  
23 Numbers represent hours after initiation of ECMO support. Data are mean±SD.  
24 Asterisks indicate significant within group difference compared to baseline levels.

25

26

1 Table.1

	<b>Healthy Control (n=4)</b>	<b>ECMO control (n=7)</b>	<b>S-ALI Control (n=7)</b>	<b>S-ALI + ECMO (n=8)</b>	<b>S-ALI + ECMO + Tf (n=14)</b>
<b>PaO<sub>2</sub> (mmHg)</b>					
Pre-ECMO	533±39	395±162	562±27	409±120	403±172
24 hr	526±23	148±40	169±148	161±48	139±24
<b>COHb (%)</b>	4.0±0.4	4.2±0.6	43.3±8.8	44.2±5.6	39.35±6.40
[range]	[3.6 – 4.3]	[3.5 – 4.7]	[30.5 – 53.6]	[35.0 – 51.4]	[25.3 – 48.4]
<b>Heart Rate</b>	108±16	102±17	114±17	103±17	100±15
<b>MAP (mmHg)</b>	120±15	108±18	85±22	81±22	89±22
<b>Inotropes</b>					
Noradrenaline (µg/min)	0	0	11.9±9.3 (n=5/7)	11.6±4.4 (n=8/8)	9.17±3.9 (n=13/14)
Dopamine (µg/min)	0	0	220±92 (n=4/7)	163±103 (n=7/8)	136±49 (n=9/14)
Vasopressin (unit/hr)	0	0	0	3.3±1.1 (n=5/8)	3.4±1.3 (n=4/14)
<b>Fluid Balance</b> @ 24hr (ml)	378±251	1289±820	4346±1270	9226±2244*	9698±2514*

2

3

Figure





