

# SYSTEMIC INFLAMMATORY RESPONSE DURING LUNG RECRUITMENT MANEUVERS IN A NEWBORN ANIMAL MODEL

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Research

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## CONFLICTS OF INTEREST

There are no conflicts of interest for any of the authors.

## ABSTRACT

**Background:** The benefits of lung recruitment maneuvers (RMs) in adult and pediatric patients undergoing anesthesia have been well documented. However, RMs may induce lung injury and pro-inflammatory changes. This study assesses the effects of airway pressure steps in lung RMs on the systemic inflammatory response, lung mechanics and arterial blood oxygenation in a healthy-lung porcine model, and evaluates the possibility of RMs extrapolation to human newborns.

**Material and Methods:** Eight healthy-lung newborn male piglets received mechanical ventilation in pressure-controlled mode. The effectiveness of the RM applied was assessed by comparing oxygenation and lung mechanics among the different pressure steps. The levels of blood cytokines: TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-10 were determined before and 60 minutes after the onset of the RM.

**Results:** Maximum positive end-expiratory pressure (PEEP) was 20cmH<sub>2</sub>O with a 35cmH<sub>2</sub>O peak pressure step. Optimum dynamic compliance-PEEP was reached in the descending branch. A significant increase in IL-1 $\beta$ , IL-6, IL-8 levels was observed after the RM. Neither TNF- $\alpha$  nor IL-10 were elevated by the RM. There was no correlation between respiratory parameters, cardiac index and cytokine plasma levels following the RM.

**Conclusions:** Although this ventilatory strategy induced a systemic inflammatory response, the effects of the RM did not seem to be clinically relevant. The RM applied improved arterial blood oxygenation and lung mechanics. This strategy could be useful in neonatal anesthesia however, further research is warranted before extrapolating this RM to human newborns.

**KEYWORDS:** anesthesia, cytokines, mechanical ventilation, recruitment maneuvers.

## INTRODUCTION

General anesthesia abolishes the sigh reflex with rapid onset of atelectasis. Studies have shown a strong correlation between atelectasis and postoperative pulmonary complications. On some occasions, about 10% of newborns need some assistance to breathe at birth.<sup>[1]</sup> The newborn is more susceptible to lung injury induced by mechanical ventilation (MV) as the lung is transitioning from being filled of fluid to air. When lung injury appears in neonates, it increases the risk for the development of chronic respiratory disease. Likewise, healthy-lung animal newborns  $\leq 48$  hours of life under general anesthesia are likely to suffer from lung collapse owing to spontaneous ventilation failure.<sup>[2]</sup> The use of lung recruitment maneuvers (RMs) may reduce postoperative pulmonary complications and improve the patient outcomes.<sup>[3]</sup>

In healthy-lung newborns who undergo general anesthesia for prolonged surgical procedures, the RMs have been proposed as a protective ventilatory strategy that reduces the collapse of lung regions and prevents lung injury secondary to the cyclic closing of alveoli. The RMs induce and/or increase lung mechanical stress during MV. It is believed that newborns are more sensitive to high-pressure RMs. However, there are very few studies of the RMs in healthy-lung children undergoing anesthesia. MV along with open lung and lung protection strategies may be beneficial in neonates to prevent alveoli collapse during anesthesia but in these patients, an RM may generate biotrauma<sup>[4]</sup>, barotrauma<sup>[5]</sup> and hemodynamic instability<sup>[6]</sup>, including bradycardia and even cardiac arrest.

MV is also associated with increased activation of inflammatory mediators, especially cytokines.<sup>[7]</sup> Currently, it is widely accepted that an elevated production of cytokines, chiefly interleukin (IL)-1 $\beta$ , IL-6, IL-8 and tumor necrosis factor (TNF)- $\alpha$ , plays a key role in initiating or perpetuating lung injury.<sup>[8,9]</sup> Three mechanisms may be responsible for this release (1) a direct trauma to the cell with disruption of the membranes, (2) a response to cyclic stretch of pulmonary cells, such as alveolar macrophages, epithelial and endothelial cells, and (3) an opening and cyclic alveolar collapse.<sup>[10]</sup> Pulmonary inflammatory response is measured by plasma cytokine levels which raise in certain pathologies and in multi-organ dysfunction.<sup>[11]</sup> A balance between pro-inflammatory cytokines (TNF- $\alpha$ , IL-1, IL-6, IL-8) and anti-inflammatory cytokines (IL-10) is a crucial factor concerning the immune response.<sup>[12]</sup>

Although the benefits of lung recruitment in anesthetized adult patients have been well documented, there is an increase in the potential risks of barotrauma and biotrauma associated with the RMs in newborns. Different studies have reported that

MV does not induce a release of cytokines into the systemic circulation.<sup>[7,13]</sup> For this reason, the present experimental pilot study has assessed the systemic inflammatory response in neonates after an RM which, to our knowledge, has not been investigated. Furthermore, this study has evaluated (1) whether a prolonged RM prevented atelectasis and improved lung function in healthy-lung newborns undergoing prolonged general anesthesia, (2) whether an RM induced cytokine high plasma levels, and (3) whether the systemic inflammatory response correlated to cardiac output, arterial blood oxygenation and lung mechanics.

## MATERIAL AND METHODS

### *Ethics*

The study was approved by the ethics committee for experimental research of La Paz University Hospital, Madrid, Spain. The experimental work was performed in accordance with the ARRIVE (Animal in Research: In Vivo Experiments) and EU Directive 2010/63/EU for animal experiments guidelines. Additionally, the prospective research was undertaken conforming to the national and institutional guidelines for the care and use of experimental animals.

### *Animals in an experimental study*

Piglets  $\leq 48$  hours of life or declared unhealthy after physical examination were excluded from the current study. Initially, ten healthy newborn male piglets of the Landrace-Large White breed,  $\leq 48$  hours of life and with a body-weight of  $3.014 \pm 43$  g were enrolled in this experimental work. Two piglets were excluded due to technical complications unrelated to MV.

### *Anesthesia and monitoring*

Sedation was induced with ketamine (10 mg kg<sup>-1</sup>) intramuscularly (IM) (Ketolar, Pfizer, Madrid, Spain) and midazolam (0.3mg kg<sup>-1</sup>) IM (Dormicum, Roche Farma, Madrid, Spain) twenty minutes before anesthesia. Anesthesia was induced with sevoflurane 6% of tidal volume via inhalational route (Sevorane, Abbott, Quebec, Canada), fentanyl (3  $\mu$ gkg<sup>-1</sup>) intravenously (IV) (Fentanest, Khern Pharma, Barcelona, Spain) and atracuriumbesylate (0.3mg kg<sup>-1</sup>) IV (AtracuriumBesylate, INIBSA, Barcelona, Spain). Anesthesia was maintained with sevoflurane 2% by inhalation and fentanyl (2mg kg<sup>-1</sup>) IV every 30 minutes. Neuromuscular blockade was maintained with atracurium (0.7mg kg<sup>-1</sup>/minute) in continuous intravenous perfusion to prevent the effects of muscle tone from influencing our results. A volume load of 0.9% physiological saline solution at 10 mL kg<sup>-1</sup> body-weight was administered to prevent low cardiac preload status and

hypovolemia that could result from the application of the RM.<sup>[14]</sup> All of the animals underwent tracheal intubation with a 3.5 mm cuffed endotracheal tube and were hemodynamically monitored using an Omicron Altea Monitor (RGB Medical Devices, Spain). Electrocardiography (ECG DII), pulse oximetry (SpO<sub>2</sub>), invasive arterial pressure in the internal carotid artery (Vygon 20 G. France), central venous pressure (CVP) in the external jugular vein (Vygon 20 G. France) and temperature were measured. The hemodynamic status of the piglets was assessed by comparing pulse, systolic, mean and diastolic arterial pressure and CVP values at each pressure step with their baseline values. Inotropes were not administered. The central temperature of the animals was maintained at 37-38°C with an electric blanket.

### **Lung Recruitment Maneuvers**

All of the newborn piglets were mechanically ventilated in pressure-controlled mode (Flow i C30 ventilator; Maquet, Solna, Sweden). Throughout the RM pressure steps, the ventilator settings used were as follows: inspiratory to expiratory ratio (I: E) of 1:1; respiratory rate at 25 breaths per minute. The piglets were relaxed and did not show auto-PEEP, and received 40% oxygen concentration throughout the study. After intubation, the baseline airway pressures (pressure step 1) were performed with zero end-expiratory pressure (ZEEP) and MIP 15cmH<sub>2</sub>O.

The recruitment maneuvers were carried out by adapting Tusman's method<sup>[15]</sup> A fixed driving pressure of 15cmH<sub>2</sub>O was maintained throughout all of the pressure steps of the RM. In the ascending branch of the RM, PEEP was increased progressively in 5cmH<sub>2</sub>O at each step every 5 minutes. The maximal pressure reached was PEEP 20cmH<sub>2</sub>O and MIP 35cmH<sub>2</sub>O (pressure step 5). In the descending branch of the RM, PEEP was reduced in 2cmH<sub>2</sub>O at each step every 2 minutes until obtaining a more accurate calculation of Max CDyn-PEEP. In this animal model, a total of 14 pressure steps (five ascending and nine descending) were performed in accordance with previous studies (Fig.1).

At each step, the following dynamic ventilatory pressure values were taken from the anesthesia workstation: tidal volume (VT); airway compliance (Cdyn); and mean airway pressure (Paw). Hemodynamic parameters were also measured. After performing the 14 pressure steps, the incremental branch of the RM was conducted a second time with a prompt decrease of PEEP and MIP until reaching the step at which maximal compliance was obtained. This method has been described in relation to the estimation of open-lung PEEP.<sup>[16,17]</sup> To detect the

occurrence of baro-volutrauma, the flow-time and pressure-time curves of the respirator were analysed, and to detect the presence of pneumothorax and subcutaneous emphysema, a radiography was required when the optimum lung opening was reached. We assessed whether a prolonged RM induced an inflammatory response, the data collected from each piglet were compared to the data gathered at the beginning of the experiment (baseline: MIP 15cmH<sub>2</sub>O, PEEP 0cmH<sub>2</sub>O) and to the results obtained 60 minutes following the onset of the RM (after achieving Optimum lung opening: Max CDyn-PEEP 2cmH<sub>2</sub>O) (Fig. 1).

### **Oxygenation evaluation**

The collection of blood samples for the study of oxygenation parameters was performed at basal pressure step 1, prior to the RM, and at pressure steps 2 to 5 of the ascending branch and at steps 6 to 14 of the descending branch. Sample analysis was carried out by gasometer (ABL 90 FLEX analyzed, Radiometer, Denmark). pH, arterial partial pressure of oxygen (PaO<sub>2</sub>), venous partial pressure of oxygen (PvO<sub>2</sub>), arterial content of oxygen, venous content of oxygen, arterial SaO<sub>2</sub> and venous O<sub>2</sub> saturation (SvO<sub>2</sub>) (%), hemoglobin (Hb), HCO<sub>3</sub> and lactate were measured.

The data obtained at each pressure step of the RM by indirect calorimetry using an integrated ventilator module in the anesthesia workstation were used to estimate oxygen consumption (VO<sub>2</sub>) and cardiac index (CI) derived from Fick's equation.<sup>[18]</sup> Hemodynamic instability was considered when there were  $\pm 20\%$  alterations in the studied parameters, compared to their baseline values.

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### **Cytokine analysis**

We used an indwelling venous line to draw a 3 ml blood sample, and stored the sample in EDTA tubes. The plasma was separated from erythrocytes by centrifugation at 3500g for 10 minutes. All of the samples were divided into aliquots and frozen at -82°C until the day of analysis. Concentrations of inflam-

matory parameters —TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10— were measured using a simultaneous porcine panel antigen standard lot: 2641108# with the Procarta immunoassay kit (Affymetrix, Inc. Santa Clara, CA, USA) by Luminex analyzer (Luminex corporation, Austin, Texas, USA).<sup>[19]</sup> Detection limits in this assay were: TNF- $\alpha$ : 150 pg/mL, IL-1 $\beta$ : 20 pg/mL, IL-6: 5 pg/mL, IL-8: 20 pg/mL, IL-10: 5 pg/mL.

### Statistical analysis

Sample size was calculated based on Cdyn (primary outcome)<sup>[16]</sup>, TNF $\alpha$ <sup>[11]</sup> and on previous studies of animal models regarding RM.<sup>[20,21]</sup> The data are expressed as mean  $\pm$  SD (95% confidence interval) or median (interquartile range) according to distribution. The normality of the variables was assessed with the Shapiro-Wilk test, and the homogeneity of the variances was estimated with the Levene's test. The mean values obtained at the different steps of RM were analyzed by One-Way Repeated Measures (ANOVA) with a Bonferroni correction. Inflammatory parameters were compared at baseline and 60 minutes after the onset of the RM by using the non-parametric Wilcoxon sign-rank test. Correlations between the inflammatory variables, oxygenation and hemodynamic parameters were assessed using Spearman's test. All of the tests were two-tailed and a P-value <0.05 was considered statistically significant. The data were analyzed with the "Statistical

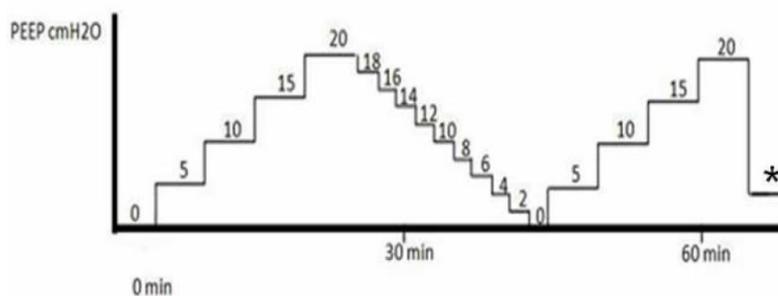
Package for the Social Sciences" (SPSS for Windows, release 18.0.0 2010, SPSS Inc, Chicago IL, USA).

### RESULTS

Table 1 includes the most important parameters of the pressure steps of the RM, as well as the comparison between the main pressure steps. Pressure step 14, (PEEP 2cmH<sub>2</sub>O and MIP 17cmH<sub>2</sub>O) exhibits a maximum increase in VT (P < 0.001) with a significant increase in PaO<sub>2</sub> (P < 0.05) and an optimum Cdyn-PEEP (2cmH<sub>2</sub>O). There were no significant changes in CI.

In addition, no changes were observed in the flow-time and pressure-time curves of the ventilator. The study of the chest radiograph showed an absence of pneumothorax or pneumomediastinum in all of the piglets after performing the RM. All of the piglets showed no complications during the time the RM was applied. They recovered from anesthesia and were employed in another experimental work.

Table 2 displays the systemic inflammatory response after the RM. There was a significant increase in IL-1 $\beta$ , IL-6 and IL-8 60 minutes following the RM, compared to the baseline levels. TNF- $\alpha$  and IL-10 levels remained unchanged. No correlation between VT, Cdyn, pH, CI and cytokine plasma levels after the RM with Max Cdyn-PEEP 2 cmH<sub>2</sub>O was detected (Fig1).



**Fig. 1.** In this animal model, in the first phase of the recruitment maneuvers (RM) a total of 14 pressure steps (five ascending and nine descending) were performed. In the second phase of RM, in the descending branch PEEP was reduced until obtaining a more accurate calculation of Max CDyn-PEEP (pressure step 14)  
PEEP: Positive end expiratory pressure; MIP: Maximal inspiratory pressure  
\* Optimum lung opening identified as Max Cdyn-PEEP (2cmH<sub>2</sub>O)

-Baseline airway pressures (pressure step 1): -zero end-expiratory pressure (ZEEP); MIP 15cmH<sub>2</sub>O.  
-Maximal pressure reached (pressure step 5): PEEP 20cmH<sub>2</sub>O; MIP 35cmH<sub>2</sub>O

**Table 1.** Comparison between the course of arterial blood oxygenation and ventilation parameters during the main steps of lung recruitment maneuvers

PEEP/MIP (cm H <sub>2</sub> O)	Step1 0/15	Step5 20/35	Step 11 8/23	Step 12 6/21	Step 13 4/19	Step 14 2/17
VT (mL)	37.7±13.5	26.1± 6 *(a); (c,d,e,f)	58.5±12 †(a,b,d,e,f)	65±12.9 *(a); †(e,f,b,c)	72±13.8 *(a,f); †(d,b,c)	73.8±13.5 *(e); †(a,d,b,c)
Cdyn(mL/cm H <sub>2</sub> O)	2.5±0.6 *(b,c); †(d,e,f)	1.4±0.3 *(a); †(c,d,e,f)	3.8±0.8 *(a); †(e,b)	4.2±0.8 †(a,b)	4.7±1 †(a,b,c)	4.9±0.8 †(a,b)
PaO <sub>2</sub> (mmHg)	243±63 *(f)	278±107	265±99	289±114	296±121	302±117 *(a)
pH	7.4 ± 0.13 *(b)	7.2 ± 0.16 *(a) †(c,d,e,f)	7.4 ± 0.19 †(b,d,e,f)	7.4 ± 0.12 *(e,f) †(b,c)	7.5 ± 0.12 *(d) †(b,c)	7.5 ± 0.11 †(b,c,d)
CI (mL/min/m <sup>2</sup> )	1.6 ± 0.74	1.7 ± 0.95	2.02 ± 1.2	1.92 ± 1.2	1.7 ± 1.21	2.03 ± 1.6

PEEP: Positive end-expiratory pressure; MIP: Maximal inspiratory pressure; Cdyn: Dynamic compliance; PaO<sub>2</sub>: Arterial partial pressure of oxygen, CI: Cardiac index

Comparison of means obtained at the different pressure steps was tested with the analysis of variance test for repeated measurements (ANOVA) with a Bonferroni correction test. The data were expressed as mean ± SD (\*P < 0.05, † P < 0.01).

a: Differences with respect to step 1; b: Differences with respect to step 5; c: Differences with respect to step 11; d: Differences with respect to step 12; e: Differences with respect to step 13; f: Differences with respect to step.

**Table 2.** Concentrations of inflammatory biomarkers at baseline and 60 minutes after the onset of lung recruitment maneuvers

IL (pg/ml)	Baseline	60m after RM onset	p
<b>IL 10</b>	21.65 (9.18-259,81)	106.27(47.05-205.5)	0.735
<b>IL1β</b>	0.59 (0.31-1.49)	3.54 (1.73-12.5)	<b>0.017</b>
<b>IL6</b>	0.43 (0.05-24.7)	49.22(29.97-176.5)	<b>0.012</b>
<b>IL8</b>	6.83 (6.83-8.53)	83.68 (40.28-142.87)	<b>0.012</b>
<b>TNF α</b>	141.65 (83.89-2426.45)	286.63 (205.39-1067.8)	0.674

RM: Lung recruitment Maneuvers; IL: Interleukin.

The data were expressed as median (interquartile range). An asymmetric distribution Wilcoxon test was used to compare baseline parameters to parameters obtained 60 minutes after the RM. P < 0.05 value was considered statistically significant.

## DISCUSSION

The current study has demonstrated that the RM applied to a MV strategy has induced an increase in some inflammatory cytokines in the newborn piglets. However, this increase has had no significant clinical repercussions on CI and ventilation parameters. The type of RM performed in our newborn porcine model improves arterial blood oxygenation and lung mechanics. To avoid confounders, we did not

administer vasopressors and performed neuromuscular blockade during anesthesia.

Cytokine plasma levels, as well as hemodynamic, oxygenation and ventilation parameters were studied in each piglet and were later compared at baseline before and after the RM to assess whether a prolonged RM might cause an inflammatory response or might influence these variables (Table 1 & 2). Topuz U et al<sup>[22]</sup> have determined the effects

of different oxygen concentrations during the RM on arterial oxygenation and respiration mechanics. The average postoperative partial arterial oxygen pressure values of the groups of patients with different concentrations of oxygen were statistically significant. In our study, we performed an RM with 40% oxygen concentration to avoid the possible negative influence by high concentrations of oxygen in respiratory mechanics and gas exchange.

The search for an efficient protocol that minimizes the risks associated with MV has led to researchers to test different strategies.<sup>[15,23-25]</sup> After lung recruitment, PEEP is optimized to maintain gas exchange by stabilizing lung volume and keeping the alveoli open. One of the validated ways of selecting optimum PEEP is the optimum level of PEEP that has been identified as maximum dynamic compliance (Max C<sub>dyn</sub>-PEEP).<sup>[16,17]</sup>

This indicates that the RM did not cause lung injury with clinical repercussion as published in a previous work by our research group.<sup>[26]</sup> The RM was performed for a much longer period of time than conventionally established to assess the impact of the RM and the inflammatory response studied by the quantification of cytokine plasma levels. Simultaneously we analyzed whether the inflammatory response influenced cardiac output, arterial blood oxygenation and lung mechanics.

There is considerable debate as to whether pure MV stress per se, without preceding lung injury, may initiate cytokine-mediated pulmonary inflammatory responses.<sup>[27]</sup> On the other hand, different studies have reported that cytokines are minimally activated in both bronchoalveolar lavage fluid and in plasma during classical MV.<sup>[7,27]</sup> The aim of the present study was to assess the inflammatory response following the RM. Low VT and PEEP appear to preserve alveolar integrity and reduce the risk of ventilator-induced lung injury with a lower release of systemic inflammatory mediators.<sup>[28]</sup> An inflammatory response has been observed, as in our study, in healthy animals only when VT is significantly higher than considered conventional.<sup>[29]</sup> Chiumello et al<sup>[30]</sup> have reported an increase in cytokine concentrations in a group ventilated with high VT and zero PEEP. It has been suggested that considerably high VT may produce a similar degree of alveolar over-distension to that observed regionally in acute respiratory distress syndrome patients receiving much lower VT.<sup>[31]</sup> Therefore, in our study, the elevation of IL-6, and perhaps of IL-8 and IL-1 $\beta$ , could have been driven by high VT. A mouse model of ventilator-induced lung injury has shown that high-VT ventilation without preceding lung injury up-regulates intrapulmonary cytokines TNF- $\alpha$ .<sup>[32]</sup> TNF- $\alpha$  appears to be involved in the pathogenesis of

both ventilator-induced lung injury and multiorgan dysfunction syndrome.<sup>[33]</sup> In contrast, TNF- $\alpha$  remained unchanged in the present study, suggesting that lung injury was only moderate in the current porcine model.

Halbertsma FJ et al<sup>[11]</sup> have conducted a study with a cohort of 7 hemodynamically stable pediatric patients with acute lung injury. An elevation of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  was observed 15 minutes following the RM. The levels of these cytokines decreased 60 minutes after the procedures, which proved the prevalence of pulmonary translocation. Moreover, the prolonged elevation of systemic TNF- $\alpha$ , IL-1 $\beta$  and IL-6 is associated with increased morbidity and mortality. However, the balance between pro-inflammatory cytokines TNF- $\alpha$ , IL-1, IL-6, IL-8 and anti-inflammatory cytokines, such as IL-10, is essential for directing the immune response. It is important to emphasize that in our study, anti-inflammatory IL-10 did not increase during the RM, which could be indicative of no significant inflammatory lung injury. Consequently, a ventilatory strategy with sufficient PEEP—optimal PEEP—and a limited MIP seems to be pivotal to avoid inflammation. No correlation between VT, C<sub>dyn</sub>, pH, CI and cytokine plasma levels was observed 60 minutes after the onset of the MR (Table 2).

One of the limitations of our study is the sample size of the experimental model that, although similar to another published by other authors<sup>[16,20,21]</sup> and adequate to prove the results obtained, it did not enable us to demonstrate whether the values of cytokines reflected biological variability. Another limitation was the lack of inclusion of a control group because this study has focused on a healthy-lung animal model and sample gathering was made before the RM (baseline: MIP 15cmH<sub>2</sub>O, zero PEEP) and 60 minutes after the onset of the maneuvers, comparing the two time-points. Furthermore, instead of studying the inflammatory response at each pressure step, we considered it more interesting to measure cytokine plasma levels after a prolonged RM. For this reason, the values of IL-10, IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  were compared to the values obtained 60 minutes after the onset of the RM, once optimum lung opening was reached (Max C<sub>dyn</sub>-PEEP 2cmH<sub>2</sub>O). In this way, it was also possible to study whether the inflammatory response correlated with VT, C<sub>dyn</sub>, pH and CI.

We have conducted a study of healthy-lung newborn male piglets, whereas most studies have been directed toward animals or patients >6 months with acute respiratory distress syndrome or ALI.<sup>[11,31]</sup> Moreover, the RM in our study was applied for a much longer period than what has been normally

used in other published animal models to assess the effects of this ventilatory strategy more accurately.

In conclusion, our findings have shown that although the RM induced an inflammatory response objectified by increasing the plasma levels of IL1B, IL6 and IL8, the RM did not cause significant inflammatory damage as no changes were detected in the anti-inflammatory cytokines. The slight inflammation produced by the RM had no clinical repercussion because it did not correlate with VT, Cdyn, PH and CI. The RM significantly improved oxygenation and ventilation parameters. For this reason, we considered that this ventilatory strategy could be useful in neonatal anesthesia. However, given the challenges for the transfer of results from animal to human trials, further research is warranted before extrapolating the RM to human newborns.

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#### STATEMENT OF AUTHORSHIP

A.M.O. and J.G.F designed the study and performed experiments; F.L.L.C. and M.J.T. collected and analysed data; M.G.C. and I.I.R. analyzed the data and revised the work; J.L.P.N. analyzed the data and drafted the manuscript. All of the authors have read and approved the final version of the manuscript..

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