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An Individualized Approach To Sustained Inflation Duration At Birth Improves Outcomes In Newborn Preterm Lambs

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Glossary

$AaDO_2$	Alveolar-arterial difference in oxygen
ABP	Arterial blood pressure
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
CBF	Cerebral Blood Flow
C_{dyn}	Dynamic respiratory system compliance
C_{RS}	Static respiratory system compliance
CI	Confidence Interval
CTGF	Connective Tissue Growth Factor
CYR61	Cysteine-rich 61
EGR1	Early Growth Response protein 1
EEV	Global end-expiratory volume measured by EIT
EIT	Electrical Impedance Tomography
ETT	Endotracheal tube
fEIT	Functional EIT
FiO_2	Fraction of inspired oxygen
FOT	Forced Oscillation Technique
FRC	Functional Residual Capacity
IL	Interleukin
IQR	Interquartile range
ΔP	Pressure amplitude
Pa_{O2}	Partial pressure of arterial oxygen
Pa_{CO2}	Partial pressure of arterial carbon dioxide
P_{AO}	Pressure at the airway opening
P_{AW}	Mean airway pressure
PIP	Peak inflating pressure
PEEP	Positive end-expiratory pressure
PPV	Positive pressure ventilation
R_{RS}	Respiratory system resistance
SEM	Standard error of the mean
SD	Standard deviation
SI	Sustained Inflation
SpO_2	Peripheral oxyhaemoglobin saturation
V_T	Tidal volume
VTV	Volume-targeted ventilation
X_{RS}	Respiratory system reactance

Abstract

A sustained first inflation (SI) at birth may aid lung liquid clearance and aeration, but the impact of SI duration relative to the volume-response of the lung is poorly understood. We compared three SI strategies; 1) variable duration defined by attaining volume equilibrium using real-time electrical impedance tomography (EIT; SI_{plat}), 2) 30s beyond equilibrium (SI_{long}) and 3) short 30s SI (SI_{30}) and 4) positive pressure ventilation without SI (no-SI) on spatio-temporal aeration and ventilation (EIT), gas exchange, lung mechanics and regional early markers of injury in preterm lambs. Fifty-nine fetal-instrumented lambs were ventilated for 60 min after applying the allocated first inflation strategy. At study completion molecular and histological markers of lung injury were analysed. The time to SI volume equilibrium, and resultant volume, were highly variable; mean (SD) 55 (34)s, coefficient of variability 59%. SI_{plat} and SI_{long} resulted in better lung mechanics, gas exchange and lower ventilator settings than both no-SI and SI_{30}. At 60 min, alveolar-arterial difference in oxygen was a mean (95% CI) 130 (13, 249) higher in SI_{30} versus SI_{long} group (2-way ANOVA). These differences were due to better spatio-temporal aeration and tidal ventilation, although all groups showed redistribution of aeration towards the non-dependent lung by 60 min. Histological lung injury scores mirrored spatio-temporal change in aeration and were greatest in SI_{30} group ($p<0.01$, Kruskal-Wallis test). An individualized volume-response approach to SI was effective in optimizing aeration, homogeneous tidal ventilation and respiratory outcomes, whilst an inadequate SI duration had no benefit over positive pressure ventilation alone.

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INTRODUCTION

The majority of extremely preterm infants require respiratory assistance in the Delivery Room (41). In part this is because many of these infants do not have the ability to generate the initial prolonged high transpulmonary pressures required to drive lung fluid from the main airways, allow alveolar aeration, establish functional residual capacity (FRC), and then maintain it during tidal ventilation, essential processes for efficient gas exchange and lung protection (19, 31). Recently, applying an initial sustained inflation (SI) at birth, consisting of an elevated pressure applied for longer than needed for usual tidal inflation, followed by sufficient positive end-expiratory pressure (PEEP), has been proposed as a method of generating the initial transpulmonary pressure needed at birth (10, 18, 20). SI have been extensively investigated in preterm animals (15, 26, 29, 32, 33, 35-38) and humans (10, 18, 34) with conflicting results. Some studies suggested SI improved aeration, FRC and cerebral oxygen delivery (29, 32, 33), whilst others failed to demonstrate any benefit over standard respiratory support with sufficient PEEP (26, 35-37). SI was associated with increased lung injury in two studies (11, 12). These data suggest the optimal SI strategy still needs to be elucidated. Consequently, current ILCOR guidelines do not recommend its routine use in newborns (24).

All studies of SI have used pre-defined pressure, duration and/or inflating volume goals (11, 12, 18, 26, 29, 32, 33, 35-38). These approaches assume that the lungs of preterm infants will behave in a consistent, predictable and uniform manner. This assumption is inconsistent with our understanding of the mechanical properties of the diseased lung (3, 23, 38). It is unlikely that a single duration, pressure or volume will be appropriate for all individuals. A SI of pre-defined duration may be too short or too

long, exposing the lung to the injurious consequences of inadequate aeration or excessively prolonged pressure. An alternative approach is to individualize a SI to the mechanical state of the lung and resultant volumetric behavior. It is well established that volume change during inflation behaves exponentially, requiring duration of five time constants to achieve steady state volume (equilibrium). Although some previous studies reported whether or not lung volume had attained steady state during the SI (32, 33, 37), all did so using post hoc volume analysis. Recently, we found that the individual volume response to a predefined 30-s SI in preterm lambs was highly variable, and in most recipients insufficient to attain volume steady state (37).

Advances in real-time imaging of the lung using electrical impedance tomography (EIT) now allow immediate display of global lung volume change at the bedside during birth transition in experimental animals (37, 38). This offers, for the first time, the ability to individualize SI application to ensure optimal aeration. We hypothesized that 1) there would be inter-subject variability in the time needed to obtain lung volume steady state (plateau) during a SI, that 2) the application of a variable duration 'volumetric SI', defined by the attainment of a real-time volume equilibrium, would result in better short-term and lung injury outcomes than a SI that was too short for an individual lung, and 3) a SI applied beyond volumetric steady state would expose the lung to the risks of regional overdistension.

The aim of this study was to compare, in preterm lambs, conventional ventilation without an SI with three SI strategies: 1) an SI with a duration determined by the achievement of lung volume equilibrium, 2) an intentionally short SI and 3) a SI prolonged beyond that required to reach equilibrium. The primary outcomes were the

time to stable lung volume (hypothesis 1) and spatio-temporal patterns of aeration and tidal ventilation, oxygenation (expressed as alveolar-arterial difference in oxygen; $AaDO_2$) and lung mechanics (hypotheses 2 and 3). In addition, haemodynamic parameters and the role of each strategy on regional early markers of lung injury and the interaction with regional aeration and ventilation were examined.

METHODS

The study was performed at the animal research facility of the Murdoch Childrens Research Institute (Melbourne, Australia) and approved by our Animal Ethics committee in accordance with the National Health and Medical Research Committee (Australia) guidelines.

Experimental Instrumentation

Border-Leicester/Suffolk lambs, gestational age 127±1 days (term ~147 days) were delivered via caesarean section under general anaesthesia to ewes exposed to betamethasone 11.4 mg IM 24 and 48 hours prior to delivery. Twin-pregnancy ewes were chosen to minimise maternal and environmental variability, and optimise reduction. A single supplier cared for ewes and mating occurred during the same season to standardise environmental factors. The lambs were born via caesarean section under general anaesthesia with isoflurane, intravenous propofol and nitrous oxide using our previously described protocol (35, 37, 38). The fetal head was first exteriorised and the right jugular vein and carotid artery cannulated, and the left carotid artery encircled with a 4 mm transit-time flow probe (Transonic Systems, Ithaca, NY). Lambs were intubated with a 4.0 cuffed endotracheal tube (ETT), the chest exposed and dried (35, 38) and fetal lung fluid passively drained to 10-15

mL/kg of anticipated birth weight before clamping the ETT. Electrical Impedance Tomography (EIT) needles were positioned equidistance around the chest in a transverse plane approximately 1 cm above the xyphisternum. The electrodes were secured in place with self-adherent bandage (Coban, 3M, St. Paul, MN) and connected to a Goe-MF II EIT system (CareFusion, Hoechberg, Germany), as previously described (4, 26, 35-38). After verification of the instrumentation, the lamb was delivered, weighed and placed supine under an infant warmer. Temperature and peripheral oxygen saturation (SpO_2) probes were positioned, vascular lines and ventilator connected and a 10 s EIT recording of the unaerated lung taken prior to un-clamping the ETT. Throughout the study continuous infusions of ketamine (4-12 mg/kg/hr) and midazolam (0.05-0.15 mg/kg/hr) were used to maintain anaesthesia, analgesia and suppress any spontaneous respiratory effort.

Measurements

The experimental protocol is summarised in Figure 1A. Peripheral oxygen saturation (SpO_2), heart rate (HR), arterial blood pressure (ABP) (HP48S monitor, Hewlett Packard, Andover, MA, US), carotid blood flow (CBF) (TS420 Perivascular Flow Module, Transonic Systems, Ithaca, NY), airway pressure (P_{AO}), gas flow and tidal volume (V_T) at the airway opening (Florian, Acutronic Medical Systems AG, Hirzel, Switzerland) were measured continuously from birth. Global and regional lung volume changes were acquired by EIT at 25 scans/s (35, 37, 38) and the unfiltered global lung volume change displayed in real-time using the Thorascan software package (CareFusion, Hoechberg, Germany). Arterial blood gases were performed at 5 min of life and every fifteen min from birth. A 5-Hz oscillatory pressure was superimposed onto the ventilation waveform for 10 s on completion of the ventilation

strategy at birth and immediately after every arterial blood gas sample to calculate lung mechanics using the forced oscillation technique (FOT) as described in Dellacà *et al* (6, 7, 43).

Ventilation Strategies at birth

Lambs were randomly assigned before the delivery to one of the following groups:

1. **Control (no-SI)** group who received positive pressure ventilation (PPV) in a volume-targeted ventilation (VTV) mode from birth without a SI. Initially, PPV was delivered with a PEEP 8 cmH_2O, inflating pressure (PIP) limit 40 cmH_2O, inspiratory time 0.4 s, rate of 60 inflations per minute and set tidal volume (V_T) of 7 mL/kg (SLE 5000 infant ventilator, SLE systems, Croydon, UK). These settings have previously been shown to be physiologically appropriate in our lamb population (35).
2. **Short, fixed-duration SI (SI_{30})** of 30 s (Figure 1B and Supplemental Video 1), which was unable to achieve lung volume plateau in our previous study (38) but described previously in preterm lambs (15).
3. **Volume-plateau SI (SI_{plat})** individualised in each lamb and consisting of a SI delivered until 10 s after a visually determined plateau by two investigators (CZ, DT) in the global EIT time-course volume signal on the Thorascan display (Figure 1B and Supplemental Video 2).
4. **Prolonged SI (SI_{long})**, also individualised in each lamb, and delivered until 30-40 s after visual observation of a plateau in the global EIT volume signal by the same investigators (Figure 1B and Supplemental Video 3).

239 All sustained inflations were delivered at 40 cmH_2O (8 L/min gas flow) using the
240 Neopuff™ Infant T-Piece Resuscitator (Fisher & Paykel Healthcare, Auckland, New
241 Zealand). On completion of the SI the lung was held at a PEEP of 8 cmH_2O for 5 s
242 prior to clamping the ETT and transferring the lamb to the SLE5000 ventilator. PPV
243 was then commenced as per the no-SI Group.
244
245 *Ventilation strategy and general management after birth*
246 All lambs were initially ventilated using 0.21 FiO_2 until the first arterial blood gas,
247 and then adjusted to maintain a SpO_2 88-95%. Ventilator settings were adjusted after
248 each arterial gas to maintain an arterial partial pressure of carbon dioxide (Pa_{CO2}) of
249 45-65 mmHg, by firstly altering V_T, and then rate if hypocarbia at V_T 5 mL/kg. Body
250 temperature was kept between 38-39°C (physiological for lambs) and hydration
251 maintained with a NaCl 0.18%, glucose 4% intravenous infusion.
252
253 At 60 min of life the animals were ventilated with 1.0 FiO_2 for 3 min, and then
254 received a lethal dose of pentobarbitone. The ETT was then disconnected to
255 atmosphere until lung collapse. A static *in vivo* super-syringe pressure-volume curve
256 was generated (maximum pressure 40 cmH_2O) to determine the static mechanical
257 properties of the respiratory system and calibrate the EIT signal (1, 26, 37). An
258 additional ten fetuses (**Fetal** Group) were euthanized at delivery as unventilated
259 controls for injury analysis comparison.
260
261 *Data processing and analysis*
262 Sp_{O2}, HR, ABP, body temperature, CBF, P_{AO}, flow and expiratory V_T were recorded
263 at 1000 Hz (PowerLab, AD Instruments, Sydney, Australia), processed and analysed

using Labchart 7 (AD Instruments, Sydney, Australia). Together with these data, EIT data were monitored in real-time and recorded (Thorascan, Carefusion, Germany) continuously for the first 15 minutes, and subsequently for 2 minutes with each arterial blood gas. EIT signals were also recorded during the static *in vivo* PV curve. PIP, PEEP and mean airway pressure (P_{AW}) were determined from the P_{AO} data, and dynamic compliance (C_{dyn}) calculated from the ΔP and V_T data. Total respiratory system reactance (X_{RS}) and resistance (R_{RS}) were computed from FOT recordings (6). X_{RS} is a measure of the elastic and inertive characteristics of the respiratory system that has been shown to be an accurate indicator of lung recruitment in our preterm lamb model (43). Static respiratory system compliance (C_{RS}) was determined from the PV curve (35). The alveolar-arterial difference in oxygen ($AaDO_2$) was calculated from the arterial blood gases.

Time-series images of the change in impedance were reconstructed from EIT data using a GREIT algorithm based on the correct anatomical shape of the lamb chest (2, 9) determined from CT chest images in pilot 128 day gestation fetal lambs (37). Data acquired immediately prior to commencing ventilation were used as reference for image reconstruction (38). Due to the elongated shape of the lamb chest, the resulting 32x32 pixel EIT images contained 586 active (non-zero) pixels, which were used for all subsequent analysis. EIT images were analysed using our previous described method after low-pass filtering to the respiratory domain with a cut-off at 130 inflations per minute (38). The expiratory trough values of the EIT signal in individual image pixels were used to determine both global and regional changes in EEV from birth (ΔEEV) in the entire cross-sectional slice (global) slice of the thorax.

The global ΔEEV signal was calibrated (mL/kg) to the change in impedance during the known-volume static PV curve (1, 17, 22).

Two types of functional EIT (fEIT) images were constructed from at least 20s of continuous artefact-free data for each time point and the spatial distribution of ventilation and aeration determined from these using the methods of Frerichs and co-workers (8). fEIT images of the end-expiratory minimum value were used to define aeration and the tidal amplitudes (minimum to maximum values) (8). Based on the CT data the uppermost 3 and lower most 7 slices were excluded, as they contained no lung tissue. This allowed for relative V_T and aeration to be determined in 22 non-dependent to dependent equal slices of the right and left hemithoraces. From these measurements, histograms of the gravity dependent distribution of V_T and aeration within the lung, expressed as a percentage of total, were created (37). Comparisons of relative regional aeration differences were made between three gravity-dependent lung regions (upper, middle and lower) of equally weighted lung tissue (that is equal number of pixels) expressed as the percentage of total aeration (%).

Sustained Inflation ΔEEV modelling

The duration of each SI was determined from the global time course ΔEEV signal and the final delivered volume (mL/kg) calculated. To determine the predicted time required to achieve a plateau lung volume for each SI a one-phase exponential model (23, 28, 38) was applied to each global EIT signal and the static time constant of the respiratory system (τ), predicted time to plateau EEV (t_{plat}, defined as 5τ) and predicted plateau EEV calculated:

$y = y_{plateau}.(1\text{-}e^{(-k.x)})$; where $y_{plateau}$ = EEV_{max} during SI, x = time (s) and k = reciprocal of τ

Lung Injury Analysis

After autopsy, total left lung protein concentration was calculated on broncho-alveolar lavage fluid (14) using the Lowry method (21). Five H&E stained sections from each of the upper, middle and lower gravity-dependent thirds of the right upper lobe (fixed at 20 cmH_2O with 4% paraformaldehyde) were scored for lung injury (n=15 total/lamb) by an investigator blinded to treatment allocation. A score out of 5 was assigned for each of 1) alveolar wall thickness, b) detached epithelial cells, c) hyaline membranes presence and d) alveolar collapse/atelectasis. On completion, 10% of slides were re-scored blinded. Injury markers were compared to the values in Fetal group. The highest and lowest score from each of the five slides in each region for each lamb were removed from subsequent analysis. Gravity dependent and non-dependent samples of the right lower lobe approximating the gravitational regions of EIT analysis were analysed by quantitative real-time PCR for known early markers of lung injury (*CTGF*, *CYR61*, *EGR1*, *IL-1β*, *IL-6* and *IL-8* mRNA)(40) using *RSP29* as the reference gene and the $2^{-\Delta\Delta CT}$ method (40).

Statistical Analysis

A sample size of 8 lambs per group would detect a clinically meaningful difference (SD) in $AaDO_2$ of 100 (100) mmHg and C_{dyn} of 0.1 (0.1) $mL.kg^{-1}/cmH_2O$, assuming a power of 0.8 and alpha error 0.05. To account for potential error in antenatal parity assessment, the role of antenatal steroids, maternal and anticipated intersubject variability in SI volume response, mating was based on 12-13 lambs per treatment

group. Data were tested for normality and analysed with one-way ANOVA, Kruskal-Wallis test or two-way repeated measures ANOVA (using time and ventilation strategy as factors), and Tukey's and Dunn's multiple comparison post-tests as appropriate. Statistical analysis was performed with GraphPad PRISM 6 (GraphPad Software, San Diego, CA) and a $p<0.05$ considered significant.

RESULTS

Fetal characteristics

59 lambs were studied. The groups were well matched for birth weight, GA, fetal lung fluid drained and fetal wellbeing (Table 1). One lamb in the no-SI group was excluded due to unrecognised oesophageal intubation. Five lambs developed pneumothoraces, one lamb at 33 min of life in the SI_{30} group, and two in each of the no-SI and SI_{30} groups after inflation to 40 cmH_2O during the static super-syringe PV curve. Two lambs in the fetal group were excluded due to fetal hypoxia.

SI characteristics

Overall SI duration was 21.4 s longer in the SI_{long} group compared to $SI_{plat,}$ but this was not statistically different (Figure 2A), with both groups being significantly longer than SI_{30}; $p<0.0001$ (one-way ANOVA). The time needed to achieve the volumetric definitions of the SI_{plat} and SI_{long} strategies was highly variable; range 36.1-131.6 s (SI_{plat}) and 76.5-145.2 s SI_{long}, (combined coefficient of variability 59%). The one-phase exponential model was able to describe all the SI data with a good fit; median (IQR) R^2 0.950 (0.777, 0.988). Using this model there was no difference in the t_{plat} between the three SI strategies. The model provided a $t_{plat} \leq 30$ s in only 9 (24%) sustained inflations.

362 ***Global and regional lung aeration***

363 At the end of SI_{30}, SI_{plat} and SI_{long} the mean (SD) ΔEEV was 36 (21), 45 (36) and 50
364 (26) mL/kg, respectively (p=0.498; one-way ANOVA, Figure 2B), which compared
365 to ΔEEV at 30s of 12 (7) mL/kg in the No-SI group ($p<0.009$ and $p<0.0003$ vs SI_{plat}
366 and SI_{long} respectively). These differences were persistent at 100 s (or immediately
367 after SI if >100 s duration). The modelling predicted that a median (IQR) 85 (75,
368 92)% of total ΔEEV occurred within the first 30 s, and the additional 30 s beyond
369 plateau a median (IQR) 3.3 (2.5, 4.8)% above EEV at t_{plat}.
370
371 Figure 3 shows the global ΔEEV over time. Strategy ($p<0.0001$) but not time
372 (p=0.508) had a significant influence on ΔEEV (two-way ANOVA). There was no
373 difference between SI_{long} and SI_{plat} groups, or between no-SI and SI_{30} groups. The use
374 of SI_{plat} or SI_{long} resulted in approximately 10 mL/kg higher ΔEEV at 5 min compared
375 to no-SI and SI_{30}; all $p<0.042$ (Tukey's post-test), and remained significantly higher
376 until 30 min (no-SI), and 15 min (SI_{30}); all permutations (all $p<0.045$). The time-
377 based decrease in SI_{plat} and SI_{long} groups did not reach statistical significance.
378
379 The relative gravity-dependent spatial distribution of aeration varied between
380 strategies at 5 min of life (Figure 4A). SI_{30} and SI_{plat} showed the most uniform
381 distribution of aeration, whilst no-SI (p=0.010 and $p<0.0001$) and SI_{long} (p=0.009 and
382 p=0.001) had significantly less relative aeration in the non-dependent (upper) regions
383 compared with middle and lower (all Tukey's post-test). Heterogeneity was greatest
384 in the no-SI group, with significantly different relative contributions to dependent
385 (p=0.004) and non-dependent (p=0.045) aeration compared to SI_{30}. There were no
386 regional differences between other strategies.

Spatial aeration patterns changed with time, with all groups demonstrating gravity-dependent redistribution of aeration towards the non-dependent lung by 60 min (Figure 4A and B) compared to 5 min (all $p<0.01$; Tukey's post-test). Within each strategy the no-SI, SI_{30} and SI_{plat} groups had the heterogeneity of aeration at 60 min, and SI_{long} the most spatio-temporal uniformity. Between strategies, relative aeration was greater in the SI_{30} and SI_{plat} within the upper region compared to no-SI ($p=0.019$ and $p=0.012$ respectively), and greater in the no-SI group compared to SI_{plat} ($p=0.013$) and SI_{long} ($p=0.042$) within the middle region at 60 min.

Gas exchange, ventilator and haemodynamic parameters

Only the SI_{plat} and SI_{long} groups achieved target SpO_2 within the first 5 min of life, with the other groups requiring up to 15 min; all $p<0.034$, (Tukey's post-test, Figure 5A). Target SpO_2 in the SI_{30} and no-SI groups was achieved at the expense of a higher $AaDO_2$ (Figure 5B); all $p<0.032$ (Tukey's post-test). ΔP was lower in the SI_{plat} and SI_{long} groups compared to no-SI and, to a lesser extent, SI_{30} during the first 15 min (Figure 5C). Before 5 min of life, the set V_T of 7 mL/kg could not be obtained in the no-SI and SI_{30} groups (Figure 5D). Pa_{CO2} was also significantly higher in the no-SI group at 5 minutes compared to the other groups (all $p<0.004$; Figure 5E). There was no difference in CBF (Figure 5F), HR or ABP (data not shown) during the entire study period.

Lung mechanics

C_{dyn} was higher in the SI_{long} and SI_{plat} groups compared to both SI_{30} and no-SI at all time points; $p<0.001$, two-way ANOVA with Tukey's post-tests (Figure 3B). R_{RS} was

lower in the SI_{plat} and SI_{long} groups at 3 min (p=0.003-0.047; Figure 3C), and SI_{long} versus SI_{30} at 5 min (p=0.010). Both SI_{long} and SI_{plat} groups showed higher X_{RS} at 3 min, and remained so for SI_{long} vs SI_{30} until 10 min (p=0.001-0.049; Figure 3D). Static C_{RS} was higher in SI_{plat} and SI_{long} compared to no-SI (Table 1).

Gravity-dependent Distribution of Tidal Ventilation

Figure 6 shows the gravity-dependent spatial distribution of V_T at 5 and 60 min. All groups behaved differently, with the no-SI group showing increased temporal heterogeneity of V_T within the lung (most gravity-dependent third p=0.016 and least-dependent p=0.046; paired t tests). Relative V_T was similar throughout the lung at 5 min following SI_{30} but significantly decreased within the dependent regions by 60 min (p=0.023). There were no temporal changes in V_T distribution within the SI_{plat} group but the non-dependent lung always contributed a significantly greater portion to total V_T (p=0.032 at 5 min and p=0.048). There were no spatio-temporal differences in V_T following the SI_{long}. The relative contribution to V_T in the most gravity-dependent third of the chest was less at 60 min in the SI_{30} group compared to SI_{long} (p=0.010, one-way ANOVA with Tukey's post-test), all other permutations of regions and strategies were not different.

Lung Injury

There was no difference in the total protein count between groups (Table 2). All groups had a higher lung injury score than the Fetal controls (all p<0.001; Kruskal-Wallis test with Dunn's multiple comparison test). Lung injury scores were higher in the SI_{30} group versus no-SI (p<0.01), SI_{plat} (p<0.0001) and SI_{long} (p<0.001), due to

variable regional differences between groups (Figure 4B and 4E). The no-SI group demonstrated greater injury scores in the middle zone compared to SI_{plat} ($p=0.040$).

All groups exhibited greater expression of all mRNA markers compared to the Fetal group (Figure 4D), except SI_{long} (*CYR61* upper region) and no-SI (*CYR61* lower). There were no differences in mRNA expression between strategies. Within each strategy there were regional differences in the expression of *EGR1* (no-SI; $p=0.042$), *CTGF* (all groups $p<0.005$ except SI_{long}, $p=0.064$), *IL8* (SI_{plat}, $p<0.034$, and SI_{long}, $p<0.001$) and *IL6* (all groups $p<0.010$ except SI_{30}, $p=0.733$); Kruskal-Wallis test, Dunn's multiple comparison.

DISCUSSION

Our preterm lamb study found that achieving volume equilibrium during a SI resulted in the best pulmonary mechanics and aeration. An inadequate SI duration provided no short-term benefit over PPV with sufficient PEEP alone. This study has important clinical implications that have not been reported previously. Whilst acknowledging the limitations inherent in the use of an animal model, we note that this is the first study to demonstrate that an individualized SI strategy using direct physiological feedback is possible and beneficial. These results suggest that predefined, standardized SI protocols based on fixed times and pressures may not always be beneficial, and have the potential to be harmful. Our findings suggest that the use of an intentionally prolonged SI maybe a better approach than one that is too short.

The need to rapidly transition from a fluid-filled to aerated lung at birth provides a sound physiological rationale for the use of a SI as the first step in infants unable to

generate adequate respiratory effort at birth (13, 39). Despite this the results of animal and human studies have been inconclusive (5, 11, 15, 18, 26, 29, 32, 33, 35-37). Notwithstanding variability in study design, all these studies adopted an *a priori* set SI duration based on time. Our data suggest that these conflicting results may be related to inter-subject variability in the time constants of the respiratory system at birth and the subsequent volume response. The high variability in final EEV attained (26) during each SI restricts the utility of targeting a pre-defined absolute lung volume, as has been previously proposed (29). In a highly variable system, utilizing the relative time-based response is the only method of ensuring steady state has been achieved. Our use of such a dynamic, individualized approach to a SI with real-time monitoring, rather than targeting static *a priori* parameters, represents an important shift in the conceptualization of managing respiratory interventions at birth. Considering the volumetric response is arguably a more translatable approach to the use of animal models, which have different lung mechanics to humans, but whose lungs follow the same mechanical concepts. In our model, obtaining lung volume steady-state during a SI influences the efficacy and safety of the intervention. Previous studies of SI at birth have not reported absolute volumes or whether lung volume equilibrium, and thus optimal aeration, was attained within each individual subject (15, 26, 29, 35, 36). We contend that our study can be considered the first to systematically investigate time as a SI parameter. The observation that the volumetric behavior of the lung at birth when exposed to an inflating pressure is exponential was not surprising (23, 28, 38), and validates the utility, and research potential, of EIT to measure ΔEEV.

SI durations of 30 s or less have been extensively explored in newly born lambs (15, 26, 35-38). In a previous study by our group, aeration was found to be still ongoing by 30 s on post hoc EIT analysis in some lambs (37). The recent availability of real-time EIT imaging allowed this observation to be further investigated in this study. A 30 s duration was likely to be sufficient for volume equilibration in only 24% of recipients, and the SI_{30} group had no clinical benefit over PPV alone. More importantly, the SI_{30} group had the worst lung injury profile within the limitations of interpretation due to study design. Preterm lung injury is associated with heterogeneous regional volume states (30, 42). Unlike other studies we compared aeration and ventilation alongside regional injury, confirming that all strategies caused complex regional lung injury patterns. The differing spatio-temporal aeration and ventilation profiles suggest that multiple injurious states, such as atelectasis, overdistension and tidal shear-forces, were likely occurring simultaneously. The gravitational changes over time (spatio-temporal changes) in aeration were the most striking finding and consistent with a previous study in preterm lambs without antenatal steroid exposure (37). Following any SI approach, the initial spatial benefits in aeration were not sustained, with all three strategies showing temporal gravity-dependent changes in aeration. The observation of more histological lung injury in the SI_{30} group, and no increase in the SI_{long} group is potentially important in this context. It is simplistic to consider that the duration of a SI, and thus aeration, will have an incremental effect on the lung. The combination of poorer initial ΔEEV, indicating partial recruitment, combined with the greatest spatio-temporal heterogeneity of aeration and ventilation, and thus risk of atelectasis, overdistension and tidal shear-forces simultaneously, would explain the higher injury profile in the SI_{30} group. Within the dynamic process of aeration and

ventilation in early life, lung protection is defined by dynamic volume state considerations, and considering it in terms of aeration or not is too simplistic.

Targeting lung volume equilibrium (or longer), irrespective of the time needed, resulted in better outcomes than a SI that did not achieve steady state lung volume, due to the longer transpulmonary pressure and better lung-liquid clearance (as evident by the initial improvements in R_{RS}). The finding that applying an intentionally too long SI did not also result in greater lung injury was unexpected. The benefits in oxygenation and lung mechanics obtained from the volumetric approaches were due to increased aeration in the least dependent regions, indicating these regions needed longer to aerate and our intention of significant overdistension may not have been achieved. Our approach of visually targeting the volumetric response globally will not identify these differences, something that could be addressed with via targeting regional volumetric stability in future studies. The SI_{long} strategy was the only strategy that did not result in any spatio-temporal differences in V_T, indicating that the subtle differences in regional aeration seen were practically relevant if not statistically significant. The lack of difference in SI duration between SI_{plat} and SI_{long} is partially explainable by the intentional individualized SI design but also suggests that the SI_{long} strategy was insufficient to cause generalized overdistension. Clinically, when clinicians are uncertain, it may be more prudent to apply a slightly longer SI rather than a shorter one.

The SI durations required in the SI_{plat} and SI_{long} to achieve volumetric, and arguably, physiological optimization of the initial aeration for lambs in our study are unlikely to be required in human infants, whose smaller lung volumes and different chest

mechanics will dictate shorter absolute time constants and thus time to volume stability. However, the concepts identified in this study, and the models used, are translatable (23, 28). The application of excessive transpulmonary pressure to the thorax, and overdistension, may have cardiovascular consequences (25). We were not able to identify any differences in heart rate, arterial blood pressure and carotid blood flow. As pulmonary artery blood flow and ductal status were not measured, our haemodynamic findings should be interpreted with caution. Pulmonary blood flow was not adversely impacted in a similar preterm lamb study comparing a 40s SI against no-SI, although global and regional SI volumes were not reported (29). The same study found that cerebral blood flow was elevated in the no-SI group and correlated with hypoxia. Our study, with larger group sizes, reassuringly did not identify any difference in cerebral blood flow. Clinically translatable measures of cerebral oxygenation, such as near-infra red spectroscopy, have been used to describe SI at birth (26) and warrant consideration in future studies, ideally in humans.

The main focus of our study was the interaction between SI strategy, spatio-temporal volume patterns, oxygenation and mechanics. Fundamentally, any clinical strategy addressing these important short-term measures also needs to be lung protective for meaningful clinical translation. Preterm lung injury is a complicated multifactorial process, and our study period and design only allowed assessment of early markers of injury, limiting interpretation. This was intentional, to allow isolation of the inflammatory events occurring in early life from those of the injurious cycle of ongoing mechanical ventilation, but the relatively short study duration, although still longer than many previous SI animal studies (26, 29, 32, 33, 36), was unlikely to be sufficient to result in post-transcription protein changes within the lung, and

559 correlation with the histology findings. We chose accepted markers of preterm lung
560 injury known to be upregulated within 30 min of injurious ventilation (11, 12, 14, 40),
561 but clear patterns for specific mechanisms of injury were not apparent. This may have
562 been influenced by the sample size of each treatment group and the large variability in
563 mRNA expression due to the multiple influences beyond those variables controlled in
564 our study. Our study was powered to assess short-term outcomes and, although
565 considerably larger than all other previous preterm respiratory transition studies (12,
566 15, 25-27, 29, 32, 33, 35-38, 40), was insufficient for evaluation of some of the
567 mRNA parameters. This highlights the difficulty in defining acute preterm lung injury
568 and the need for new approaches. Despite these limitations, the spatial injury findings
569 are potentially important, especially when considered with the EIT data. In addition
570 our sample size of 12/group would allow detection of a 1.8 (2.0) difference in lung
571 injury score. The observation that injury changes were more prominent in the
572 dependent lung, the region most susceptible to temporal volumetric changes, is in
573 keeping with our understanding of lung injury in the surfactant deficient lung. This
574 suggests that our integrated regional volume state, mechanics and injury methodology
575 could be used in longer-term studies primarily designed to consider injury pathways.
576
577 This study has some additional limitations. To limit confounding factors between
578 groups the lambs were anaethestised and ventilated with cuffed endotracheal tubes,
579 not a common clinical scenario but consistent with other similar animal studies at
580 birth (15, 26, 27, 29, 32, 33, 36-38, 40). Measuring dynamic regional lung volumes
581 during birth transition is difficult. Although limited to the neonatal research setting,
582 EIT offers the only practical solution at present, and our study demonstrates potential
583 utility to guide (42) rather than simply monitor therapy, but is not without well-

584 described limitations (16). Applying individual EIT electrodes is a time consuming
585 and operator dependent process that has limited clinical utility. However, three new
586 EIT systems are now commercially available. These use a single non-adhesive belt
587 and have very short application times that would potentially allow for use in the
588 Delivery Room during non-invasive ventilation. Although the calibration of EIT
589 signals to known volume measurements is validated (1, 22), we only calibrated the
590 global signal (17) and limited our interpretation of regional volumetric behaviour to
591 relative rather than absolute changes.
592
593 In conclusion, our study suggests that the high variability and unpredictability of the
594 mechanical properties of the lung limits the efficacy of the current standardized
595 approaches to a sustained inflation at birth. An individualized approach to SI, tailored
596 to the volumetric response of the lung over time to an applied pressure was effective
597 in optimizing aeration, homogeneous tidal ventilation and bedside respiratory
598 outcomes, with potentially less lung injury. The optimal strategy to delivering a
599 sustained inflation to the preterm lung, and the most suitable real-time monitoring
600 system to guide it, still need to be further investigated. In the meantime, this study
601 suggests a SI that is as long, or longer, than the time required for volume equilibrium
602 at birth may confer less risk of injury than one that is too short.
603
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607

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Competing interests: Politecnico di Milano University, the institution of E.Z. and R.L.D., owns a patent on the use of forced oscillation technique for the detection of lung volume recruitment/derecruitment. The other authors have no competing interests to declare.

622 **Figure Legends**

623 **Figure 1. A.** Overview of experimental protocol. SI, sustained inflation as per study
624 allocation or PPV; ABG, arterial blood gas; FOT, forced oscillation technique
625 measurement of lung mechanics; M, Measurement of physiological, ventilator,
626 haemodynamic and EIT parameters. * Measurements were taken immediately after SI
627 was completed or from birth (no-SI). **B.** Representative tracing of the real-time global
628 EIT time-course volume signal for each of the three SI groups. Unclamping of the
629 ETT, and commencement of the SI, is indicated with the solid arrow. The point of
630 signal plateau, as determined visually, in the SI_{plat} and SI_{long} groups is indicated with
631 the dashed arrow and grey dashed horizontal line. EIT data is presented as shown on
632 the Thorascan software in uncalibrated (arbitrary) units (AU). Real-time videos of
633 each tracing are available in the Online Supplement (Videos 1-3).

634

635 **Figure 2. A**. Actual SI duration and time to t_{plat} (determined from modelling) during
636 the SI_{30} (white box), SI_{plat} (light grey hatched) and SI_{long} (dark grey). The time to
637 stable ΔEEV during SI_{long} was mean (SD) 60 (30) s. *$p<0.0001$ SI_{30} vs SI_{plat} and
638 SI_{long}. **B**. ΔEEV from birth for SI_{30}, SI_{plat}, SI_{long} and no-SI (white hatched), using
639 patterns as A, at 30 s of life (*30 s*), the end of the SI if applicable (*Final*), during the
640 first 10 inflations immediately post the SI (*Post SI*), 100 s of life, or immediately after
641 SI if SI had not completed at 100 s (*100 s*). The mean (SD) ΔEEV at t_{plat} was 39 (19),
642 45 (36) and 42 (19) mL/kg for SI_{30}, SI_{plat} and SI_{long} respectively ($p=0.498$, one-way
643 ANOVA). Box represents 5-95th CI and mean (line) and whiskers minimum and
644 maximum. *$p=0.009$ and $p=0.0003$, No-SI vs SI_{plat} and SI_{long}.

645

Figure 3. A. ΔEEV from birth over time for no-SI (black circles), SI_{30} (open diamonds), SI_{plat} (grey diamond with dot) and SI_{long} (dark grey diamonds) groups. Change in C_{dyn} (**B**), R_{RS} (**C**) and X_{RS} (**D**) using the same symbols as **A**. All data mean ± SD. $p<0.05$; * SI_{long} vs no-SI and SI_{30}, † no-SI vs SI_{long}, ‡ no-SI vs SI_{long} and SI_{plat}, § SI_{30} vs SI_{long} and SI_{plat}, # SI_{plat} vs no-SI and SI_{30}, ** SI_{30} and No-SI vs SI_{plat} and SI_{long},

Figure 4. A. fEIT images of the regional gravity-dependent distribution of aeration (expressed as a % of total aeration) at 5 min and 60 min in each of the 22 equally sized slices for the right (dark bars) and left (white bars) hemithorax. The least gravity dependent slices of the thorax are at the top of each histogram and the most dependent at the bottom. Dashed and dotted lines delineate the upper (U), middle (M) and lower (L) equally weighted (by lung tissue) gravity-dependent regions. Data mean and SD. * $p<0.05$ against no-SI for that region, † $p<0.05$ within strategy against both other regions (one-way ANOVA with Tukey's post-tests). **B.** Change in relative regional aeration from 5 min at 60 min of life in the upper (most gravity non-dependent; black bars), middle (grey bars) and lower (most gravity dependent; white bars) equally sized thirds of the lung. Corresponding lung injury score (H&E sections) shown using circles with same colour scheme for regions. Data mean and SD. * $p<0.05$ between strategies for that region, † $p<0.05$ within strategy against both other regions (one-way ANOVA with Tukey's post-tests). **C.** Expression of *EGR1*, *CYR61*, *CTGF, IL-1β*, *IL-6* and *IL-8* mRNA in the non-dependent (black bars) and dependent (white bars) regions. mRNA data median and IQR. *$p<0.05$ between regions for that strategy, † Fetal vs all other strategies for region, ‡ vs Fetal for region (Kruskal-Wallis test with Dunn's multiple comparison). **D.** Representative haematoxylin and eosin stained right upper lobe lung tissue sections in an unventilated fetus (upper

671 image) and a mechanically ventilated lamb with lung injury (lower image)
672 demonstrating hyaline membranes (arrowheads) and detached epithelial cells
673 (arrows).
674
675 **Figure 5.** Change in Sp_{O2} (**A**), $AaDO_2$ (**B**), ΔP (**C**), V_T (**D**), Pa_{CO2} (**E**) and CBF (**F**) for
676 the four recruitment strategies using the same symbols as Figure 2. The first time
677 points for SpO_2, ΔP and V_T represent data at 100 s (no-SI and SI_{30}) or during the first
678 10 inflations after the SI (SI_{plat} and SI_{long}). All data mean ± SD, except SpO_2 (mean ±
679 SEM). * $p<0.05$ no-SI vs SI_{long}, † SI_{30} vs SI_{long}, ‡ SI_{30} vs SI_{plat}, ** no-SI vs SI_{plat} and
680 SI_{long}, *** no-SI vs all SI strategies, # SI_{30} vs SI_{plat}.
681
682 **Figure 6.** fEIT images of the regional gravity-dependent distribution of V_T
683 (expressed as a % of total V_T) at 5 min and 60 min using the same format and
684 orientation as Figure 4A. The relative contribution of the equally weighted most and
685 least-dependent thirds of each lung slice to total V_T are shown in each histogram. All
686 data mean and SD. Specific p values are listed in the text with *$p<0.05$ (details in
687 results) between gravity-dependent region for a strategy at that time point and
688 †$p<0.05$ within gravity-dependent region between 5 and 60 min for a strategy.
689
690

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Table. 1. Study groups characteristics

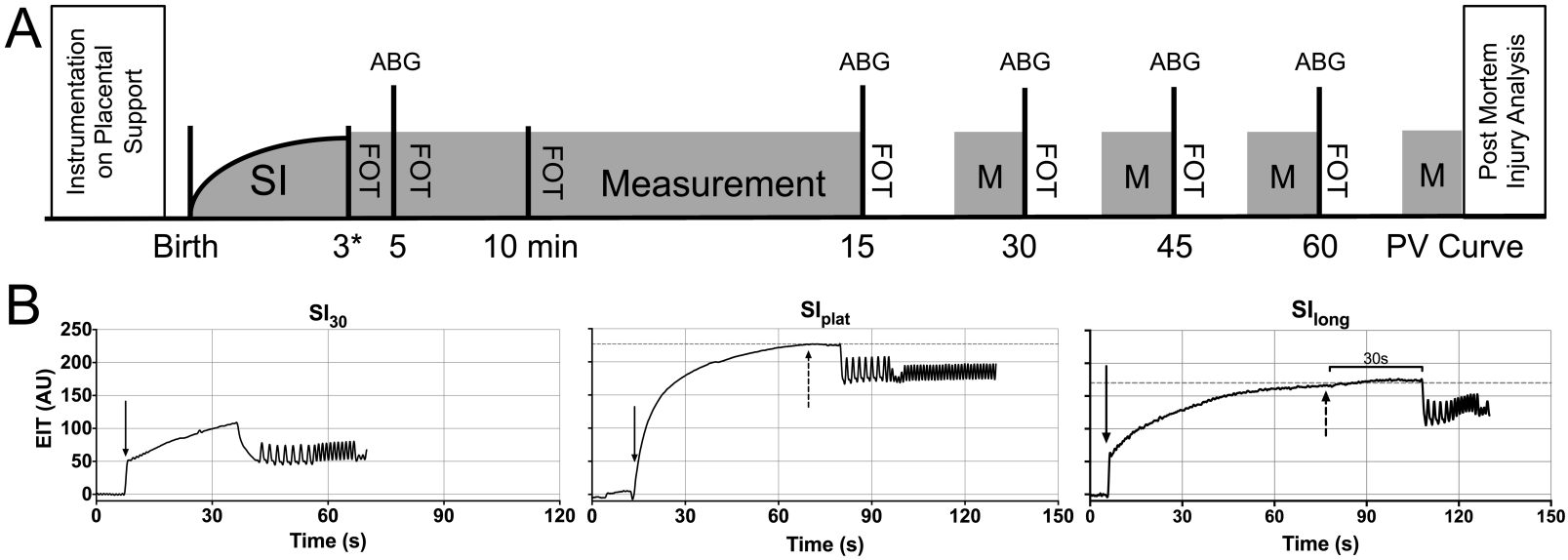
	no-SI	**SI_{30}**	**SI_{plat}**	**SI_{long}**	**Fetal**
Number	12	13	12	12	10
GA (days)	127 (1.00)	127 (0.86)	127 (0.79)	127 (0.80)	127 (0.68)
Female n (%)	5 (42%)	6 (46%)	7 (58%)	6 (50%)	6 (60%)
First born (n)	7 (64%)	7 (54%)	5 (42%)	6 (50%)	3 (30%)
Birth Weight (g)	3169 (519)	3119 (439)	3042 (420)	3016 (391)	2765 (432)
Fetal Lung Fluid (mL/kg)	15.9 (8.6)	17.5 (6.0)	16.5 (10.0)	16.0 (8.5)	N/A
Arterial Cord pH	7.33 (0.04)	7.35 (0.05)	7.33 (0.06)	7.34 (0.05)	7.20 (0.19)
Arterial Cord Pa_{O2} (mmHg)	22.0 (5.1)	21.9 (5.4)	22.7 (3.7)	23.0 (5.0)	21.4 (14.7)
Static C_{RS} ($mL.kg^{-1}/cm\ H_2O$)	0.89 (0.23)*	1.02 (0.26)	1.18 (0.37)	1.21 (0.26)	N/A

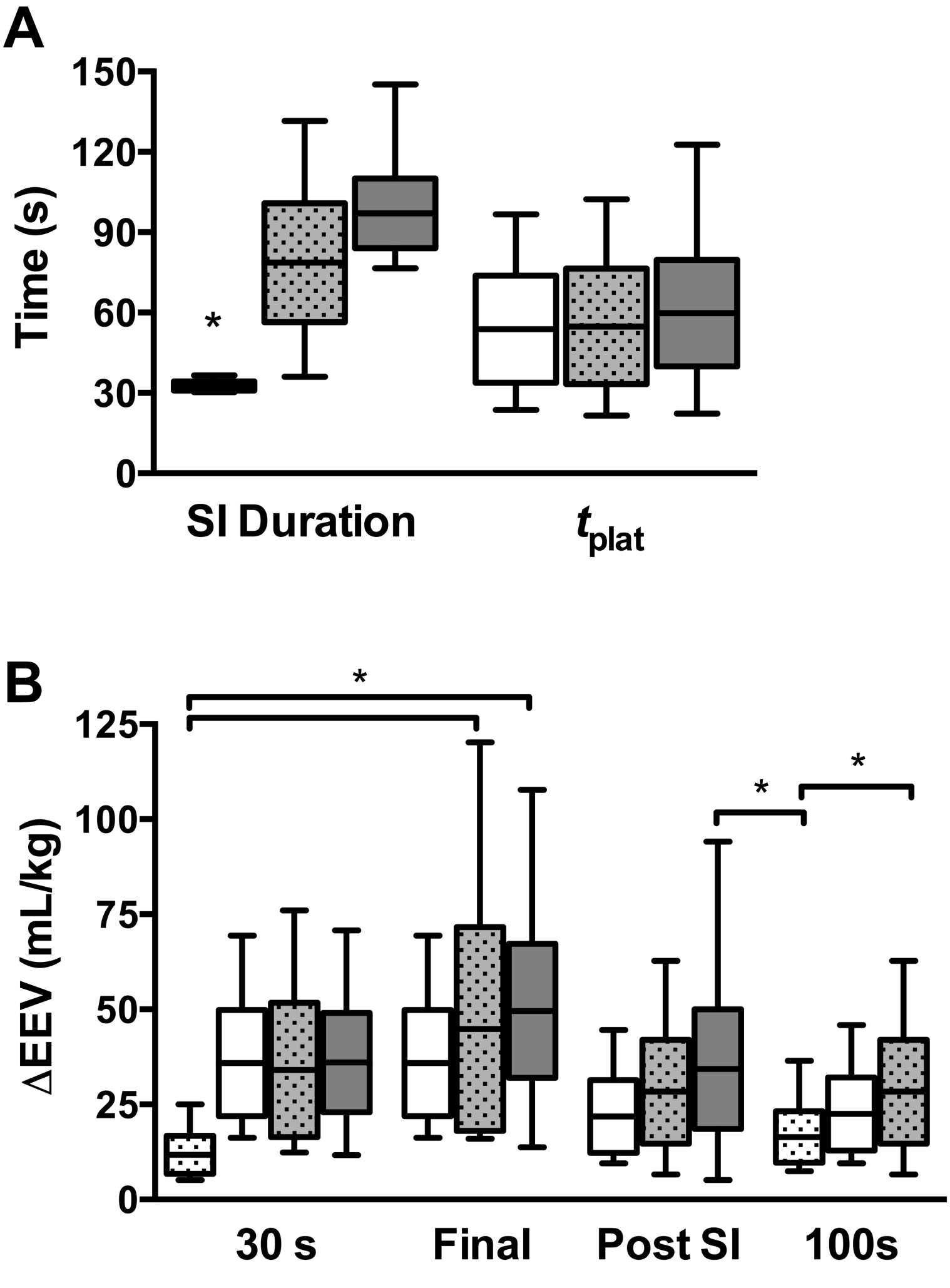
All data mean (SD). *p=0.038 no-SI vs SI_{plat} and SI_{long} (1-way ANOVA).

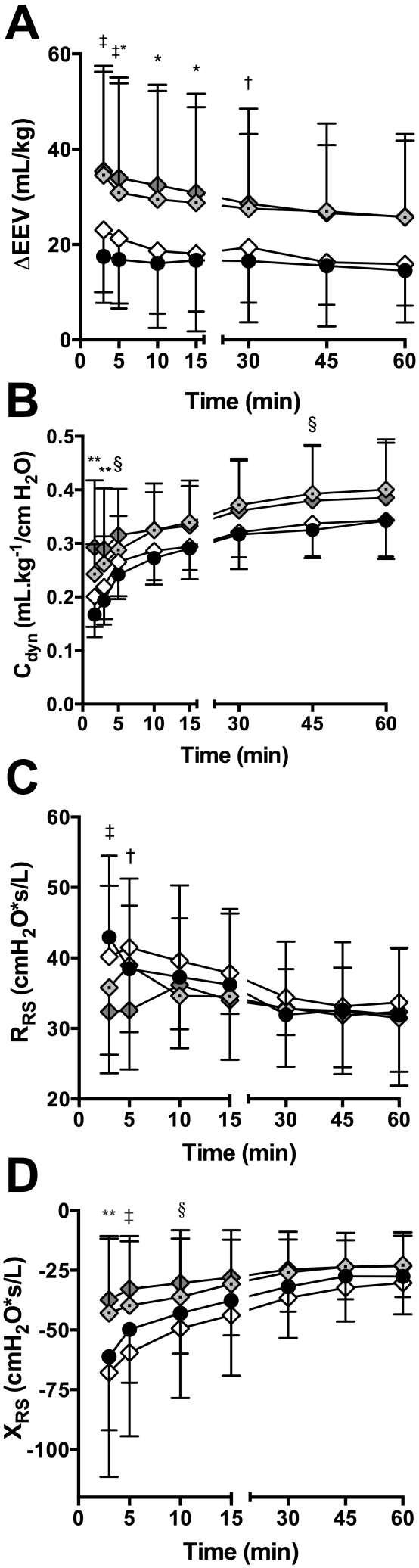
Table 2. Total Protein and Histological Injury Data by gravity-dependent lung region

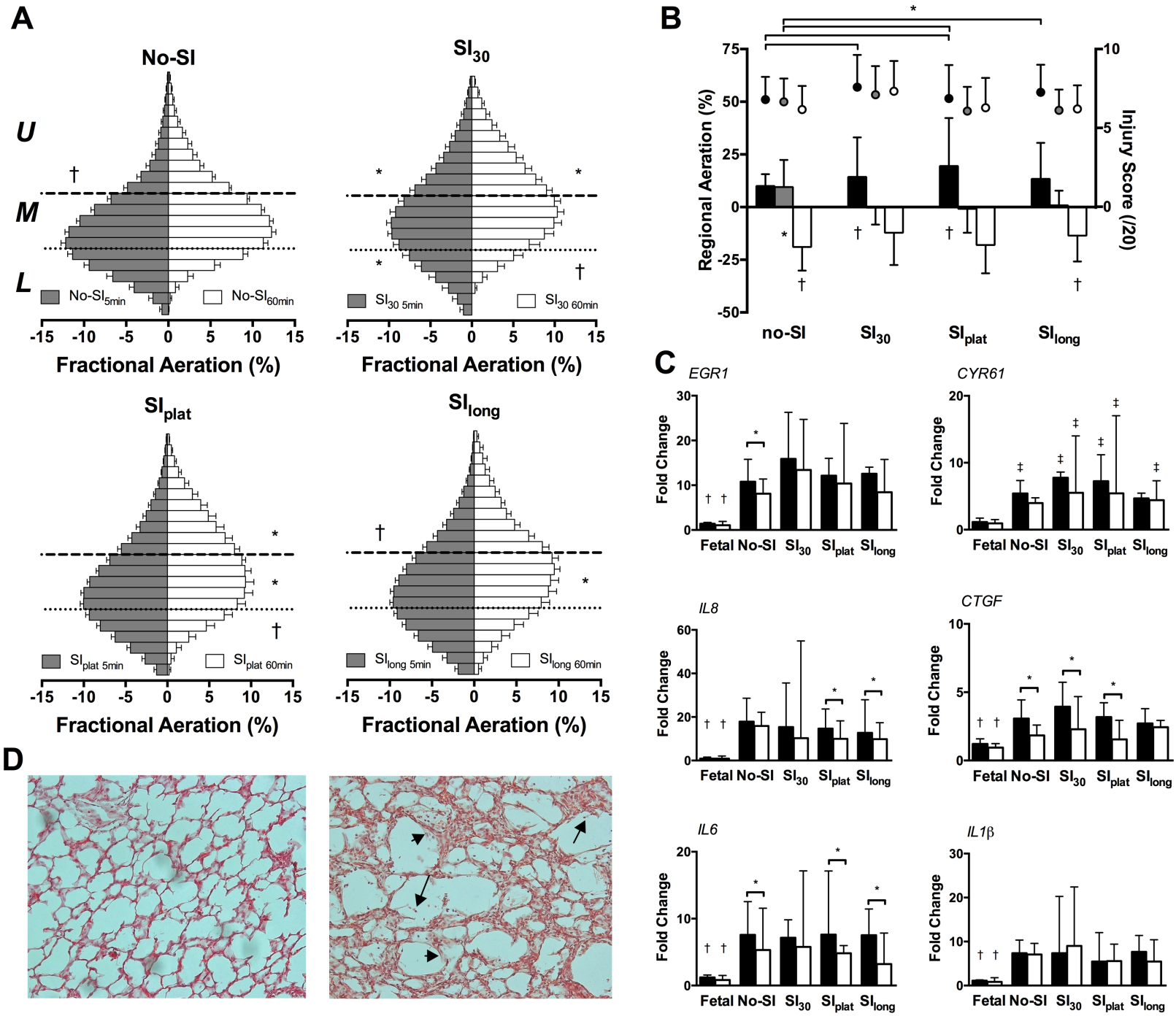
	UVC			No-SI			SI_{30}			SI_{plat}			SI_{long}		
Total Protein (mg/mL)	0.54 (0.26)			0.72 (0.46)			0.89 (0.54)			0.66 (0.55)			0.74 (0.73)		
Total Score (/20)	5.6 (1.4)*			6.5 (1.5)*			7.3 (1.9)*†‡§			6.4 (1.9)*			6.5 (1.6)*		
Regional Injury	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**	**U**	**M**	**L**
n	50	50	50	55	55	55	65	65	65	60	60	60	50	50	50
Total Score (/20)	5.3 (1.3)	6.0 (1.4)	5.4 (1.5)	6.8 (1.4)*	6.7 (1.5)*†	6.2 (1.5)*	7.6 (2.0)*†‡	7.1 (1.8)*†‡	7.3 (1.9) *†‡§	6.9 (2.1)*	6.1 (1.5)	6.3 (1.9)*	7.3 (1.7)*	6.1 (1.7)	6.2 (1.5)*
Alveolar Wall Thickness (/5)	2.7 (0.7)	2.6 (0.7)	2.6 (0.8)	3.1 (0.8)*	3.3 (0.7)*	3.1 (0.7)*	3.3 (0.9)*	3.2 (1.0)*	3.4 (1.0)*†	3.0 (0.7)*	3.0 (0.7)*	3.1 (1.0)*	3.3 (0.9)*	3.2 (0.7)*	3.3 (0.7)*
Detached Epithelial Cells (/5)	1.6 (0.8)	2.0 (0.9)	1.8 (0.9)	2.3 (0.8) *†‡#	2.2 (0.8)	1.9 (1.0)	2.9 (1.4)*‡§	2.6 (1.3)*†‡	2.5 (1.5) *†‡§	2.3 (1.5)*	1.8 (1.0)	1.8 (1.1)	2.7 (1.1)*	1.6 (0.9)	1.7 (1.0)
Hyaline Membranes (/5)	0.3 (0.5)	0.6 (0.6)	0.3 (0.5)	0.8 (0.6)*†‡	0.6 (0.5)‡	0.6 (0.5)*	0.7 (0.5)*	0.7 (0.5)‡	0.8 (0.5) *†‡§	0.4 (0.5)	0.6 (0.5)	0.5 (0.5)*	0.5 (0.5)	0.4 (0.5)	0.4 (0.5)
Atelectasis (/5)	0.7 (0.6)	0.7 (0.6)	0.8 (0.7)	0.6 (0.5)	0.6 (0.7)	0.5 (0.6)	0.7 (0.7)	0.6 (0.8)	0.6 (0.6)	1.2 (1.0) *‡§#	0.7 (0.7)	0.8 (0.7)	0.8 (0.5)	0.9 (0.7)§#	0.7 (0.7)§#

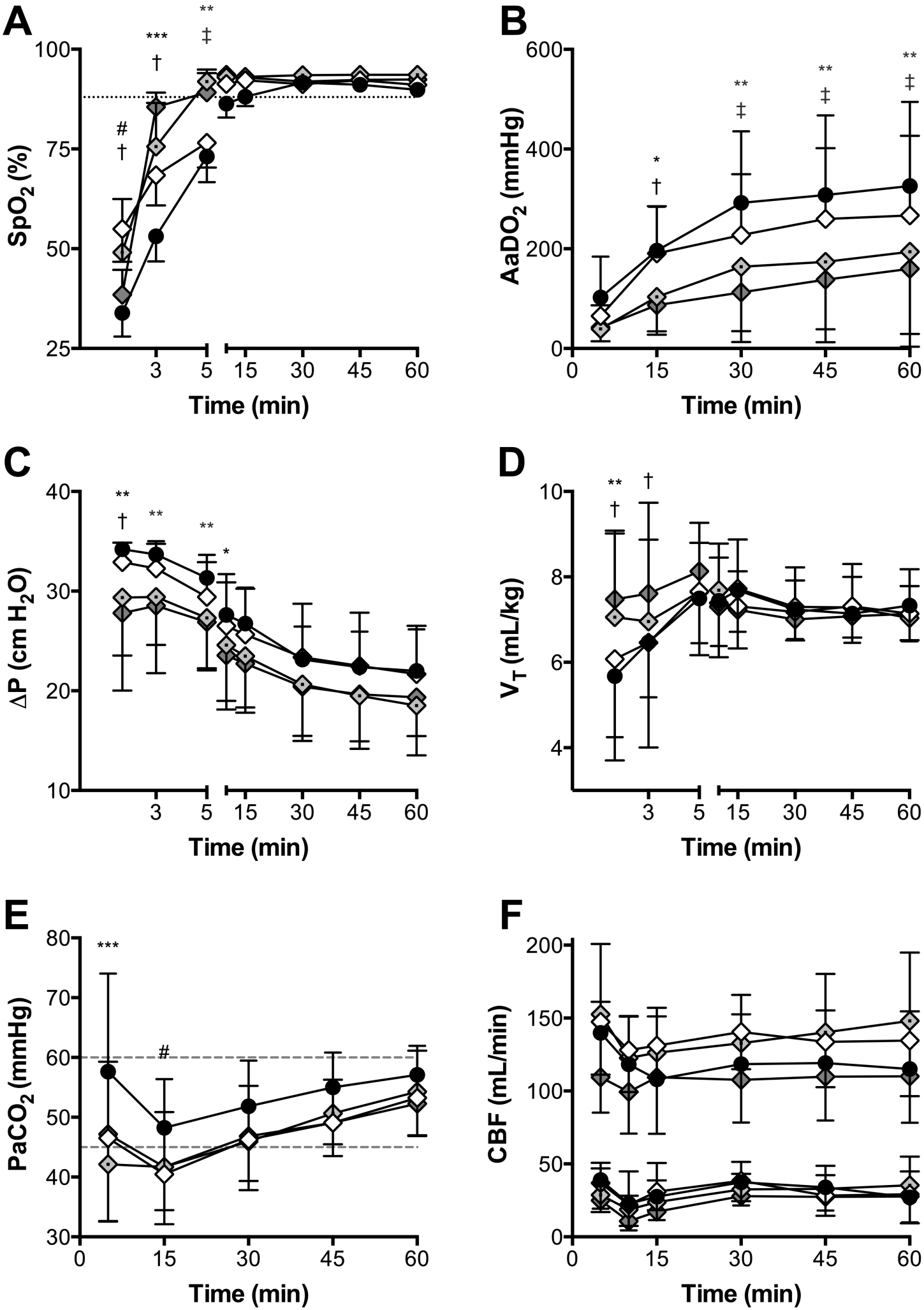
Abbreviations: **U**; Upper, **M**; Middle, **L**; Lower. All data mean (SD). *p<0.05 vs UVC, §vs no-SI, #vs SI_{30}, †vs SI_{plat}, ‡vs SI_{long}; one-way ANOVA with Tukey post tests.

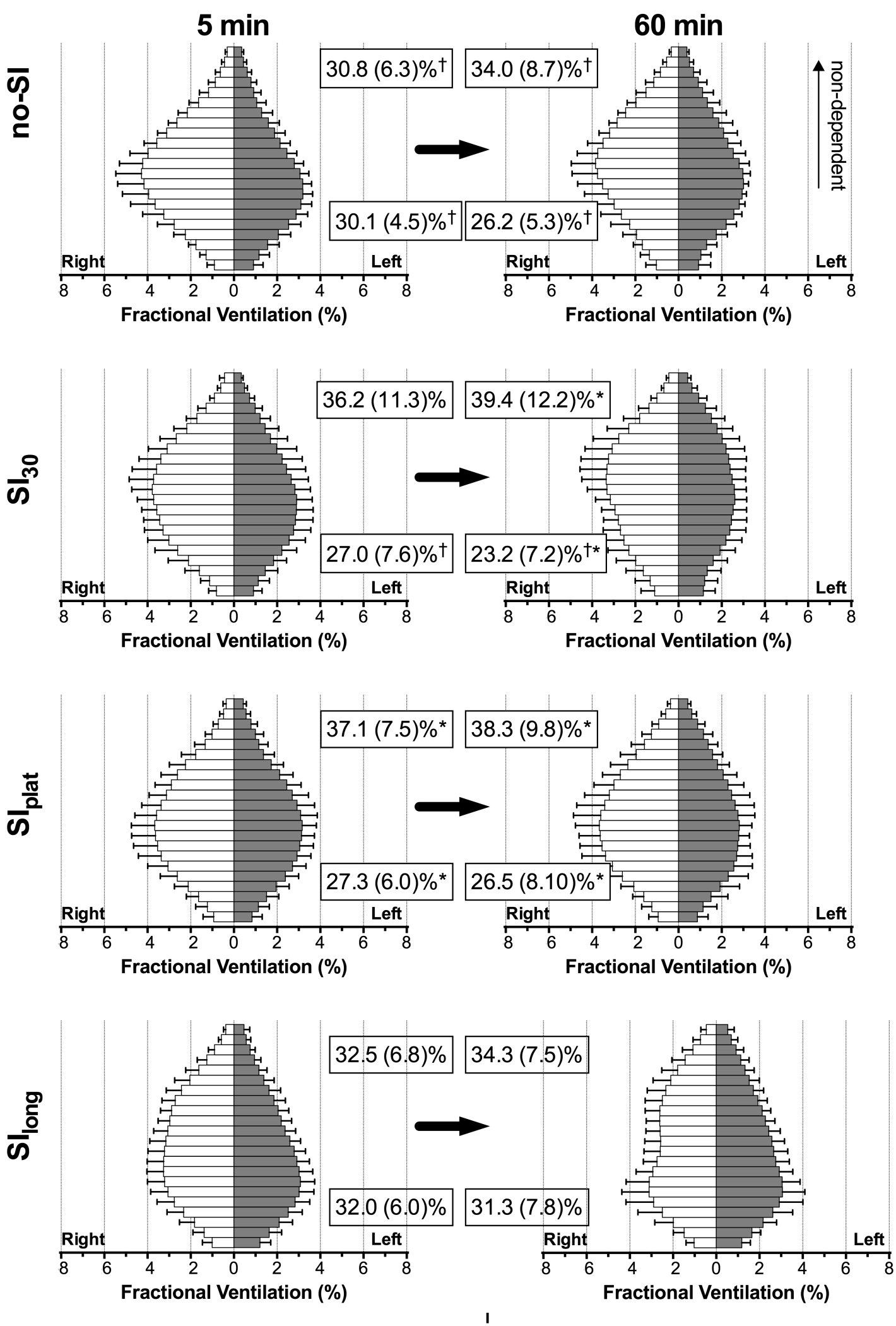

A
Instrumentation on Placental Support
SI
FOT
ABG
FOT
FOT
Measurement
ABG
FOT
M
ABG
FOT
M
ABG
FOT
M
M
Post Mortem Injury Analysis
Birth
3*
5
10 min
15
30
45
60
PV Curve
B
SI30
SIplat
SIlong
30s
EIT (AU)
Time (s)
Time (s)
Time (s)


A
150
120
90
60
30
0
Time (s)
*
SI Duration
t_{plat}
B
125
100
75
50
25
0
ΔEEV (mL/kg)
*
*
*
30 s
Final
Post SI
100s


A
ΔEEV (mL/kg)
Time (min)
B
C_{dyn} (mL.kg^{-1}/cm H_2O)
Time (min)
C
R_{RS} (cmH_2O*s/L)
Time (min)
D
X_{RS} (cmH_2O*s/L)
Time (min)


A
No-SI
SI30
U
M
L
No-SI5min
No-SI60min
SI30 5min
SI30 60min
SIplat
SIlong
SIplat 5min
SIplat 60min
SIlong 5min
SIlong 60min
-15 -10 -5 0 5 10 15
Fractional Aeration (%)
B
Regional Aeration (%)
Injury Score (/20)
75
50
25
0
-25
-50
10
5
0
no-SI
SI30
SIplat
SIlong
C
EGR1
CYR61
IL8
CTGF
IL6
IL1β
Fold Change
Fetal No-SI SI30 SIplat SIlong
D


A
B
C
D
E
F
SpO2 (%)
AaDO2 (mmHg)
ΔP (cm H2O)
VT (mL/kg)
PaCO2 (mmHg)
CBF (mL/min)
Time (min)
100
75
50
25
600
400
200
0
40
30
20
10
8
6
4
80
70
60
50
40
30
150
3
5
15
30
45
60
#
*
**
***
†
‡


5 min
60 min
no-SI
30.8 (6.3)%†
34.0 (8.7)%†
30.1 (4.5)%†
26.2 (5.3)%†
non-dependent
Right
Left
8 6 4 2 0 2 4 6 8
Fractional Ventilation (%)
SI30
36.2 (11.3)%
39.4 (12.2)%*
27.0 (7.6)%†
23.2 (7.2)%†*
SIplat
37.1 (7.5)%*
38.3 (9.8)%*
27.3 (6.0)%*
26.5 (8.10)%*
SIlong
32.5 (6.8)%
34.3 (7.5)%
32.0 (6.0)%
31.3 (7.8)%