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# Lung ultrasound features and relationships with respiratory mechanics of evolving BPD in preterm rabbits and human neonates

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**Running title:** Preterm rabbits share lung ultrasound appearance with infants with evolving BPD

**Running head:** Preterm rabbits resemble lung ultrasound features of human BPD

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**ABSTRACT** - Evolving bronchopulmonary dysplasia (BPD) is characterized by impaired alveolarization leading to lung aeration inhomogeneities. Hyperoxia-exposed preterm rabbits have been proposed to mimic evolving BPD; therefore, we aimed to verify if this model has the same lung ultrasound and mechanical features of evolving BPD in human neonates. Semi-quantitative lung ultrasound and lung mechanics measurement were performed in twenty-five preterm rabbits (28 days of gestation) and twenty-five neonates (mean gestational age  $\approx$ 26 weeks) with evolving BPD. A modified rabbit lung ultrasound score (rLUS) and a validated neonatal lung ultrasound score (LUS) were used. Lung ultrasound images were recorded and evaluated by two independent observers blinded to each other's evaluation. Lung ultrasound findings were equally heterogeneous both in rabbits as in human neonates and encompassed all the classical lung ultrasound semiology. Lung ultrasound and histology examination were also performed in 13 term rabbits kept under normoxia as further control and showed the absence of ultrasound and histology abnormalities compared to hyperoxia-exposed preterm rabbits. The inter-rater absolute agreement for the evaluation of lung ultrasound images in rabbits was very high (ICC: 0.989 (95%CI: 0.975-0.995);  $p < 0.0001$ ), and there was no difference between the two observers. Lung mechanics parameters were similarly altered both in rabbits and human neonates. There were moderately significant correlations between airway resistances and lung ultrasound scores in rabbits ( $\rho=0.519$ ;  $p=0.008$ ) and in neonates ( $\rho=0.409$ ;  $p=0.042$ ). In conclusion, the preterm rabbit model fairly reproduces the lung ultrasound and mechanical characteristics of preterm neonates with evolving BPD.

## **NEW AND NOTHEWHORTY**

We have reported that hyperoxia-exposed preterm rabbits and human preterm neonates with evolving BPD are the same lung ultrasound appearance, and that lung ultrasound can be fruitfully applied on this model with a brief training. The animal model and human neonates also presented the same relationship between semiquantitative ultrasound-assessed lung aeration and airway resistances. In conclusion, this animal model fairly reproduce evolving BPD as it is seen in clinical practice.

## 66 INTRODUCTION

67 Bronchopulmonary dysplasia (BPD) is characterized by an impaired lung development due to the  
68 aberrant reparative response to both pre- and post-natal lung injury.(1) From a parenchymal  
69 perspective, developing BPD is an evolutive and regionally heterogeneous disorder characterized by  
70 impaired alveolarization leading to structural lung tissue modifications and lung aeration  
71 inhomogeneities.(1) Given its multifactorial nature, mimicking BPD in animal models remains a  
72 challenging task. Ideally, an animal model of BPD should include a preterm birth and some possible  
73 lung injury triggers, such as invasive ventilation, inflammation or oxidative stress. Additionally,  
74 animal care should be as similar as possible to that provided in neonatal intensive care units  
75 (NICU).(2) Moreover, animals should be supported for long periods, since the chronic respiratory  
76 disease of preterm infants cannot be resumed in a punctual diagnosis at a given time point. In fact,  
77 preterm infants are rather affected by a chronic respiratory failure representing a *continuum* evolving  
78 over time from early life to infancy and characterized by persistent airway and pulmonary vascular  
79 disease: this is expressed by the “chronic pulmonary insufficiency of prematurity” concept.(3)  
80 For instance, lambs, pigs, baboons or monkeys are effective to mimic the clinical reality in detail,  
81 albeit they may be less cost-effective compared with small laboratory animals.(4, 5) Newborn rodents  
82 can be delivered at term during the saccular stage of lung development, which is morphologically  
83 similar to the human premature.(6) Nonetheless, these animals are difficult to ventilate and lack the  
84 significant impact of preterm birth. Therefore, they cannot reproduce the complex reality of neonatal  
85 critical care and the translatability of this model to human BPD can be questioned. Large animals and  
86 rodents also have different survival, with the former surviving for longer times, which impacts the  
87 type of studies that can be performed and their costs. Rabbits represent an excellent compromise to  
88 model BPD since they have many offspring, can be delivered prematurely and allow some perinatal  
89 manipulations, making the model closer to the actual neonatal care.(2, 7) For instance, preterm  
90 rabbits are fed artificially with milk formula and can be exposed to hyperoxia to induce the  
91 alveolarization arrest similar to what occurs in infants with evolving BPD.(8, 9)

92

93 From the imaging point of view, the heterogeneous parenchymal abnormalities of BPD can be  
94 accurately described by lung ultrasound, which is characterized by a steady loss of lung aeration over  
95 time.(10) By this means, early lung ultrasound performed at 7-14 days of post-natal age can predict  
96 the occurrence of BPD at 36 weeks post-menstrual age.(10) From a mechanical point of view, BPD is  
97 characterized by a mixed pattern (obstructive and restrictive) with heterogeneous distribution and a  
98 certain variability according to clinical severity.(11) The lung ultrasound features of hyperoxia-  
99 exposed preterm rabbits are unknown. Therefore, the present study compared the lung ultrasound and  
100 mechanical features of preterm rabbits and human neonates with evolving BPD to validate the  
101 hyperoxia-exposed preterm rabbit as a reliable model of BPD.

102

## 103 **MATERIALS AND METHODS**

104

105 This project was divided into two phases: 1) an experimental model of evolving BPD in hyperoxia-  
106 exposed preterm rabbits, and 2) data collection in extremely preterm infants with evolving BPD.

### 107 *Experimental model and animal care*

108 Time-mated New Zealand White female rabbits were purchased from Charles River Laboratories  
109 (Miserey, France). Pregnancy was confirmed with ultrasounds at 12-14 days after the artificial  
110 insemination. Subsequently, they were maintained with *ad libitum* food and water until caesarean  
111 section was performed. At 28 days of gestation, which is equivalent to the saccular stage of human  
112 lung development,(2, 7) does ( $3.7 \pm 0.4$  kg of body weight) were sedated with medetomidine 2 mg/kg  
113 (Domitor<sup>®</sup>, Orion, Espoo, Finland). Ten minutes later, the animals received 25 mg/kg of ketamine  
114 (Imalgene 1000<sup>®</sup>, Merial, France) and 5 mg/kg of xylazine (Rompun<sup>®</sup>, Bayer, Leverkusen,  
115 Germany). When adequate sedation was reached, the abdomen was shaved and does were euthanized  
116 with pentothal sodium (50 mg/kg; MSD Animal Health, Madison-NJ, USA). All drugs were given  
117 intramuscularly. The abdomen was immediately opened through a low midline abdominal incision,  
118 the uterus was exposed, and pups were extracted. They were dried, stimulated and placed in the

119 incubator at 32°C, with 95% oxygen and 50-60% relative humidity. After 1h, the surviving pups  
 120 were weighed, numbered and placed on soft bedding for 7 days with no treatment. Housing material  
 121 was changed daily. Pups were fed twice a day through 3.5 Fr esophageal tubes (Vygon, Ecouen,  
 122 France) transiently introduced for the feeding which lasted few minutes. A milk replacer (Day One<sup>®</sup>,  
 123 Protein 30%, Fat 50%; FoxValley, Aurora-IL, USA) dissolved in water (250 mg/mL) was given. The  
 124 volume of each feed was increased from 40 mL/kg/day on the day of birth, to 50 mL/kg/day on day  
 125 1, 75 mL/kg/day on day 2, and 100 mL/kg/day from day 3 to day 7. Pups were stimulated twice daily  
 126 to urinate before feeding. A mixture of pre- (fructo-oligosaccharide and acacia) and pro-biotics  
 127 (*Saccharomyces cerevisiae*) were added for all 7 days (Bio-Lapis<sup>®</sup>; Probiotics International, UK; 25  
 128 mg/mL), whereas immunoglobulins (15 mg/mL) were added only for the first 3 days (Col-o-Cat<sup>®</sup>,  
 129 SanoBest, Hertogenbosch, Netherlands). On day 2, vitamin K was intramuscularly given (0.25  
 130 mg/kg, Izokappa<sup>®</sup>, Brescia, Italy). These rabbits generally open eyelids around 7-10 days of life,  
 131 which is similar to what happens in extremely preterm human neonates.(12, 13)  
 132 A control group of newborn rabbits without lung injury was also included. This consisted of term  
 133 rabbits naturally delivered on the 31<sup>st</sup> day of gestation, which is their physiological term of pregnancy  
 134 and correspond to human lung development's alveolar stage .(12) After birth, pups were maintained  
 135 with their mothers at room air in individual cages until day 4 in order to reach the age equivalence  
 136 with preterm pups at day 7. At the end of all experiments, newborn rabbits were euthanatized as  
 137 described above. The protocol was approved by the Italian Ministry of Health review board  
 138 (n°744/2017) and met all the local and European regulations on animal research. This study phase  
 139 followed ARRIVE guidelines.(14)

#### 140 *Semi-quantitative lung ultrasound in rabbits*

141 On days 7 and 4 in preterm and term rabbits, respectively, the animals were anesthetized with  
 142 isoflurane (3% in pure oxygen). The lung ultrasound procedure in adult rabbits has been already  
 143 described.(15) Here, we adapted the technique to apply it to newborn rabbits and particularly to  
 144 preterm rabbits. Each lung was scanned using a hand-held ultrasound tablet equipped with a “hockey

145 stick” and a micro-linear, high-resolution probe (18 MHz, 2.5 cm length, Sonoscanner U-LITE<sup>®</sup>  
146 EXP, Sonoscanner, Paris, France). Since the probe was relatively large compared to the rabbit chest,  
147 the evaluation was done on the whole hemithorax (i.e. without division in smaller chest areas) as  
148 performed by Raimondi et al. in neonates scanned with a broadband linear probe.(16) A simplified  
149 rabbit lung ultrasound score (rLUS) was adapted from the previously validated neonatal lung  
150 ultrasound score (LUS).(17) rLUS and LUS were based on the same lung ultrasound semiology, and  
151 all images were evaluated according to the classical lung ultrasound scoring system assigning 0-to-3  
152 points.(17, 18) If the rabbit lung appeared homogenous, a single picture (300 dpi) per lung was  
153 captured (one picture for the left and one for the right lung); if the lung appearance was not  
154 homogenous, additional pictures were recorded to capture all ultrasound features, which were  
155 averaged for each rabbit. Ultrasound scans were performed both transversally and longitudinally,  
156 with automatic gain adjustment, using the dedicated software feature of the ultrasound device; no  
157 harmonics were used. Depth and focus were adjusted according to chest size and the sign of  
158 interest.(19) Illustrative pictures of lung ultrasound procedure and findings, as well as rLUS  
159 calculation, are shown in **Fig.1**. All images per each rabbit were independently evaluated by the  
160 investigator (CCas) performing the ultrasound and animal care, who received a short formal lung  
161 ultrasound training, as described elsewhere (20), and by a skilled neonatologist with several years of  
162 lung ultrasound experience (DDL), who did not participate in animal care and performed the  
163 evaluation blinded to any animal data.

#### 164 *Lung mechanics in preterm rabbits*

165 Preterm rabbits also underwent pulmonary function tests immediately after lung ultrasound.  
166 Pulmonary function was studied with the forced oscillation technique, using the FlexiVent<sup>TM</sup> system  
167 equipped with module 2 (Scireq, Montreal, Canada), as we previously described.(21) A tracheostomy  
168 was performed and an 18-gauge needle was inserted in the trachea and tied. Pups were connected to  
169 the FlexiVent ventilator (tidal volume: 10 mL/kg, frequency: 120 breaths/min, positive end-  
170 expiratory pressure (PEEP): 3 cmH<sub>2</sub>O).(21) In addition to total inspiratory capacity, airway resistance

171 (Raw), dynamic compliance (C<sub>dyn</sub>), lung tissue damping and elastance were measured using the  
172 Prime-8 and Snapshot-120 perturbation programs, respectively, as previously described.(22) All  
173 measurements were performed until three consistent values (with a minimum  $R^2 > 0.95$ ) were obtained  
174 and averaged.

### 175 **Histology**

176 Right after the sacrifice, the trachea was cannulated with a 20-gauge catheter. Lungs were removed  
177 and fixed with 10% buffered formalin (Sigma-Aldrich, St.Louis-MI, USA) for at least 4 hours under  
178 constant pressure (25 cmH<sub>2</sub>O) using a custom-made fixation device to ensure homogenous fixation  
179 pressures. Subsequently, the lungs were first left in formalin for at least 24h, then transferred to 70%  
180 ethanol, embedded in paraffin, and stained with eosin and hematoxylin (H&E) following standard  
181 histology protocols. A section for each lung was used to evaluate the mean linear intercept (Lm).(23)  
182 Lm was evaluated in 40 fields per section to cover the entire section area by calculating the number  
183 of intersections between the reference line and the alveolar tissue using a micrometric scale; Lm  
184 value of the right and left lung were then averaged and used in the analysis.(24, 25)

### 185 **Patients**

186 Neonates were enrolled between 2018 and 2020 in a prospective cohort study performed in an  
187 academic tertiary referral NICU if they fulfil all the following criteria: 1) gestational age  
188  $\leq 28^{+6}$  weeks; 2) invasive ventilation and oxygen supplementation at 14<sup>th</sup> day of postnatal life for  
189 ongoing hypoxemic respiratory failure; 3) invasive ventilation for at least 10 days in the first 2 weeks  
190 of life. Exclusion criteria were: 1) severe hemodynamic instability (defined as any need for  
191 inotropes); 2) need for surgery; 3) air leaks; 4) grade III-IV intraventricular hemorrhage; 5)  
192 congenital lung anomalies; 6) major congenital malformations or chromosomal abnormalities. In  
193 addition, all enrolled neonates had to qualify for BPD diagnosis at 36 weeks post-menstrual age  
194 according to the National Institute for Child Health and Development criteria(26) otherwise, they  
195 would have been excluded *a posteriori*. Data were collected in real-time by attending physicians in  
196 charge of patients, and no procedure was performed solely for the study participation. In fact, lung



197 ultrasound and mechanics evaluation are part of our routine care and are serially performed in long-  
 198 term ventilated neonates with evolving BPD.(10, 27) Data were anonymously collected in a  
 199 dedicated and secured database, respecting all local and European privacy regulations. The protocol  
 200 was approved by the local ethical committee (SRLF16/58), and written informed consent was  
 201 obtained from parents/guardians upon NICU admission. This study phase followed STROBE  
 202 guidelines.(28)

### 203 *Semi-quantitative lung ultrasound in preterm neonates*

204 We performed lung ultrasound on the 14<sup>th</sup> day of postnatal life (while all neonates were invasively  
 205 ventilated) as lung ultrasound score peaks and has the highest diagnostic accuracy to predict BPD at  
 206 this time-point.(10) We calculated the classical neonatal LUS on 6 chest areas (3 per each side),  
 207 assigning 0-to-3 points to each area (total score going from 0 to 18), based on lung ultrasound  
 208 semiology patterns, as previously described.(17) Transversal and longitudinal scans were performed  
 209 on all chest areas with a “hockey stick”, micro-linear, high-resolution probe (15 MHz; CX-50 Philips  
 210 Healthcare, Eindhoven, The Netherlands), as previously reported.(18) Exams were accomplished in a  
 211 standardized manner when ventilation was optimized and infants were in a calm state, using the same  
 212 technical settings described above for rabbits. No sedation was needed to perform lung ultrasound.  
 213 Clinicians performing ultrasound had at least 1 year of experience in daily use of lung  
 214 ultrasound,(19) as this is the first-line imaging technique in our NICU.(27)

### 215 *Lung mechanics in preterm neonates*

216 Neonates were intubated with appropriately sized endotracheal tubes, lightly sedated as previously  
 217 described.(29) Time-cycled, pressure-regulated, assisted-controlled ventilation was provided with the  
 218 following targets: 5-6 ml/kg tidal volume, 7.4 pH, and 35-65 mmHg PaCO<sub>2</sub>. PEEP was set between 5  
 219 and 8 cmH<sub>2</sub>O. A maximal peak pressure of 22 cmH<sub>2</sub>O was allowed, FiO<sub>2</sub> was as low as possible to  
 220 guarantee a peripheral hemoglobin saturation between 90% and 95% and PaO<sub>2</sub> between 50 and 70  
 221 mmHg. The flow trigger was set at the maximal possible sensitivity without auto-triggering.  
 222 Inspiratory time and flow were set looking at flow and pressure tracings and aiming to avoid any gas-

trapping and optimize synchrony. All patients were on continuous flow neonatal ventilators equipped with a low dead-space, hot-wire anemometers. Airway pressure and flow were both measured at the Y-piece (30) to maximize the accuracy of lung mechanics assessment. Ventilators were subjected to serial technical quality controls.(31) Sensors were calibrated before each use and manufacturers' recommendations were always followed. Lung mechanics was evaluated within 1h from lung ultrasound, considering Raw and Cdyn shown by a breath-to-breath analysis on the ventilator screen. Measurements were performed when neonates were stable, after airway suctioning, and following the previously described technique.(32) In detail, spontaneous breathing was temporally avoided by increasing mechanical rate, while the flow was decreased to 5L/min to reach a quasi-static situation. In these conditions, when we observed gas leaks to be <5% and normal-appearing pressure-volume and flow-volume loops, Raw and Cdyn were averaged on 10 mechanical breaths showing identical tracing.

### Statistics

We enrolled 25 preterm rabbits as in previous studies describing lung mechanics and pathology of this experimental model.(33) To be consistent, we aimed to enroll the same number of neonates with evolving BPD in the second study phase. We also enrolled 13 term rabbits as further controls. Data were tested for normality with the Shapiro-Wilk test and expressed as mean (standard deviation) or median [interquartile range], as appropriate. Data sets were compared with Student or Mann-Whitney test, respectively. The conformity of lung ultrasound interpretation, that is, the inter-rater absolute agreement between the two independent observers, was evaluated using the intra-class correlation coefficient (ICC).(34) rLUS and LUS were correlated with lung mechanics measures in preterm rabbits and neonates, respectively, using Spearman correlation coefficient ( $\rho$ ). Results were also graphically shown using scatter plots analyzed with local (smoother) regression with 95% Epanechnikov kernel;  $p < 0.05$  values were considered statistically significant. Analyses were performed using SPSS version 27.

## 249 RESULTS

250

251 Details of preterm rabbits and human neonates recruited in the first and second project phase are

252 summarized in **Tab.1**. All enrolled neonates were invasively ventilated at the fourteenth day of life

253 with a mean airway pressure of 15 (1.8) cmH<sub>2</sub>O. They were ventilated for ongoing respiratory failure

254 due to evolving BPD, and no infant had other concomitant reasons to need invasive ventilation: the

255 total duration of invasive ventilation was 13 [11-16] days. During their NICU stay, 6 (24%) neonates

256 needed postnatal steroids to facilitate extubation, and 17 (68%) were treated at least once with

257 nebulized salbutamol for spells due to bronchospasm. All neonates qualified for BPD diagnosis at 36

258 weeks' gestation and were eventually discharged from the NICU: eight (32%) infants had mild, eight

259 (32%) had moderate, and nine (36%) had severe BPD. **Tab.2** shows lung mechanics variables: Raw

260 and Cdyn were respectively above and below the normal values, both in preterm rabbits and

261 neonates.

262

263 Lung ultrasound findings were heterogeneous in hyperoxia-exposed preterm rabbits and

264 encompassed normally aerated zones, zones with interstitial-alveolar pattern and others with total

265 loss of aeration. More than one picture per lung was recorded in 17 (68%) preterm rabbits (an

266 average of 2.9 (0.8) lung ultrasound images were taken per rabbit), as the lung appearance was not

267 homogenous (**Fig.1-2**). The inter-rater absolute agreement for the evaluation of lung ultrasound

268 images in preterm rabbits (ICC: 0.989 [95%CI: 0.975-0.995];  $p<0.0001$ ) was very high, and there

269 was no difference when rLUS was calculated by the animal lab investigator (1.6 [0.9-2]) or the

270 independent expert rater (1.6 [1-2];  $p=0.462$ ; **Fig.2**). All preterm rabbits completed the experimental

271 protocol; lung ultrasound lasted 2-3 minutes per rabbit and no issue was noticed during the

272 procedure. Lung ultrasound findings were also heterogeneous in neonates with evolving BPD: **Fig.3**

273 shows the similarities between the main lung ultrasound abnormalities in hyperoxia-exposed preterm

274 rabbits and neonates with evolving BPD.

275

276 **Fig.4** shows the relationship between lung ultrasound scores and Raw or Cdyn in preterm rabbits and  
 277 neonates. Raw significantly correlated with rLUS ( $\rho=0.519$ ,  $p=0.008$ ,  $R^2=0.20$ ) and LUS ( $\rho=0.409$ ,  
 278  $p=0.042$ ,  $R^2=0.29$ ) (**Fig.4, Panel A and C**). There was no significant correlation between rLUS and  
 279 any other mechanics parameter in rabbits (Cdyn:  $\rho=0.067$ ,  $p=0.750$ ; inspiratory capacity:  $\rho=0.463$ ,  
 280  $p=0.259$ ; tissue damping:  $\rho=0.327$ ,  $p=0.190$ ; elastance:  $\rho=0.067$ ,  $p=0.750$ ), nor between LUS and  
 281 Cdyn ( $\rho=0.205$ ,  $p=0.325$ ) in neonates.

282

283 **Fig.5** reports the differences between hyperoxia-exposed preterm rabbits and control rabbits  
 284 delivered at term and treated in normoxia. Typical histological findings of experimental BPD were  
 285 observed in preterm rabbits with extensive inflammation and airspace simplification (**Fig.5A-D**).  
 286 This is confirmed by Lm, which was higher in hyperoxia-exposed preterm rabbits compared to term  
 287 animals (**Fig.5G**). Illustrative images show that the lungs of preterm rabbits were poorly aerated,  
 288 compared to control animals (**Fig.5E-F**), and this is confirmed by rLUS, which was always zero in  
 289 term rabbits and significantly higher than in hyperoxia-exposed preterm rabbits.

290

## 291 **DISCUSSION**

292 Our study adds novel findings to the knowledge about experimental BPD models: we show that the  
 293 hyperoxia-exposed preterm rabbit model has similar lung ultrasound features and relationships with  
 294 lung mechanics (that is, correlation between lung ultrasound scores and Raw, lack of correlation with  
 295 other parameters) of evolving BPD in human neonates.

296

297 To our knowledge, this is the first use of lung ultrasound in an experimental model of BPD. Evolving  
 298 BPD in neonates is characterized by a non-homogeneous pattern composed of consolidated, tissue-  
 299 like zones with significant loss of aeration, zones with a more or less severe interstitial-alveolar  
 300 pattern, and zones with a normal aeration (10, 35). These features were also found in rabbits with  
 301 experimental BPD. Interestingly, these ultrasound findings were coherent with those obtained by

302 micro-CT scan demonstrating altered lung aeration in preterm rabbits,(36) and this is fully consistent  
303 with data showing a close correlation between lung ultrasound score and CT-measured lung  
304 aeration.(37, 38) The similarity can be attributed to the shared pathophysiology, such as impaired  
305 alveolarization and airway development typical of both human BPD and the experimental BPD-like  
306 phenotype generated in the rabbit model.(39, 40) Of note, lung ultrasound appearance looks similar  
307 in preterm rabbits and human neonates, while lung development is also known to be comparable (41)  
308 and we can estimate to have a similar developmental stage in preterm rabbits and human neonates  
309 studied at 7 and 14 days of postnatal age, respectively. (12, 13, 41)

310

311 Semi-quantitative ultrasound assessment of lung aeration has been successfully applied in small  
312 preterm rabbits, with similar probes, frequencies and procedures used in preterm neonates,  
313 confirming the reliability of our findings. Interestingly, relatively novice and experienced operators  
314 achieved similar lung ultrasound scores, exactly as observed in NICU care when operators of  
315 different degrees of expertise performed lung ultrasound.(19) These data expand those showing the  
316 feasibility of lung ultrasound in adult rabbit models of acute respiratory distress syndrome (15) or in  
317 preterm rabbits with primary surfactant deficiency at birth (42) and represent the first example of  
318 ultrasound-assessed lung aeration in a model of chronic, evolutive lung injury with a mixed  
319 obstructive/restrictive pattern.

320

321 Our findings also show consisting similarities in the relationship between ultrasound-assessed lung  
322 aeration and mechanics. Lung ultrasound scores correlated with Raw both in preterm rabbits and  
323 human neonates, while no other significant correlation was found with any other lung mechanics  
324 parameter. Infants with BPD have variably decreased compliance and increased resistances, leading  
325 to non-uniform ventilation and time-constant (43) and this inhomogeneity is well described by our  
326 heterogeneous lung ultrasound findings. In addition, established BPD seems to be particularly  
327 characterized by small airway obstruction, (44) and this may explain the observed relationship

328 between Raw and the lung ultrasound scores. When resistances are higher due to small airway  
329 obstruction, lung parenchyma is less aerated, resulting in higher lung ultrasound scores. Small airway  
330 obstruction may also explain the lack of correlation between lung ultrasound scores and Cdyn as  
331 significantly obstructed lung zones are less ventilated and cannot contribute to the Cdyn calculation.  
332 The lack of correlation between rLUS and other mechanical parameters in rabbits may be due either  
333 to the non-homogeneous lung injury pattern and the relatively low resolution of lung ultrasound,  
334 which cannot provide further details in these small animals. Correlation findings are analogous in the  
335 experimental model and in neonates with evolving BPD, confirming the equally impaired lung  
336 mechanics (33, 36) as results of similar histology abnormalities.(39, 40) In fact, all lung mechanics  
337 parameters showed in **Tab.2** are significantly impaired compared to term rabbits with no lung  
338 disease.(33) The analyses performed in term rabbits as further control corroborate our findings. In  
339 fact, histology clearly shows typical features of lung injury mimicking BPD in hyperoxia-exposed  
340 preterm,(33) while these were absent in term rabbits. Also, lung ultrasound findings were  
341 heterogeneous in preterm rabbits as in human neonates with evolving BPD,(10) while lung  
342 ultrasound appearance was always normal in term rabbits, exactly as it is always observed in term  
343 neonates without any respiratory disorder.(45)

344

345 This study has some limitations. The rabbit model is suitable but obviously cannot reproduce all the  
346 features of evolving BPD. The effect of invasive ventilation is not considered, since preterm rabbits  
347 were spontaneously breathing while the neonates were intubated. Other injuries such as fetal  
348 inflammation or infections, that may add to the complexity of BPD development, are also not  
349 considered in this model. Moreover, rabbits are not exposed to prenatal steroids, while hyperoxia is  
350 avoided in neonates as much as possible. Studying a cohort of infants with evolving BPD serves as  
351 “control” of the experimental model, further to the control rabbits delivered at term and unexposed to  
352 hyperoxia.(33) The studied sample size is relatively small but is represented by a well-described  
353 experimental model and by a homogeneous population of infants with severe ongoing respiratory

354 failure due to evolving BPD. The choice of BPD definition and a given time-point to perform LUS  
355 may be seen as limitations. However, we only used the classical BPD definition since recent analyses  
356 performed using multiple definitions have yielded similar diagnostic accuracy for LUS to predict  
357 BPD.(35) Also, we only performed LUS at 14 days of postnatal age, because LUS predictivity for  
358 BPD is best, while rabbit and human lung development are quite similar at this time-points.(10, 12)  
359 Lung ultrasound has been applied for the first time in this experimental model and its use could be  
360 improved with experience or smaller probes with higher resolution, albeit using the same devices and  
361 protocols as in neonatal lung ultrasound guaranteed a fair comparison of resulting images. The  
362 application of a given mean airway pressure by invasive ventilation during lung mechanics  
363 measurements in rabbits can provide a certain alveolar recruitment and improve the lung aeration  
364 compared to spontaneous breathing: as lung ultrasound and mechanics measurements in rabbits are  
365 performed during spontaneous breathing and invasive ventilation, respectively, this may have  
366 partially influenced results. However, this is unavoidable to perform lung mechanics measurements  
367 and ventilation has been applied with low positive end-expiratory pressure: therefore, its influence, if  
368 any, should have been minimal. Our population consisted of BPD neonates who survived and  
369 obviously those who died during the NICU hospitalization might have worse lung mechanics, which  
370 might influence results. However, mortality directly due to BPD is uncommon in newborn  
371 populations similar to ours (46) and we wanted to mimic the majority of patients with evolving BPD.  
372

### 373 CONCLUSIONS

374 Lung ultrasound features are similar in the preterm rabbit model of experimental BPD and in human  
375 neonates with evolving BPD. Lung ultrasound is easy to be used even in small animals and with short  
376 training. We found an inverse correlation between airway resistance and ultrasound-assessed lung  
377 aeration both in preterm rabbits and neonates. The preterm rabbit model fairly reproduces the lung  
378 ultrasound and mechanical characteristics of preterm neonates with evolving BPD.

379

## 380 DISCLOSURE

381 DDL received research and educational grants from Chiesi Pharmaceuticals spa and ABBVIE inc. He  
382 served as lecturer for, Chiesi Pharmaceuticals spa, ABBVIE inc and Getinge. Finally, has been a  
383 member of advisory boards for Chiesi Pharmaceuticals spa, ABBVIE inc, Airway Therapeutics and a  
384 consultant for OPHIREX inc. These companies produce surfactants or surfactant components but had  
385 no role at all in this project.

386 NY received travel grants from Chiesi Pharmaceuticals spa. This company produce surfactants or  
387 surfactant components but had no role at all in this project.

388 CCas, CC, MS and FS are employees of Chiesi Pharmaceuticals spa.

389

## 390 ABBREVIATION LIST

391 BPD: broncho-pulmonary dysplasia; Cdyn: dynamic compliance; CRIB-II: critical risk index for  
392 babies; ICC: Intra-class correlation coefficient; LUS: neonatal lung ultrasound score; NICU: neonatal  
393 intensive care units; PEEP: positive end-expiratory pressure; Raw: airway resistance; rLUS: rabbit  
394 lung ultrasound score.

395

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**Figure 1. Lung ultrasound procedure in newborn rabbits.** Both lungs were entirely scanned in each animal (**Panel A**) and, since the probe was relatively large compared to the animal chest, the evaluation was done on the whole hemithorax (i.e., without any division in chest areas (16)) both transversally (**Panel B<sub>1</sub>**) and longitudinally (**Panel B<sub>2</sub>**). rLUS was calculated according to the classical semiology pattern used for the neonatal lung ultrasound score (17), that is, assigning 0-to-3 points (**Panel C-F**). A 0 indicates A-pattern (defined by the presence of A-lines only, white triangles, representative of normally aerated zones); 1, B-pattern (defined as the presence of  $\geq 3$  well-spaced B-lines, white arrows, indicative of zones with interstitial edema); 2, severe B-pattern (defined as the presence of crowded and coalescent B-lines, white arrows with parentheses, with or without consolidations limited to the subpleural space, that is zones with alveolar edema); and 3, extended consolidations (that is zones with total loss of aeration; white star). If the lung appearance was not homogenous, several pictures were registered to capture all lung ultrasound features and their evaluation was averaged. Pictures refer to a hyperoxia-exposed preterm rabbit, but the same technique was used both in preterm and term rabbits.

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**Figure 2. Conformity of lung ultrasound features interpretation to calculate rLUS.** Observer 1 and 2, respectively, indicate the investigator performing animal care and the neonatologist expert in lung ultrasound who independently evaluated the lung ultrasound images. Horizontal lines depict medians and 25<sup>th</sup>-75<sup>th</sup> percentiles and analyzed with intra-class correlation coefficient and Mann-Whitney test. Experiments were performed in 25 hyperoxia-exposed preterm rabbits. rLUS is a dimensionless number. **Abbreviations:** rLUS: rabbit lung ultrasound score.

569

**Figure 3. Illustrative pictures of abnormal lung ultrasound features in hyperoxia-exposed preterm rabbits and human neonates with evolving BPD.** Pictures on the left- and right-side have been taken from rabbits and infants, respectively. Panels A and B show a B-pattern (interstitial); panels C and D show a severe B-pattern (alveolar) and panels E and F show extended consolidation

574 with loss of lung aeration. Lung ultrasound findings were very and equally heterogeneous both in  
 575 preterm rabbits and in neonates.

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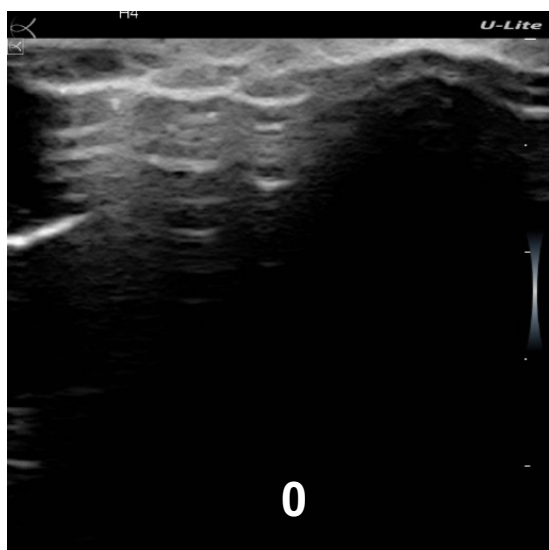
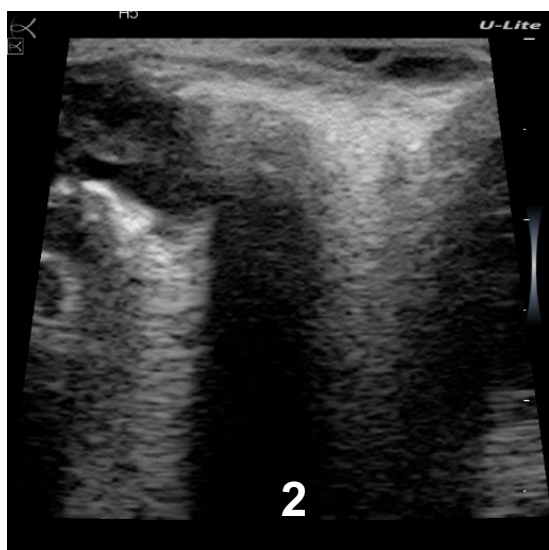
577 **Figure 4. Relationship between lung ultrasound scores and lung mechanics measurements in**  
 578 **hyperoxia-exposed preterm rabbits (n=25; panel A-B) and neonates with evolving BPD (n=25;**  
 579 **panel C-D).** Spearman correlation coefficients and their *p*-values are shown. The hatched curve  
 580 shows the tendency and represents the best fitting data curve generated with local smoother  
 581 regression. Panel A and B represent the correlation between rLUS and airway resistance or dynamic  
 582 lung compliance, respectively. Panel C and D represent the correlation between the classical neonatal  
 583 LUS and airway resistance or dynamic lung compliance, respectively. LUS and rLUS are  
 584 dimensionless numbers. **Abbreviations:** Cdyn: dynamic lung compliance; Raw: airway resistance;  
 585 LUS: lung ultrasound score; rLUS: rabbit lung ultrasound score.

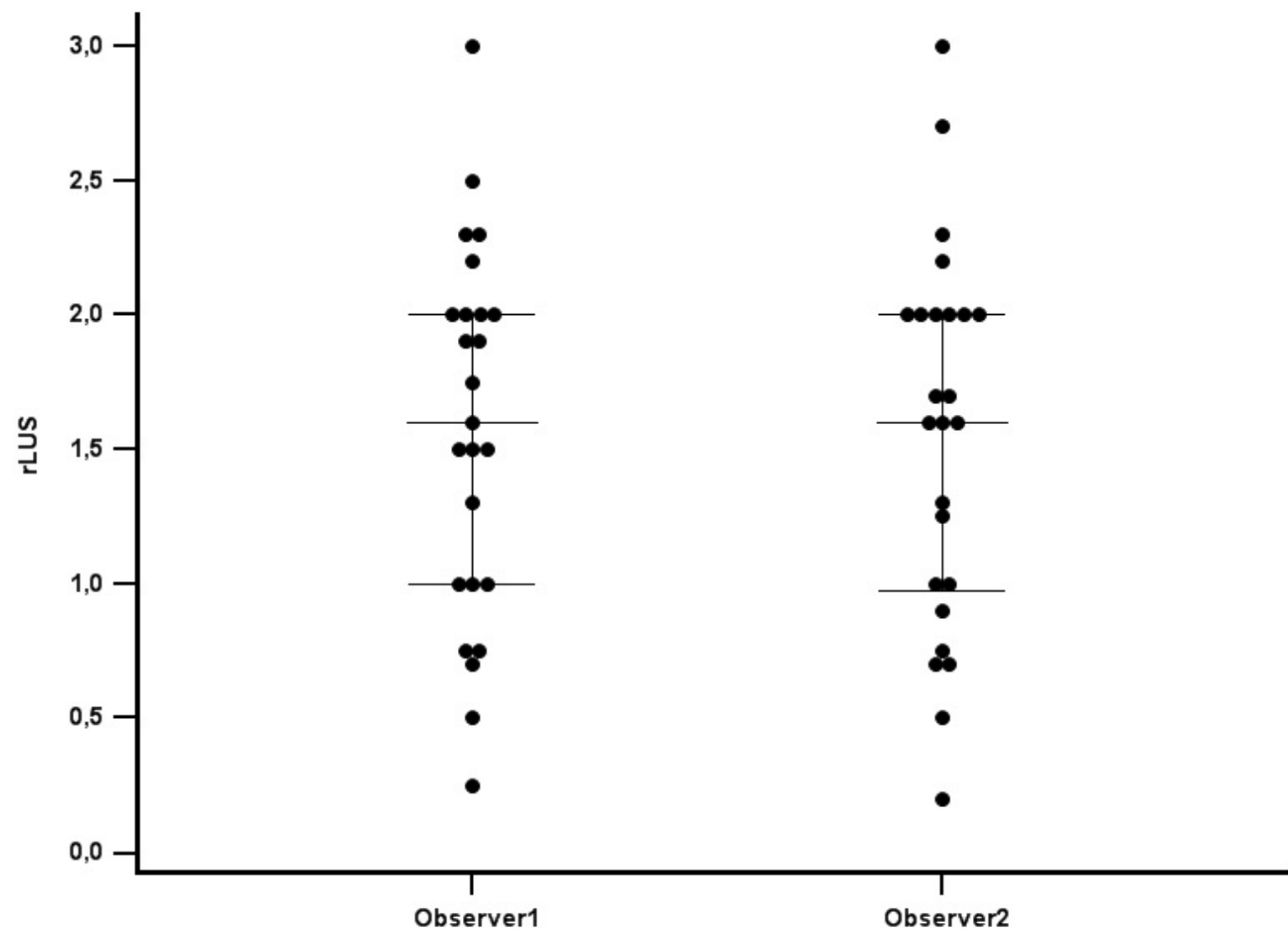
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587 **Figure 5. Histology and lung ultrasound features in hyperoxia-exposed preterm rabbits (left**  
 588 **side; n=25) and term rabbits kept in normoxia (right side; n=13).** Illustrative hematoxylin and  
 589 eosin staining of a sagittal section of the right lung from one animal per group are shown with 2x  
 590 (panel A-B) and 10x magnification (panel C-D). There are clear differences between the groups, with  
 591 hyperoxia-exposed preterm rabbits showing inflammatory cell infiltration, larger alveoli, and  
 592 thickened septa. Panel E-F show illustrative lung ultrasound images from one animal per group: the  
 593 hyperoxia-exposed preterm rabbit has an alveolar pattern with confluent B-lines, while the control  
 594 rabbit has a normal lung appearance with only A-lines. Panel G shows morphometric and lung  
 595 ultrasound score data in the two groups. rLUS is a dimensionless number. **Abbreviations:** Lm: mean  
 596 linear intercept; rLUS: rabbit lung ultrasound score.

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**A****B<sub>1</sub>****B<sub>2</sub>****C****D****E****F**

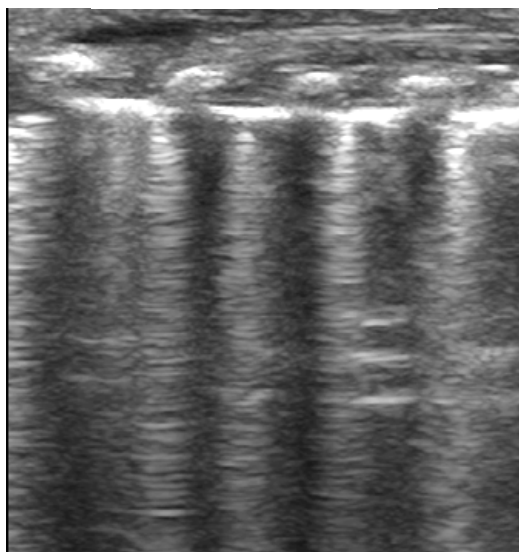




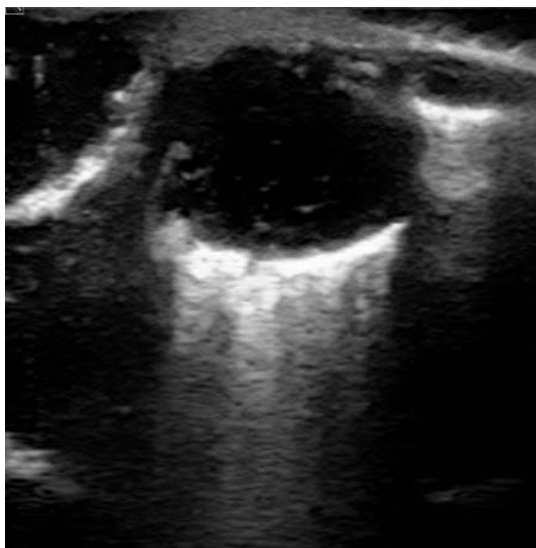
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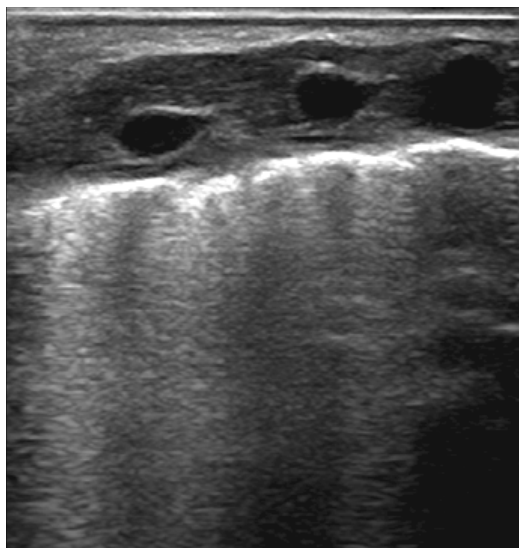
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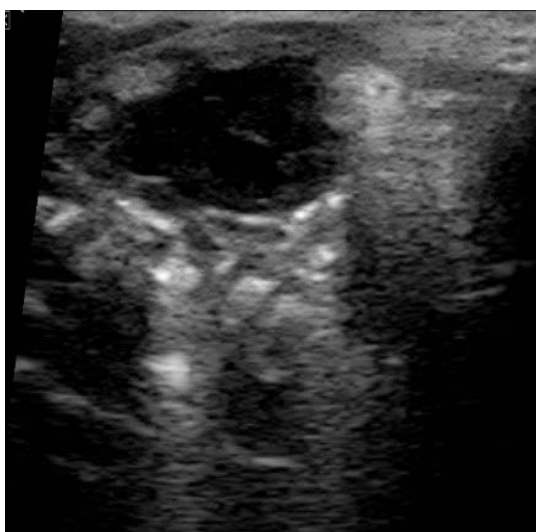
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**D**

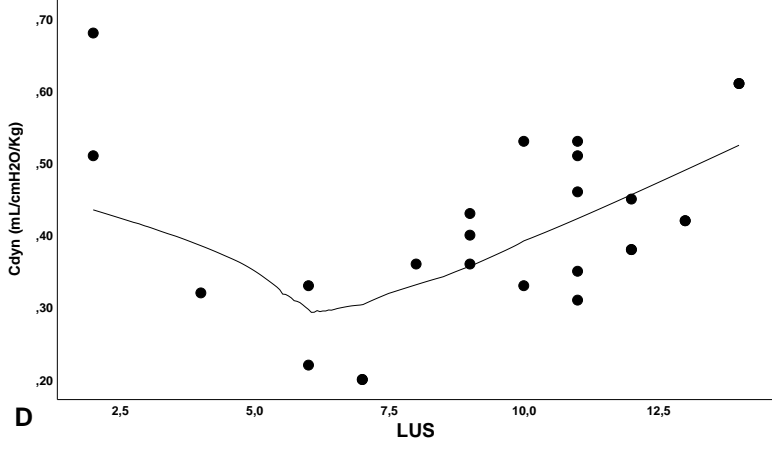
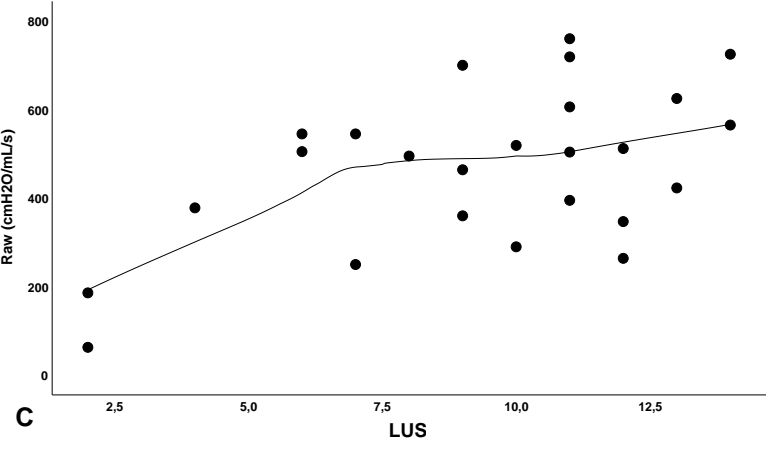
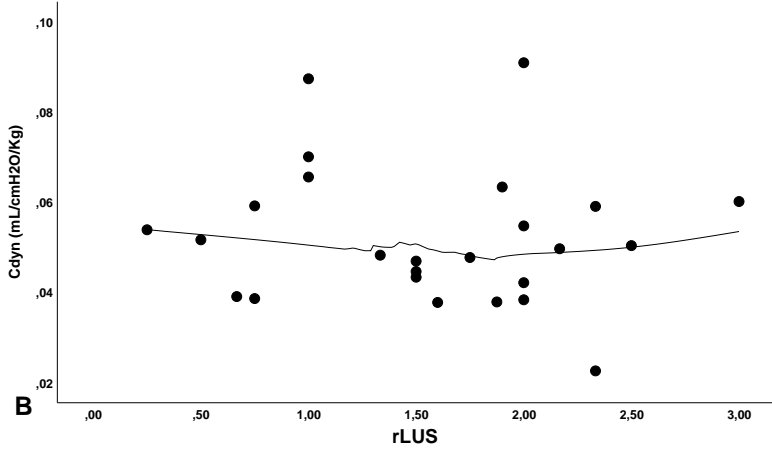
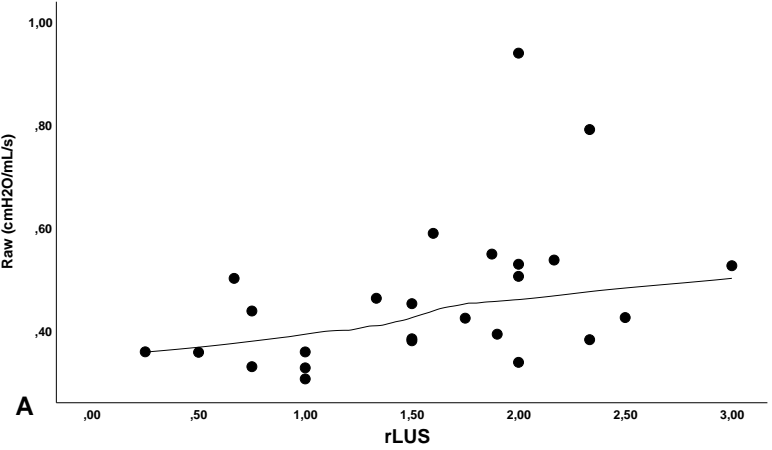


**E**



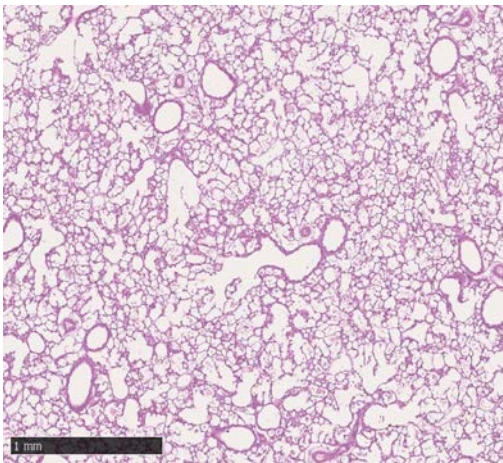
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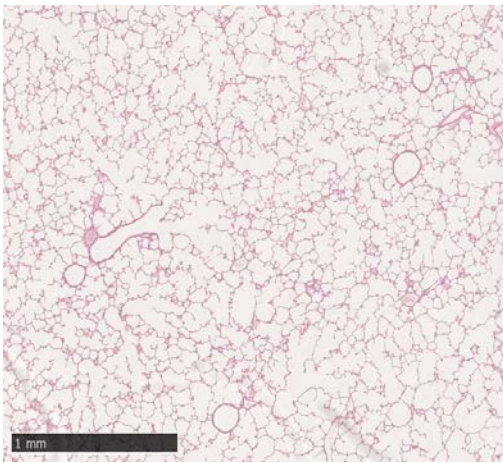




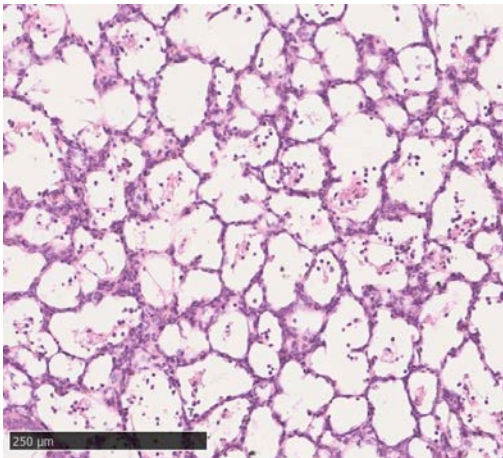
**A**



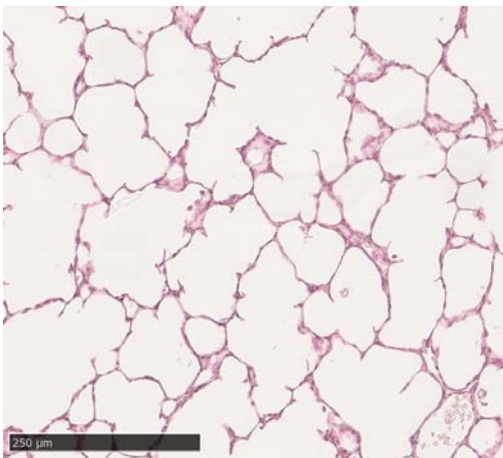
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**C**



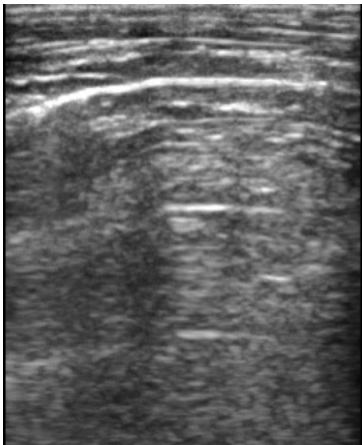
**D**



**E**



**F**



<b>G</b>	<b>Preterm (hyperoxia-exposed)</b>	<b>Term (normoxia)</b>	<b><i>p</i></b>
<b>Lm (μm)</b>	84 (4.9)	72 (5.5)	<0.0001
<b>rLUS</b>	1.6 [1-2]	0 [0-0]	<0.0001

**Table 1. Characteristics of hyperoxia-exposed preterm rabbits and human neonates.** Data are expressed as mean (standard deviation), median [interquartile range] and number (%). Weight-D7 and Weight-D14 indicate the weight at the 7<sup>th</sup> and 14<sup>th</sup> day of life, respectively. rLUS-D7 and LUS-D14 indicate the rabbit and the neonatal lung ultrasound score at the 7<sup>th</sup> and 14<sup>th</sup> day of life, respectively. Apgar, CRIB-II, LUS and rLUS are dimensionless numbers. Prenatal steroids prophylaxis is considered if complete, that is, if two 12mg betamethasone doses were administered 24h apart and within 7 days before the delivery.

**Abbreviations:** CRIB-II: critical risk index for babies; LUS: neonatal lung ultrasound score; NICU: neonatal intensive care units; rLUS: rabbit lung ultrasound score.

<b>Preterm rabbits (n=25)</b>	
Birth weight (grams)	37 (1.01)
Weight-D7 (grams)	49.3 (1.44)
rLUS-D7	1.5 [0.25-3]
<b>Preterm neonates (n=25)</b>	
Gestational age (weeks)	25.8 (1.01)
Birth weight (grams)	759 (115)
Weight-D14 (grams)	898 (125)
Prenatal steroids prophylaxis	17 (68%)
Male Sex	14 (63.6%)
5' Apgar score	9 [5.5-8.5]
CRIB-II score	12 [11-13]
LUS-D14	7 [8-11]
NICU stay (days)	101 [96-130]

**Table 2. Lung mechanics variables in preterm rabbits and neonates.** Data are expressed as mean (standard deviation). **Abbreviations:** Cdyn: dynamic compliance, Raw: airway resistance.

<b>Preterm rabbits (n=25)</b>	
Raw (cmH <sub>2</sub> O/mL/sec)	0.5 (0.1)
Cdyn (mL/cmH <sub>2</sub> O/kg)	1.07 (0.3)
Inspiratory capacity (mL/kg)	20.6 (6.2)
Tissue damping (cmH <sub>2</sub> O/mL)	3.1 (0.7)
Elastance (cmH <sub>2</sub> O/mL)	12 (2.9)
<b>Preterm neonates (n=25)</b>	
Raw (cmH <sub>2</sub> O/L/sec)	470 (176)
Cdyn (mL/cmH <sub>2</sub> O/kg)	0.32 (0.1)