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## Surfactant-secreted phospholipase A2 interplay and respiratory outcome in preterm neonates

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**Authors' contributions:** A/Prof. De Luca designed and managed the study as group coordinator, helped in data collection and organization of the experiments, transferred the samples, performed the analysis of all the data and wrote the manuscript. Drs.Shankar, Autilio, Raschetti, Vedovelli, Payré and Jeammet, performed the experiments and/or collected all the data and critically reviewed the manuscript for important intellectual content. Moreover, Dr. Shankar organized the experiments, helped with the data analysis and writing of the first manuscript draft. A/Prof. Cogo and Proff. Perez-Gil, Carnielli and Lambeau helped to design the experiments, helped to analyse the data and critically reviewed the manuscript for important intellectual content. Prof.Touqui conceptualized and designed the study, coordinated and supervised it and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

59 **ABSTRACT**

60           Secreted phospholipase A2 hydrolyses surfactant phospholipids and is crucial for the  
61 inflammatory cascade; preterm neonates are treated with exogenous surfactant but the interaction  
62 between surfactant and phospholipase is unknown. We hypothesize that this interplay is complex  
63 and the enzyme plays a relevant role in neonates needing surfactant replacement. We aimed to: 1)  
64 identify phospholipases A2 isoforms expressed in preterm lung; 2) study the enzyme role on  
65 surfactant re-treatment and function and the effect of exogenous surfactant on the enzyme system;  
66 3) verify if phospholipase A2 is linked to respiratory outcomes. In bronchoalveolar lavages of  
67 preterm neonates, we measured enzyme activity (alone or with inhibitors), enzyme subtypes,  
68 surfactant protein-A and inflammatory mediators. Surfactant function and phospholipid profile were  
69 also tested. Urea ratio was used to obtain epithelial lining fluid concentrations. Follow-up data were  
70 prospectively collected. Subtype-IIA is the main phospholipase isoform in preterm lung, although  
71 subtype-IB may be significantly expressed. Neonates needing surfactant retreatment have higher  
72 enzyme activity ( $p=0.021$ ) and inflammatory mediators ( $p$  always  $\leq 0.001$ ), and lower amounts of  
73 phospholipids ( $p$  always  $< 0.05$ ). Enzyme activity was inversely correlated to surfactant adsorption  
74 ( $\rho=-0.6$ ;  $p=0.008$ ; adjusted  $p=0.009$ ), total phospholipids ( $\rho=-0.475$ ;  $p=0.05$ ) and  
75 phosphatidylcholine ( $\rho=-0.622$ ;  $p=0.017$ ). Exogenous surfactant significantly reduced global  
76 phospholipase activity ( $p<0.001$ ), subtype -IIA ( $p=0.005$ ), and increased  
77 dioleoylphosphatidylglycerol ( $p<0.001$ ) and surfactant adsorption ( $p<0.001$ ). Enzyme activity  
78 correlated with duration of ventilation ( $\rho=0.679$ ,  $p=0.005$ ; adjusted  $p=0.04$ ), respiratory morbidity  
79 score at 12 months postnatal age ( $\tau_b=0.349$ ,  $p=0.037$ ; adjusted  $p=0.043$ ), but was not associated  
80 with mortality, bronchopulmonary dysplasia or other long-term respiratory outcomes.

81

82 **KEY WORDS:** phospholipase A2, surfactant, RDS, prematurity, lung injury, newborn infant

83

84

## 85 INTRODUCTION

86 Surfactant is a cornerstone of neonatal critical care and has significantly improved  
87 clinical outcomes of preterm neonates with respiratory distress syndrome (RDS), that is, primary  
88 surfactant deficiency related to prematurity, which is treated with one or, less often, multiple  
89 surfactant doses (38). Surfactant is a complex mixture of phospholipids (90%) and proteins  
90 (10%) and its adsorption at the air/liquid interface is essential to create a surfactant layer able to  
91 lower alveolar surface tension. Despite surfactant replacement, preterm neonates may still  
92 experience negative long-term outcomes requiring a significant burden of care (38).

93 Secreted phospholipases A2 (sPLA2; phosphatide 2-acylhylolase, EC3.1.1.4) are the  
94 main enzymes which hydrolyze surfactant phospholipids and participate in the first step of the  
95 arachidonic acid inflammatory cascade. Different sPLA2 subtypes exist with various substrate  
96 specificity. Increased levels of sPLA2 are associated with the occurrence of acute respiratory  
97 distress syndrome (ARDS) (47) and sPLA2 correlates with lung function and clinical outcomes,  
98 both in adults and children affected by ARDS (11, 30). ARDS is characterized by a complex  
99 qualitative and quantitative injury of endogenous surfactant, but the exogenous surfactant may  
100 also be damaged if it is administered in an environment with increased levels of sPLA2 and other  
101 inflammatory agents (29, 47).

102 Despite exogenous surfactant being routinely used in preterm infants, there are no data  
103 about its interactions with sPLA2 in humans. We only know that sPLA2 is expressed  
104 irrespective of gestational age and that its activity correlates with ventilatory requirements and  
105 lung compliance (10, 41). Preterm neonates may sometimes need repeated surfactant  
106 administrations. Therefore, they represent the ideal setting to study the sPLA2-surfactant  
107 interactions and their possible consequences. We hypothesize that sPLA2 may play a relevant  
108 role in neonates needing surfactant replacement. Our aims were: 1) to identify sPLA2 subtypes  
109 expressed in preterm neonates with RDS; 2) to study the role of sPLA2 on surfactant retreatment  
110 and function, and, conversely, the effect of exogenous surfactant on sPLA2 and its inhibitors; 3)  
111 to investigate the relationship between sPLA2 and respiratory outcomes. Some partial results of

112 this study have been previously presented as abstracts at the 58<sup>th</sup> annual congress of the  
113 European Society for Pediatric Research (Venice, Italy – Oct 30, Nov 3 2017).

114

## 115 **MATERIALS AND METHODS**

### 116 *Design and hypothesis*

117 This was a prospective, translational, pragmatic, international, long-term, cohort study,  
118 which is part of a larger project investigating the role of sPLA2 in various pediatric respiratory  
119 disorders (45). STrengthening the Reporting of OBservational studies in Epidemiology  
120 guidelines were followed (48). The study has been conducted in four laboratories collaborating  
121 with one tertiary academic neonatal intensive care unit (NICU). We hypothesize that: 1) preterm  
122 lungs may express at least the four sPLA2 isotypes previously found in adult lungs (26); 2)  
123 sPLA2 activity affects the need for surfactant and its function and that surfactant replacement  
124 influences sPLA2 activity; 3) sPLA2 may influence respiratory outcomes. The study protocol  
125 was approved by the local institutional review board (n.13/046) and informed consent was  
126 obtained from parents upon NICU admission.

### 127 *Patients*

128 Eligible babies were inborn preterm neonates with gestational age  $\leq 32$  weeks needing  
129 intubation and surfactant replacement for RDS (45). Exclusion criteria were: 1) lung or airway  
130 malformations; 2) any need for surgery; 3) chromosomal abnormalities, genetic syndromes or  
131 complex congenital malformations; 4) septic shock, as defined earlier (18); 4) other previously  
132 defined neonatal lung disorders: a] meconium or blood aspiration syndrome (11,12); b]  
133 pulmonary hemorrhage or maternal blood aspiration (39); c] congenital pneumonia (17); d] any  
134 type of neonatal ARDS (16). Finally, babies enrolled but having bloody bronchoalveolar lavage  
135 (BAL) samples were excluded from the study.

136 Neonates received full prenatal care, including antenatal steroids, delayed cord clamping  
137 and MgSO<sub>4</sub>, when appropriate, following current guidelines (44,49,50). Respiratory care was  
138 provided, as described earlier (17). In short, neonates are started on variable-flow, nasal mask-

139 delivered continuous positive airway pressure (CPAP; 6 cmH<sub>2</sub>O) in the delivery room and  
140 continued during the transfer to the NICU, which is adjacent to the delivery suite. Surfactant  
141 (200 mg/kg poractant- $\alpha$ , CUROSURF<sup>®</sup>, Chiesi Farmaceutici, Parma, Italy) was administered in  
142 the NICU, when CPAP failed, based on inspired oxygen fraction (FiO<sub>2</sub>) thresholds derived from  
143 the European guidelines (44) and using intubation-surfactant-extubation technique. However,  
144 neonates were kept ventilated if they had hemodynamic troubles needing inotropes or severe  
145 anemia (hemoglobin  $\leq$ 8g/L) or if oxygenation was not rapidly improved by surfactant  
146 administration (4). Anyhow, invasive ventilation was shortened as much as possible and non-  
147 invasive ventilatory techniques were extensively applied following a strict protocol (17). A  
148 second 100 mg/kg poractant- $\alpha$  dose was administered, if the FiO<sub>2</sub> was higher than the threshold  
149 FiO<sub>2</sub>, at least 10h after the first administration. Retreatment was not performed earlier, as the  
150 median half-life of dipalmitoylphosphatidylcholine (DPPC) is 10h in preterm neonates requiring  
151 multiple surfactant doses (5).

## 152 *Sampling*

153 Non-bronchoscopic BAL was performed following European Respiratory Society advices  
154 (9) and using a well standardized technique (10). In short, 1 mL/kg 0.9%NaCl (37°C) was  
155 injected through a Y-piece connected to the endotracheal tube to keep the patients ventilated  
156 during the lavage. Sedation was provided as described earlier (17). BAL was performed before  
157 the first surfactant dose and repeated after at least 10h from the administration, only if, according  
158 to clinical conditions, the neonate still needed invasive ventilation at that time. If this was the  
159 case, BAL was performed as soon as the neonate needed airway suction for secretions removal.  
160 BAL samples were immediately divided: 0.2 mL were sent for standard microbiological cultures,  
161 as per our policy of microbiological surveillance. The remaining volume was immediately  
162 centrifuged (800g;10'). Supernatant was divided in several tubes and frozen (-80°C). Tubes were  
163 sent in dry ice to the different laboratories; because of their small volume, each sample was not  
164 used for each assay. All measurements were performed in duplicate and laboratory investigators  
165 were blinded to patients' clinical data. Due to the small volume of BAL samples, it was not

166 possible to perform all assays and experiments with each sample. However, we used a fair mix  
167 of patients' samples for each assay, paying attention to be representative of the whole  
168 population, according to its basic characteristics.

#### 169 *sPLA2 activity*

170 Total sPLA2 activity was measured as previously described (35). Briefly, 30  $\mu$ L of BAL  
171 samples were incubated with substrate ( $^3$ H-OA-labeled *Escherichia coli* membrane  
172 phospholipids) at 37°C in 150  $\mu$ L of assay buffer (0.1 M Tris [pH 8], 1 mM CaCl<sub>2</sub>, 0.2% sodium  
173 azide). After incubation, the released  $^3$ H-OA-labelled free fatty acids were counted and the  
174 sPLA2 activity was expressed as % of radioactivity. This assay has an average sensitivity of 0.1  
175 pmol (i.e.: can detect the activity of as low as 1 pg of enzyme) (35).

#### 176 *Differential sPLA2 isoforms assays*

177 sPLA2 activity was also measured as above, but in the presence of various sPLA2 inhibitors as  
178 described earlier (20). Characteristics of the inhibitors are described in the **supplementary data**  
179 **(Figshare - 10.6084/m9.figshare.12148686)**. Control recombinant human sPLA2-IB, -IIA, -V  
180 and -X were prepared (43) and time-resolved fluoroimmunoassays for these subtypes were  
181 performed (32). Moreover, concentrations of these subtypes were measured using specific  
182 ELISA (Biomatik, Cambridge, Canada; Cayman Chemical, Ann Arbor, USA; Cusabio  
183 Technology, Houston, USA; Cloud Clone Corp., Katy, USA, respectively) and normalized for  
184 total proteins.

#### 185 *Inflammatory mediators and other assays*

186 Multiple inflammatory mediators were measured using customized Luminex assay  
187 (ThermoFisher scientific, Waltham, MA, USA) (28). Surfactant protein A (SP-A) was measured  
188 with ELISA (BioVendor, Brno, Czech Republic). Total proteins and urea in BAL were measured  
189 using Lowry's and colorimetric methods, respectively (27,28). Epithelial lining fluid  
190 concentrations were obtained by correcting for serum-to-BAL urea ratio, using urea measured on  
191 the first day of life with routine blood tests. Measurements were expressed as epithelial lining

192 fluid concentrations, unless otherwise indicated. sPLA2 was also normalized for SP-A, as it is a  
193 main natural inhibitor (47).

#### 194 ***Phospholipid profile***

195 Total phosphatidylcholine was assayed with an enzymatic method (31). A BAL volume  
196 containing 50  $\mu\text{g}$  of phosphatidylcholine was dried to analyze the phospholipid profile with  
197 LC/MS, as described earlier (40) and data were normalized to specific standards per each  
198 analyzed lipid (purity= $\pm 2\%$ ; error $< 5\text{ppm}$ ; Avanti Polar Lipids; Merck KGaA, Darmstadt,  
199 Germany). Lyso-phosphatidylcholine and lyso-phosphatidylethanolamine have been also  
200 normalized for phosphatidylcholine and phosphatidylethanolamine amount. Limit of detection  
201 for the various phospholipids are reported in the **supplementary methods**.

#### 202 ***Surfactant adsorption test***

203 Samples were diluted to a phosphatidylcholine concentration of 0.043 mg/mL  
204 (corresponding to a total of 3  $\mu\text{g}$  of surfactant in a volume of 70  $\mu\text{L}$ ), using 5 mM Tris buffer  
205 (pH 7.4), containing 150 mM NaCl. Surfactant adsorption was then measured as previously  
206 described and reported as relative fluorescence unit (RFU) (1).

#### 207 ***Clinical follow up***

208 Clinical data were recorded in real time during the NICU stay. Broncho-pulmonary  
209 dysplasia (BPD) was defined according to common definition (22). According to our routine  
210 clinical protocol, patients were followed with regular visits for 24 months and follow-up data  
211 were collected on a dedicated database. The clinician taking care of the follow-up was blinded to  
212 laboratory data. Data collected as long-term endpoints were: respiratory infections, use of  
213 respiratory drugs, need for re-hospitalization for respiratory disorders and home oxygen therapy.  
214 Moreover, respiratory morbidity during the first year of life was evaluated using a respiratory  
215 morbidity score, which is known to correlate with late respiratory outcomes (33). In detail, we  
216 scored 0 if there was no cough, wheezing or need of respiratory drugs; 1 or 2 if there were  $\leq 3$  or  
217  $\geq 4$  days or nights of cough, wheezing or need of respiratory drugs, respectively, during the 12<sup>th</sup>  
218 month of life.

219 **Statistics**

220 A convenience sample size of 60 babies was originally previewed (45) and we  
221 subsequently enlarged the enrolment to have more samples allowing for the different  
222 experiments. Data were tested for normality with Shapiro-Wilk test and expressed as mean  
223 (standard deviation) or median [interquartile range]. Comparisons were conducted with two  
224 sized Mann-Whitney, Wilcoxon or Kruskal-Wallis test, as appropriate. Correlation analyses  
225 using Spearman ( $\rho$ ) or Kendall ( $\tau_b$ ) coefficients were also performed, as appropriate.

226 Relationship between surfactant adsorption and sPLA2 activity has been investigated using  
227 multiple curve estimation procedure to find the best- fitting model;  $R^2$  values were considered to  
228 assess the model goodness- of- fit and the model with the highest  $R^2$  was chosen (6).

229 Correlations between sPLA2, surfactant adsorption and clinical outcomes were adjusted for  
230 gestational age or birth weight using partial correlation after Log-normalization ( $_{adj}r$ ) or using  
231 multinomial regression with backward method, as appropriate. SPSS 15.0 (SPSS, Chicago, IL,  
232 USA) was used and  $p < 0.05$  was considered to be significant. Dataset used are available to  
233 readers on reasonable request.

234

235 **RESULTS**

236 Eighty-three preterm neonates were eligible; BAL procedure was uneventful and lasted 4  
237 (1.5)'. Five patients had bloody BAL samples and were excluded. Mean retrieved volume was 1.5  
238 (0.1) mL. Basic data of neonates finally studied are shown in **Tab.1**.

239 ***sPLA2 subtypes identification***

240 The concentration of sPLA2-IIA was the highest amongst enzyme subtypes, followed by  
241 that of sPLA2-IB while other enzyme subtypes were much less present (**Fig.1A**). Time-resolved  
242 fluoroimmunoassays overall detected sPLA2-IIA and -IB in a similar amount of samples, that is,  
243 in 25 (69.4%) and 24 (66.6%) BALs, respectively; sPLA2-V was never detected and sPLA2-X  
244 was detected in 2 (5.6%) BAL samples (n=36) (data not shown). **Fig.1B** depicts results of  
245 enzymatic activity assays in the absence and presence of various sPLA2 inhibitors for four

246 illustrative patients with different subtype expression profiles: sPLA2-IIA appears to be the  
247 predominant subtype in some cases (patients n.1 and 2), sPLA2-IB also contributes to the  
248 enzyme activity (patient n.3), but other phospholipases may also be present (patient n.4).  
249 Consistently, when incubating samples with increasing varespladib concentrations, the  
250 enzymatic activity was almost completely or only partially blocked (residual activity  $\approx 10\%$  for  
251 patients n.1-2 and  $\approx 45\%$  for patient n.3; **(Figshare - supplementary results-**  
252 **110.6084/m9.figshare.12148686)**. Pooling down the data of all samples (n=12) treated with  
253 different sPLA2 inhibitors and analyzed under the same experimental conditions, varespladib  
254 was the more efficacious inhibitor (overall  $p < 0.0001$ ; **Fig.2**).

### 255 ***Role of sPLA2 on surfactant retreatment and function***

256 Neonates needing surfactant retreatment showed higher sPLA2 total activity and higher  
257 expression of sPLA2-IIA (**Tab.2**) in their epithelial lining fluid before receiving any surfactant  
258 treatment. Consistently, these neonates also had more lung tissue inflammation, as they showed  
259 higher inflammatory mediators (**Tab.2**). There were significant positive correlations between  
260 sPLA2-IIA and TNF $\alpha$  ( $\rho = 0.505$ ;  $p = 0.027$ ;  $n = 19$ ) and between sPLA2-IIA and GM-CSF  
261 ( $\rho = 0.416$ ;  $p = 0.006$ ;  $n = 19$ ).

262 Neonates needing multiple surfactant administrations had significantly lower total  
263 phospholipids and various phospholipid species in their epithelial lining fluid (prior to any  
264 surfactant treatment) (**Fig.3**). Lyso-phospholipids were only present in small amounts: lyso-  
265 phosphatidylcholine levels were similar in neonates receiving one or two surfactant doses (one  
266 dose:  $0.14 [0.1-0.47]$  eq nmol; two doses:  $0.08 [0.04-0.25]$  eq nmol;  $p = 0.515$ ), while lyso-  
267 phosphatidylethanolamine levels tended to be higher in neonates requiring surfactant retreatment  
268 but this was not significant (one dose:  $0.03 [0.02-0.1]$  eq nmol; two doses:  $0.81 [0.03-2]$  eq  
269 nmol;  $p = 0.074$ ); lyso-phosphatidylglycerol was detectable only in two patients, thus no statistics  
270 was performed. Mean gestational age was not significantly different in neonates receiving one  
271 ( $28.5 (2.4)$  weeks) or two surfactant doses ( $27.6 (2.1)$  weeks;  $p = 0.08$ ).

272 There was a significant inverse correlation between sPLA2 activity and the adsorption of  
273 total surfactant available in the first day of life, that is, endogenous and exogenous pool, in  
274 babies undergoing BAL before and after surfactant replacement ( $\rho = -0.6$ ;  $p = 0.008$ ;  $n = 18$ ; **Fig.3**).  
275 This correlation remains significant upon adjustment for gestational age ( $_{adj}r = -0.61$ ;  $p = 0.009$ ).  
276 There were also significant correlations between sPLA2 activity and total phospholipids  
277 ( $\rho = -0.475$ ;  $p = 0.05$ ;  $n = 17$ ) or phosphatidylcholine ( $\rho = -0.622$ ;  $p = 0.017$ ;  $n = 29$ ) and between  
278 sPLA2-IIA and lysophosphatidylethanolamine ( $\rho = 0.686$ ;  $p = 0.002$ ;  $n = 17$ ), all measured prior to  
279 any surfactant treatment.

### 280 *Effect of exogenous surfactant on sPLA2 and its natural inhibitors*

281 In neonates undergoing BAL before and after surfactant replacement (**Fig.4**), poractant- $\alpha$   
282 significantly reduced sPLA2 activity and sPLA2-IIA expression and significantly increased  
283 dioleoylphosphatidylglycerol (DOPG). Surfactant administration did not significantly change  
284 SP-A levels ( $p = 0.247$ ). sPLA2 total activity significantly correlated with the concentration of  
285 sPLA2-IIA both before ( $\rho = 0.89$ ;  $p < 0.0001$ ;  $n = 34$ ) and after surfactant replacement ( $\rho = 0.826$ ;  
286  $p < 0.0001$ ;  $n = 40$ ).

### 287 *Relationship between sPLA2 and respiratory outcomes*

288 The ratio between the sPLA2 activity measured before and after surfactant replacement  
289 was considered to better describe the enzymatic activity present during the first day of life.  
290 Enzyme activity correlated with the duration of invasive ventilation ( $\rho = 0.679$ ;  $p = 0.005$ ;  $n = 15$ )  
291 and this remained significant upon adjustment for gestational age ( $_{adj}r = 0.46$ ;  $p = 0.04$ ;  $n = 15$ ). As  
292 12 babies died and 18 were lost to follow up (for family relocations), 48 patients were finally  
293 followed-up after NICU discharge. sPLA2 activity was not significantly different between  
294 patients followed (1.7 [0.6-9.5]  $\text{nmol} \cdot \text{min} \cdot \text{mL} / \text{ng}$ ) or lost to follow up (1.4 [0.4-3.3]  
295  $\text{nmol} \cdot \text{min} \cdot \text{mL} / \text{ng}$ ;  $p = 0.665$ ). The sPLA2 activity measured in the first day of life significantly  
296 correlated with the respiratory morbidity score ( $\tau_b = 0.349$ ;  $p = 0.037$  and  $p = 0.043$  upon  
297 multivariate adjustment). There are no significant differences in sPLA2 between patients

298 grouped for mortality, BPD or any other follow-up data (Figshare - **supplementary results-2 -**  
299 **Figshare - 10.6084/m9.figshare.12148686**).

## 300 **DISCUSSION**

301 This study addressed three open key points in human surfactant biology. We found that:  
302 1) sPLA2-IIA is the main subtype expressed in the preterm lung and its concentration is higher  
303 than other enzyme isoforms. sPLA2-IB or other phospholipases may also be significantly  
304 expressed and contribute to the enzyme activity, while sPLA2-V and -X are marginal. However,  
305 varespladib is able to inhibit most of the enzymatic activity. 2) Preterm neonates needing  
306 repeated surfactant administrations have significantly higher sPLA2 activity, sPLA2-IIA  
307 expression and inflammation. Neonates needing re-treatment have a different phospholipid  
308 profile which correlates to sPLA2 activity, while the enzymatic activity negatively influences  
309 surfactant adsorption. The administration of one poractant- $\alpha$  dose reduces sPLA2 activity and  
310 sPLA2-IIA expression through, at least partially, the increment of DOPG. 3) sPLA2 activity in  
311 the first day of life correlates with short-term ventilatory needs and with long-term respiratory  
312 morbidity score.

313 These data are interesting because they have been produced in a homogeneous population  
314 of surfactant-treated human neonates affected by RDS treated with a homogeneous ventilatory  
315 policy. This represents an ideal clinical model to study sPLA2-surfactant interactions and a  
316 pragmatic picture of common NICU patients. Moreover, these findings are consistent with the  
317 data produced so far, mainly in animal models (29, 46), and foster future steps in research and  
318 development.

319 The presence of sPLA2-IIA as the main alveolar subtype (29) and the expression of other  
320 subtypes in airway cells were already known (42). Our sampling technique may also recover  
321 some material from the airways (9) and this may explain the finding of small amounts of sPLA2-  
322 V and -X, which are unlikely to play a relevant role, given their marginal concentrations. It is  
323 difficult to clarify the relative importance of sPLA2-IB, although it seems to influence the  
324 enzyme inhibition profile, at least in some cases. However, sPLA2-IIA is the main pulmonary

325 subtype even in expectoration specimens obtained without any deep sampling in cystic fibrosis  
326 patients (36). The presence of sPLA2-IB is consistent with data showing its production by  
327 activated alveolar macrophages in adults or children with ARDS (11, 23) or when lung tissue  
328 inflammation is relevant (24). In fact, some of our patients may have been exposed to fetal  
329 inflammation since it triggers preterm labor (19). Fetal inflammation, if significant, is known to  
330 impair response to surfactant replacement, negatively affecting respiratory outcomes (2) and this  
331 might be mediated by the increased presence of sPLA2.

332         There seems to be three different inhibition profiles, as *ex vivo* treatment with varespladib  
333 (the sPLA2 inhibitor with largest clinical experience (25)), inhibits sPLA2 activity in some  
334 cases, while inhibition is less evident in others for the presence of sPLA2-IB or other  
335 phospholipases. Nonetheless, the importance of sPLA2-IIA is also confirmed by the strong  
336 correlation between this isoform and the total enzymatic activity. As varespladib is particularly  
337 able to inhibit sPLA2-IIA, -V and -X in the nanomolar range (14), the different inhibition profile  
338 is likely to be due to the presence of sPLA2-IB or other phospholipases, which are less sensitive  
339 to varespladib. These data confirm that inhibiting sPLA2-IIA is a potential target to protect  
340 surfactant and reduce lung tissue inflammation (46). This is consistent with our previous  
341 experiments showing *ex vivo* potency of varespladib in mixed populations of neonates and  
342 infants, with various respiratory disorders and higher levels of lung inflammation (41,42), where  
343 varespladib was efficient at higher doses. This may be caused by the higher protein content  
344 related to the inflammation, since the inhibitor may bind to proteins preventing it to reach the  
345 sPLA2 catalytic site, or to the different assay conditions (13).

346         The need for surfactant retreatment seems to be associated to sPLA2 and lung  
347 inflammation, as retreated neonates had significantly higher enzyme activity and sPLA2-IIA and  
348 cytokines concentrations. This is consistent with the dual role of sPLA2, which regulates the first  
349 step of the inflammatory cascade and hydrolyses surfactant phospholipids (26). Moreover,  
350 correlations between sPLA2-IIA, TNF $\alpha$  and GM-CSF confirm our previous findings, as TNF $\alpha$   
351 is the main inducer of sPLA2-IIA gene transcription in alveolar macrophages (43,44).

352 The link between sPLA2 and need for surfactant retreatment is confirmed by the  
353 differences in lipid profile between patients receiving one or two doses, and by the correlations  
354 between sPLA2 and various phospholipids. In fact, babies needing more surfactant have less  
355 phospholipids both in terms of total amount and various species; furthermore, the higher is the  
356 sPLA2, the lower are total phospholipids and phosphatidylcholine. Interestingly, the amount of  
357 sPLA2-IIA is also correlated to lysophosphatidylethanolamine. As phosphatidylethanolamine is  
358 a preferential substrate for sPLA2-IIA (21), this correlation also confirms the main role played  
359 by this subtype in the endogenous surfactant hydrolysis. Furthermore, sPLA2 activity negatively  
360 influences the efficiency of surfactant in terms of air/liquid interface adsorption, since there is an  
361 inverse relationship between surfactant adsorption and sPLA2 activity. This can be explained  
362 since sPLA2 can modify the phospholipid profile, as shown by correlations between sPLA2 and  
363 phospholipids. Our data show this negative effect on the total surfactant available on the first day  
364 of life (endogenous plus exogenous pool), as measurements were done in babies undergoing  
365 BAL before and after surfactant replacement. Thus, the relationship between sPLA2 and  
366 surfactant adsorption is evident within a relatively narrow range of values, as surfactant  
367 adsorption is overall concentrated on intermediate values with only few negative ones. These  
368 points indicate a globally poor surfactant function and they actually represent the few sickest  
369 babies who do not have a good surfactant function even after surfactant replacement (in fact,  
370 four of them out of six needed surfactant retreatment). We unfortunately did not have a  
371 population large enough to reliably distinguish the effect of sPLA2 on endogenous and  
372 exogenous surfactant. Moreover, more datapoints would be needed to better detail the decay of  
373 surfactant function with increasing sPLA2 activity.

374 One dose of exogenous surfactant increased the air/liquid interface adsorption and  
375 reduces both sPLA2 activity and sPLA2-IIA expression. Surfactant also increased DOPG, which  
376 was almost undetectable in our patients before surfactant replacement. The relative absence of  
377 DOPG in preterm neonates with RDS seems logical, as DOPG is only a minor component of  
378 endogenous surfactant and these patients have primary surfactant deficiency. DOPG and SP-A

379 are important, as they are natural modulators of sPLA2 activity and expression (47). Poractant- $\alpha$   
380 contains small amounts of DOPG (34) but it is SP-A-free, thus its administration resulted in an  
381 increase of DOPG only.

382 sPLA2 activity in the first day of life correlates with short-term ventilatory needs and this  
383 is consistent with a previous pilot study in a mixed population of term and preterm neonates with  
384 various respiratory disorders (10). Analogous correlations with similar short-term respiratory  
385 outcomes have been found in adults and children affected by ARDS (4,5). We add here the  
386 correlation between the sPLA2 activity on the first day of life and long-term respiratory  
387 outcomes in a homogeneous population of preterm babies with RDS. In fact, neonates exposed  
388 to higher sPLA2 activity early in life have a worse respiratory score at 12<sup>th</sup> month of age. This  
389 may be related to the need for a longer and/or more aggressive ventilation, increasing the risk of  
390 secondary lung injury; the confirmation of this hypothesis would require a dedicated and larger  
391 follow-up study. These results are however interesting since the respiratory morbidity score is  
392 known to be more predictive of later respiratory performance than the diagnosis of BPD (33).

393 It is unlikely that our results would be due to the effect of gestational age. In fact: 1) in  
394 our population, gestational age does not significantly differ between babies needing one or  
395 multiple surfactant doses; 2) correlations between sPLA2 and surfactant efficiency or respiratory  
396 outcomes remain significant upon multivariate adjustments. Consistently, we already knew that  
397 the expression of sPLA2 is unrelated to gestational age and that correlations between sPLA2 and  
398 short term respiratory outcomes were unrelated to patients' age (4,7).

399 We acknowledge some study limitations. Ours is a pragmatic study willing to integrate  
400 clinical and biological data obtained with several techniques. Due to the low samples volume,  
401 not all techniques were applied to every sample: this is a common issue of studies on newborn  
402 BAL specimens, but these studies still represent an useful and pragmatic tool to study neonatal  
403 lung biology. Animal studies may provide larger samples volume, but these specimens are  
404 coming from controlled experimental situations that are far from the clinical reality. Given the  
405 low cellularity of our samples, we could not study other phospholipases. Some data suggest that

406 cytosolic phospholipase might be more involved in inflammation than sPLA2 (29), but the  
407 relative contribution of different phospholipases to the inflammation during neonatal respiratory  
408 failure should be further investigated. We performed non-bronchoscopic BAL and limitations of  
409 this technique are well-known (9): however, this technique is the only one applicable to preterm  
410 neonates and these samples are more suitable to describe the alveolar biology than tracheal  
411 aspirates (7). Phospholipases are not the only inflammatory mediators able to injure surfactant:  
412 our findings may also be partially due to other agents, however, sPLA2 has a well-recognized  
413 role in surfactant injury and our findings are consistent with the literature data (26). Quadratic  
414 regression performed better than others to describe the relationship between sPLA2 activity and  
415 surfactant adsorption, however there is no pathobiological reason to prefer quadratic over linear  
416 regression. It is physiopathologically sound to think that, at low sPLA2 activity value, surfactant  
417 adsorption tends to plateau but more datapoints are needed to clarify this. We analyzed BALs  
418 taken after a relatively short ventilation but we cannot totally exclude that the ventilation itself  
419 might have also influenced surfactant function and local inflammation, as shown in animal  
420 models (46); however, our patients are all ventilated with a homogenous gentle ventilation policy  
421 (17). Our population is large enough to study relationship between the sPLA2 system, surfactant  
422 efficiency and various perinatal-neonatal variables. This could not have been presented in an  
423 unique paper: we preferred a pragmatic design, analyzing a homogeneous population of preterm  
424 babies including all cofactors commonly influencing the clinical picture (such as  
425 chorioamnionitis or antenatal steroids). Future works should also investigate if perinatal  
426 cofactors may trigger the production of sPLA2-IB, as this might identify subgroups of patients  
427 with a different inhibition profile and therapeutic possibilities. The study was subjected to a  
428 certain follow-up drop out and other variables (such as nutritional or environmental factors), may  
429 have influenced the clinical outcomes. This part of results should be considered preliminary for a  
430 larger cohort long-term study. Moreover, ours is the first study investigating the relationship  
431 between sPLA2 and long-term respiratory outcomes and results are consistent with those shown  
432 for short term outcomes (10, 11, 30).

433 In conclusion, sPLA2-IIA seems to be the main sPLA2 subtype in the lung of preterm  
434 neonates with RDS and linked to the need for surfactant retreatment. Surfactant administration  
435 partially inhibits sPLA2 activity. sPLA2 activity may negatively influences surfactant  
436 adsorption, short- and long-term respiratory outcomes. sPLA2 may be a potential target to  
437 protect surfactant and increase efficacy of surfactant replacement.

438

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445 and had final responsibility for the decision to submit for publication.

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609 **Table 1. Basic details of studied neonates.** Data are expressed as mean (standard deviation),  
 610 median [interquartile range] or number (%), as appropriate. Antenatal steroids were considered  
 611 complete when two doses of 12 mg-betamethasone were given, 24h apart from each other and at  
 612 least 24h before the delivery. The respiratory morbidity score describes the respiratory condition  
 613 during the first year of life.(33) Apgar, CRIB-II and respiratory morbidity scores are  
 614 dimensionless numbers. Abbreviations: BAL: broncho-alveolar lavage; BPD: broncho-  
 615 pulmonary dysplasia; CRIB-II: Critical Risk Index for Babies-II.

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<b>Neonates</b>	<b>78</b>
Gestational age (weeks)	28 (2.2)
Birth weight (grams)	1168 (333)
Complete antenatal steroids	49 (62.8%)
Cesarean section	44 (56.4%)
Male sex	44 (56.4%)
5' Apgar score	8 [7-9]
CRIB-II score	9.2 (3.9)
Postnatal age at 1 <sup>st</sup> BAL (h)	4 [1-5]
Postnatal age at 2 <sup>nd</sup> BAL, if any (h)	12 [10-14]
Need for surfactant re-treatment	32 (41%)
Duration of invasive ventilation (h)	96 [24-192]
In-hospital mortality	12 (15.4%)
BPD	31 (39.7%)
Respiratory morbidity score	0 [0-1]

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620 **Figure 1. Identification of sPLA2 subtypes responsible for enzyme activity in BAL samples.**

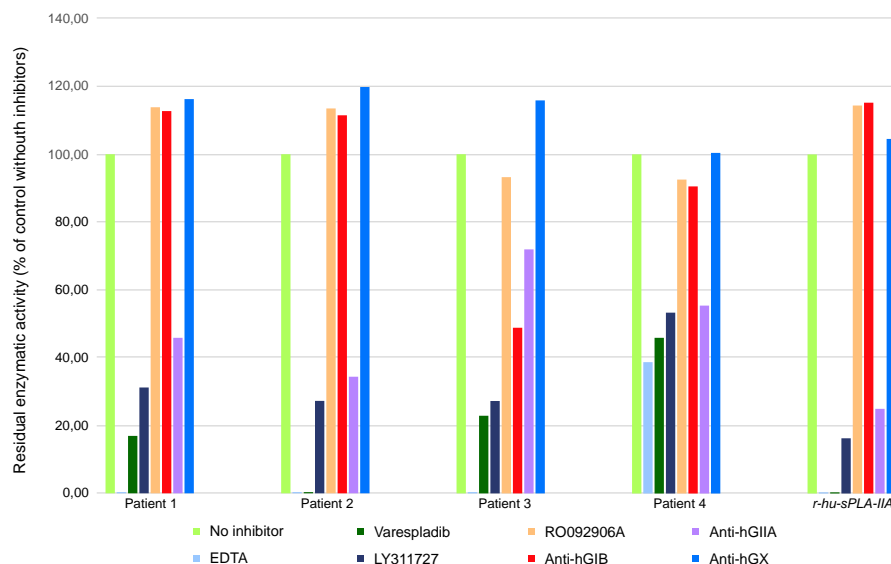
621 Panel A shows the results of ELISA normalized for total proteins (n=36). Panel B shows  
 622 inhibition of enzymatic activity for four illustrative patients versus human sPLA2-IIA by  
 623 different sPLA2 inhibitors. Results in panel B are expressed as % of sPLA2 residual activity  
 624 after the addition of various inhibitors as listed in the legend; recombinant human sPLA2-IIA is  
 625 used as control and the first column represents the raw sPLA2 global activity without any  
 626 inhibitor. Characteristics of each inhibitor are described in the online supplement. Experiments  
 627 have been performed in BAL samples obtained prior to any surfactant administration. All  
 628 measurements have been performed in duplicate. Abbreviations: anti-hGIB: rabbit polyclonal  
 629 antibody anti-sPLA2-IB; anti-hGIIA: rabbit polyclonal antibody anti-sPLA2-IIA; anti-hGX:  
 630 rabbit polyclonal antibody anti-sPLA2-X; BAL: broncho-alveolar lavage; EDTA:  
 631 ethylenediaminetetraacetic acid; *r-hu*-sPLA2-IIA: recombinant human sPLA2-IIA; sPLA2:  
 632 secretory phospholipase A2.

633

A

Enzyme subtype	(pg/mg)
sPLA2-IIA	239 [60-678]
sPLA2-IB	4 [0.8-11]
sPLA2-V	1.7 [0.4-3.5]
sPLA2-X	0.01 [0.008-0.011]

B



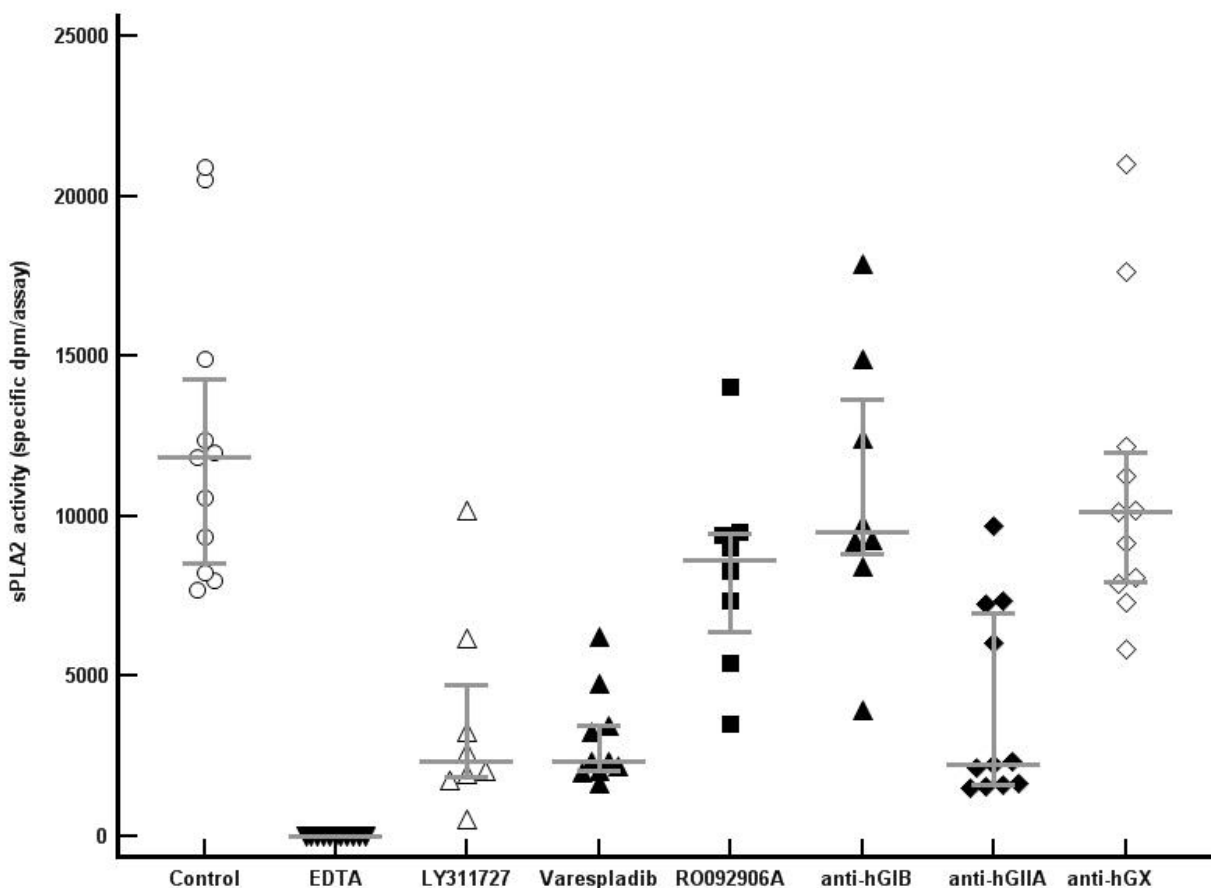
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638 **Figure 2. sPLA2 activity of BAL samples and inhibition with various sPLA2 inhibitors**  
 639 **under the same experimental conditions.** Results have been obtained in an unique assay  
 640 session performed on BAL samples obtained prior to any surfactant administration (n=12). All  
 641 measurements have been performed in duplicate. Results are expressed as specific radioactivity  
 642 (dpm). Grey lines represent median and interquartile range. Control represents sPLA2 activity  
 643 measured in BAL samples prior to the addition of any inhibitor. Characteristics of each inhibitor  
 644 are described in the online supplement. Data were compared with Kruskal-Wallis and Mann-  
 645 Whitney tests. sPLA2 activity obtained with varespladib addition is lower than that obtained  
 646 with RO092906A ( $p=0.043$ ), anti-hGIB ( $p<0.001$ ) and anti-hGX antibodies ( $p<0.001$ ) and is  
 647 lower than control ( $p<0.001$ ). Abbreviations: anti-hGIB: rabbit polyclonal antibody anti-sPLA2-  
 648 IB; anti-hGIIA: rabbit polyclonal antibody anti-sPLA2-IIA; anti-hGX: rabbit polyclonal  
 649 antibody anti-sPLA2-X; BAL: broncho-alveolar lavage; EDTA: ethylenediaminetetraacetic acid;  
 650 sPLA2: secretory phospholipase A2.



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653 **Table 2. Epithelial lining fluid sPLA2 and inflammatory mediators in neonates receiving**  
654 **one (n=46) or two (n=32) poractant- $\alpha$  doses.** Measurements have been performed in BAL  
655 samples obtained prior to any surfactant administration. Surfactant treatments have been  
656 managed according to our protocol;(17) more details in the text. All measurements have been  
657 performed in duplicate. Data are reported as median [interquartile range] and compared with  
658 Mann-Whitney test. Epithelial lining fluid concentrations have been obtained correcting for  
659 serum-to-BAL urea ratio; sPLA2 total activity and sPLA2-IIA are also normalized for surfactant  
660 protein-A. Abbreviations: BAL: broncho-alveolar lavage; GM-CSF: granulocyte macrophage-  
661 colony stimulating factor; IL1- $\beta$ : interleukine-1 $\beta$ ; IL6: interleukine-6; sPLA2: secretory  
662 phospholipase A2; TNF $\alpha$ : tumor necrosis factor- $\alpha$ .

663

	<b>Single dose (46)</b>	<b>Two doses (32)</b>	<b><i>p</i></b>
GM-CSF (pg/mL)	261 [70-367]	832 [352-1514]	<b>&lt;0.0001</b>
TNF $\alpha$ (pg/mL)	128 [77-324]	5481 [152-20867]	<b>0.005</b>
sPLA2 activity (nmol x min x mL/ng)	0.9 [0.3-2.5]	3.6 [0.8-13.3]	<b>0.021</b>
sPLA2-IIA (ng/ng)	57.4 [13-198]	270 [34-2259]	<b>0.039</b>
IL1- $\beta$ (ng/mL)	1.05 [0.4-2.2]	41.6 [15.2-211.4]	<b>0.01</b>
IL6 (ng/mL)	18.2 [10.1-54.9]	101.5 [55.7-204.1]	<b>0.012</b>
Total proteins (mg/mL)	17 [7.3-28.5]	23.3 [15.6-34]	<b>0.05</b>

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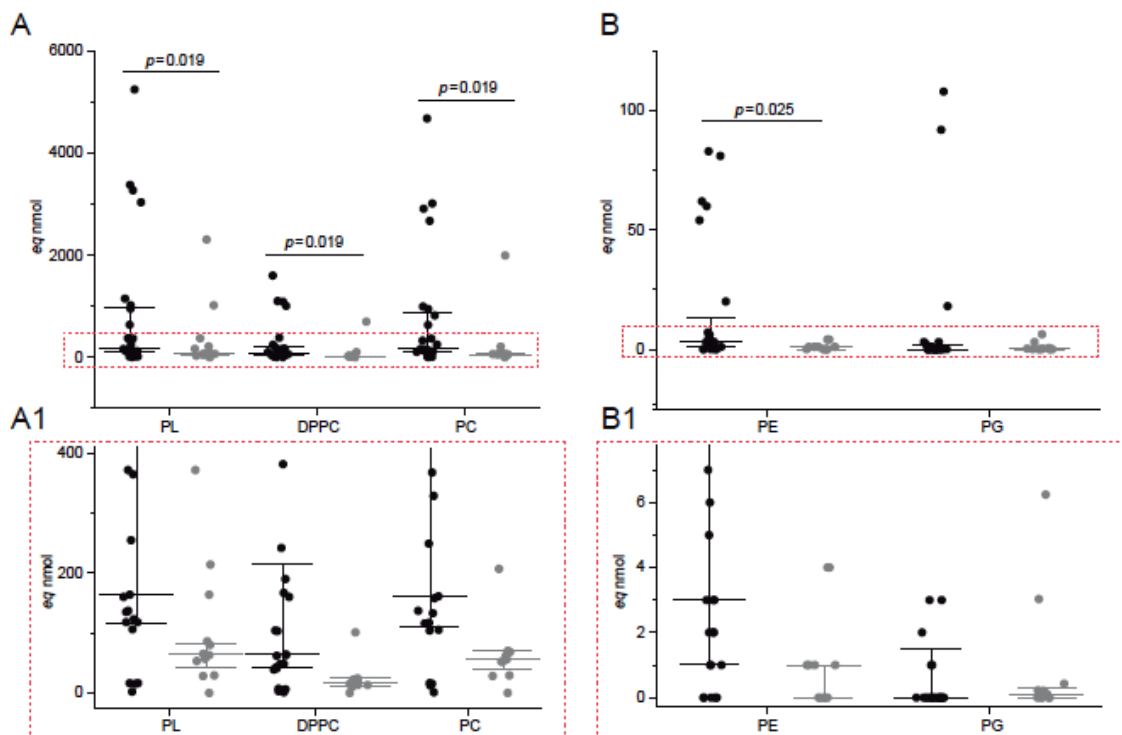
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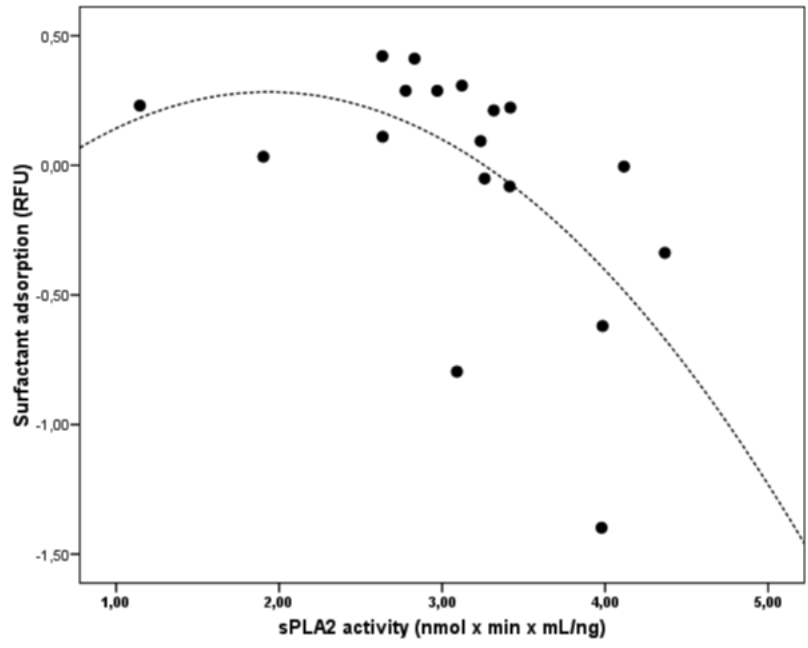
668 **Figure 3. Epithelial lining fluid phospholipid profile of neonates receiving one or two**  
 669 **poractant- $\alpha$  doses.** Measurements have been performed in BAL samples obtained prior to any  
 670 surfactant administration. Surfactant treatments have been managed according to our  
 671 protocol;(17) more details in the text. All measurements have been performed in duplicate. Panel  
 672 A and B represent zwitterionic and anionic lipids, respectively; to increase visibility, the data  
 673 clouds within the hatched red lines are depicted and zoomed in panel A1 and B1, respectively.  
 674 Black and grey dots represent measurements in neonates receiving one (n=21) or two (n=12)  
 675 surfactant doses, respectively. Horizontal thin lines represent median and interquartile range.  
 676 Epithelial lining fluid concentrations have been obtained correcting for serum-to-BAL urea ratio.  
 677 Data were compared with Mann-Whitney test and significant  $p$ -values are shown on top of the  
 678 data. Abbreviations: BAL: broncho-alveolar lavage; DPPC: dipalmitoylphosphatidylcholine;  
 679 LysoPC: lyso-phosphatidylcholine; Lyso-PE: lyso-phosphatidylethanolamine; PC: total  
 680 phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PL: total  
 681 phospholipids.

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686 **Figure 4. Correlation between adsorption of surfactant available in the first day of life**  
687 **(endogenous plus exogenous pool) and epithelial lining fluid sPLA2 activity in neonates**  
688 **undergoing BAL before and after surfactant replacement (n=18).** The adsorption of  
689 surfactant available on the first day of life is measured as surface accumulation of fluorescently  
690 labelled surfactant (read at 9 min as relative fluorescence unit) and reported as ratio of the values  
691 measured in BAL samples obtained after and before surfactant (200 mg/kg poractant- $\alpha$ )  
692 replacement. sPLA2 activity is expressed (Log scale) as epithelial lining fluid corrected for  
693 serum-to-BAL urea ratio and normalized for surfactant protein-A content. All measurements  
694 have been performed in duplicate. The hatched line is the best-fitting data curve generated with  
695 quadratic regression ( $Y = -0.015^xX^2 - 0.28^xX + 1.8$ ). Correlation analysis was performed  
696 calculating Spearman coefficient. Abbreviations: BAL: broncho-alveolar lavage; RFU: relative  
697 fluorescent units; sPLA2: secretory phospholipase A2.  
698



**Figure 5. Effect of one poractant- $\alpha$  administration on epithelial lining fluid sPLA2, its natural inhibitors and surfactant function.** Measurements have been performed in BAL samples obtained before the first surfactant dose (PRE) and after at least 10h from the administration (POST). All measurements have been performed in duplicate. Panel A, B, C, D and E represent sPLA2 activity (n=55), sPLA2-IIA (n=55), SP-A (n=27), DOPG (n=36) and surfactant adsorption (n=34), respectively. Grey horizontal lines represent medians and interquartile range. Panel A, B, C and D show epithelial lining fluid concentrations obtained correcting for serum-to-BAL urea ratio; sPLA2 activity is also normalized for surfactant protein-A content. Adsorption of surfactant (Panel E) is measured as surface accumulation of fluorescently labeled surfactant (read at 90' as relative fluorescence unit). Data were compared with Mann-Whitney test and significant  $p$ -values are shown on top of the data. Abbreviations: BAL: broncho-alveolar lavage; DOPG: dioleoylphosphatidylglycerol; RFU: relative fluorescent units; SP-A: surfactant protein-A; sPLA2: secretory phospholipase A2.

