Available online at www.sciencedirect.com

ScienceDirect

Biomedical Journal

journal homepage: www.elsevier.com/locate/bj

Review Article: Special Edition

Techniques to evaluate surfactant activity for a personalized therapy of RDS neonates

Chiara Autilio [a,b,*]

[a] *Department of Biochemistry and Molecular Biology and Research Institute Hospital October 12 (imas12), Faculty of Biology, Complutense University, Calle Jose Antonio Novais 12, Madrid, Spain*
[b] *Clinical Pathology and Microbiology Unit, San Carlo Hospital, Via Potito Petrone, Potenza, Italy*

Dr. Chiara Autilio

ARTICLE INFO

Article history:
Received 22 June 2021
Accepted 1 November 2021
Available online 7 November 2021

Keywords:
Lung surfactant
RDS prediction
CPAP failure

ABSTRACT

According to both European and American Guidelines, preterm neonates have to be treated by nasal continuous air pressure (CPAP) early in the delivery room. The administration of surfactant should be reserved only for babies with respiratory distress syndrome (RDS) with increased oxygen requirement, according to different thresholds of FiO_2. However, these oxygenation thresholds do not fully take into consideration the lung physiopathology and mechanics or the lung surfactant biology of RDS neonates. Since surfactant replacement therapy (SRT) seems to be more effective if it is initiated within the first 3 hours after birth, the use of a reliable bench-to-bedside biological test able to predict as soon as possible the necessity of SRT will help optimise individualised therapies and personalise the actual collective strategy used to treat RDS neonates. With this in mind, in the present review several quantitative and qualitative biological tests to assess the surfactant status in RDS neonates are introduced as potential candidates for the early prediction of SRT requirement, summarising the state-of-the-art in the evaluation of surfactant activity.

The combination of early continuous positive air pressure (CPAP) and selective surfactant therapy is more effective than only administrating surfactant preparations in decreasing both death and bronchopulmonary dysplasia of preterm neonates [1]. On this basis, the latest American and European guidelines strongly recommend the administration of selective surfactant treatments after early CPAP failure [2,3] and this most often occurs in extremely preterm neonates [4].

Surfactant replacement in preterm infants treated with CPAP should be started only when certain oxygen requirements are reached [3]. However, the best time for surfactant administration and how to predict which neonates will need the replacement therapy is still not defined. When the threshold of FiO_2 exceeds 0.30 for all babies with a clinical diagnosis of neonatal respiratory distress syndrome (RDS), the latest European guidelines suggest starting surfactant

* *Corresponding author*. Department of Biochemistry and Molecular Biology and Research Institute Hospital October 12 (imas12), Faculty of Biology, Complutense University, Jose Antonio Novais 12, Av. Séneca, 2, 28040 Madrid, Spain.
E-mail address: cautilio@ucm.es.
Peer review under responsibility of Chang Gung University.
https://doi.org/10.1016/j.bj.2021.11.001

Table 1 Values of diagnostic accuracy for candidate techniques to predict RDS or CPAP failure due to surfactant need in preterm neonates with RDS. An averaged sensibility and specificity for each test is shown together with the corresponding possible advantages and drawbacks.

to predict RDS				
	Sensitivity (%)	Specificity (%)	Advantages	Drawbacks
L/S ratio	91	79	- precise - sensitive - assays major surfactant lipid	- technically complex - does not test surfactant quality - interference by blood and meconium
PG	100	50	- quick - simple	- technically complex/coarse, depending on the assay - does not test surfactant quality
SP-A	88–100	83–93	- major surfactant protein	- does not test surfactant quality
to predict CPAP-failure in RDS neonates				
	Sensitivity (%)	Specificity (%)	Advantages	Drawbacks
LBC	70	67	- quick - simple	- does not test surfactant quality - interference by blood and meconium
SMT	71	75	- quick - simple - tests surfactant quality	- dilution problem - inter/intra-observing variability - possible interference by contaminants
SAT	95	70	- quick - simple - tests surfactant quality	- needing a fluorimeter - possible interference by contaminants

administration as early as possible [3]. However, these arbitrary thresholds do not accurately reflect the real oxygenation status and the lung conditions during RDS, since they do not take into account either the physiopathology and surfactant biology or the lung mechanics.

Interestingly, the outcome of preterm neonates seems to improve when the early surfactant replacement occurs within 2–3 hours after birth [5]. Thus, a surfactant replacement therapy (SRT) with exogenous surfactant preparations should be given as early as possible in CPAP-treated preterm babies in order to maximise the efficacy [5,6]. This generates a dilemma for neonatologists since they need to predict, within a short timeframe, which neonate is at risk to fail CPAP and therefore will need surfactant, and which one can progress only with CPAP. However, a reliable tool to predict the need for surfactant administration in this time window is still not available to date.

Lamellar bodies are oblong organelles whose content is secreted by the alveolar type II cells. The secreted content (lamellar body-like particles, LBPs) consists of multimembranes of highly packed surfactant phospholipids and proteins, which unfold at the respiratory surface during the compression/expansion cycles of breathing, thus promoting surfactant lining of the alveolar air-liquid interface and a concomitant reduction of surface tension [7]. Lamellar bodies can be found in lung lavages, amniotic fluids and gastric aspirates as LBPs. The greater the number of particles in those fluids, the more mature the foetal lungs are. Theoretically, the surface-active properties and the amount of the main surface-active lipids in these lamellar-body-like structures can reflect their capability to open up correctly and adsorb at the air-liquid interface in the neonatal lung. With this in mind, biological tests to assess the quantity and quality of surfactant from these LBPs, if quick and easy, could be good candidates as a point-of-care technique for use at the bedside and guide replacement therapy [8]. However, none of the currently available lung maturity assays are used yet in clinical practice to predict CPAP failure in RDS neonates.

Based on these considerations, the present review summarises the main surfactant biological tests available, both for quantitative and qualitative analyses, which may represent possible candidates to predict CPAP failure and requirement of SRT in RDS neonates [Table 1]. These tests may be easy to use at the bedside, providing quickly available results for rapid and timely decision making.

Quantitative tests

Lecithin/sphingomyelin ratio

The lecithin/sphingomyelin ratio (L/S ratio) was the first test developed to assess lung maturity. Historically, it detects the ratio in amniotic fluid between phosphatidylcholine, the major lung surfactant component which increases in amount along gestation, and sphingomyelin, a cell-derived component that does not tend to vary throughout pregnancy [9]. After amniocentesis, the sample is centrifuged and commonly analysed by Thin Layer Chromatography (TLC). In normal pregnancy, the value should be 2.5 or higher at 35 weeks' gestation, whereas inferior ratios are related to lung immaturity. A similar alternative with improved point-of care spectroscopic technique was proposed by employing a tip-column with a cation-exchange resin and mass spectroscopy to isolate choline-containing phospholipids – including lecithin and sphingomyelin – and calculate the L/S ratio from the intensity of six lecithin peaks and sphingomyelin 34:1 by LC-MS/MS and MALDI-TOF [10]. This method shows a 100% concordance with TLC to determine the L/S ratio in LBPs, but it has not been tested for RDS occurrence and CPAP failure.

Contamination of the samples by urine, blood, meconium or vaginal secretions strongly interfere with testing, resulting in false values. Moreover, the procedure has some risk, is time-consuming, prone to error and technically complex. Thus, the L/S ratio has been essentially abandoned because of

these problems, beside the adverse events associated with diagnostic amniocentesis if performed to measure L/S ratio in high-risk pregnancies. Indeed, this method provided minimal additional benefits when a combination of pre-natal steroids, early surfactant therapy and improved clinical management were adopted.

Interestingly, the presence of L/S was investigated to predict RDS also in tracheal aspirates, raising the threshold of L/S ratio to 3 with 91% of accuracy [11]. However, the quality, the dilution and homogeneity of the samples along with its recovery still remain a relevant issue.

More recently, a very quick alternative point-of-care spectroscopic method has been proposed to measure L/S ratio in LBPs isolated by centrifugation from fresh gastric aspirates [12,13]. This technique is based on mid-infrared spectroscopy of L/S ratio with a detection time of around 10–15 min [14]. This method shows high diagnostic sensitivity (91% (95% CI: 78–97)) and specificity (79% (95% CI: 59–92)) to predict RDS, but it has not yet been investigated to predict CPAP failure in a diagnostic accuracy study.

Phosphatidylglycerol and Surfactant Protein A

Of the minor phospholipids of lung surfactant, phosphatidylglycerol (PG) is an essential component, playing a role in both surface-active properties and immunomodulatory functions of the surfactant system [15]. It appears at around weeks 35–36 of gestation, shortly after phosphatidylcholine and thus its presence can be considered as an indicator of pulmonary maturity in amniotic fluids. The levels of PG can be tested by TLC, enzymatic assays or a quicker qualitative agglutination test by using antibodies to detect the lipid as visible agglutinates. Apart from bacteria-contaminated vaginal pool specimens that may lead to false positive results [16], PG quantification is not affected by any interference by contaminants such as blood or meconium [17–19]. Indeed, as for amniotic fluids, PG is predominantly present in surfactant and lung tissue.

PG amount in amniotic fluids can predict RDS within 3 days of birth with an accuracy of 93%, when tested in combination with L/S ratio [20]. Conversely, the assay alone shows a lower specificity (around 50%), although a higher diagnostic sensitivity compared to L/S (100% vs 90.2%, respectively) [21].

When coupled with a low L/S ratio, the absence of PG in tracheal aspirates also seems to be very useful to predict RDS with a positive result predictive value of 89% [11]. Moreover, regarding gastric aspirates, PG was detected at very low levels from neonates of 25–29 weeks of gestation without any apparent sex or age differences in its proportion [22].

Surfactant Protein A (SP-A) is one of the two hydrophilic proteins of lung surfactant. It is mostly required for the innate immune defence against potential lung pathogens, harmful inspired particles and allergens [15]. The concentration of SP-A in amniotic fluid is very low before 30 weeks of gestation but starts to increase significantly from the 34th week onwards [23]. Thus, similarly to PG, its quantification in amniotic fluids by using enzyme-linked immunoassays was proposed as a reliable test to predict RDS [23,24]. In this regard, the reported sensitivity, specificity and accuracy were 88.3–100%, 83–92.6% and 72.4–88%, respectively [25,26].

SP-A was also found significantly lower in tracheal aspirates collected in RDS neonates when compared to infants ventilated for other reasons with a sensitivity of 87% and a specificity of 81% to diagnose RDS [27]. Contamination by meconium or blood was not reported to affect results. However, especially in the case of tracheal aspirates, the presence of cholesterol and bile acids may facilitate the fluidification of DPPC-ordered domains, reducing the SP-A association to these complexes [28] and possibly its recovery in the lung surfactant system.

Unfortunately, PG and SP-A seem to correlate better with gestational ages rather than incidence of RDS. Moreover, as expected, the methods to quantify these parameters are coarse, technically complex and/or unsuitable as point-of-care assay (ELISA), although less cumbersome than spectrometry. Therefore, they are no longer taken into consideration, and have not been employed to predict CPAP failure in RDS neonates.

Lamellar Body Count

The aforementioned techniques provide information about the amount of certain surfactant phospholipid species (phosphatidylcholine and phosphatidylglycerol) or proteins (SP-A), but they cannot represent a real estimation of the available exogenous surfactant in the form of LBPs, the genuine form of surfactant secreted by type II pneumocytes. Conversely, Lamellar Body Count (LBC) can be considered a quick and easy quantitative biological test, which may detect the real amount of secreted LBPs as they are counted by automated platelet counters, considering the similar diameter of human LBPs to platelets (1–5 μm) [29]. LBC can be performed in non-bronchoscopic bronchoalveolar lavages, amniotic fluids and gastric aspirates with greater accuracy than the L/S ratio and PG in the prediction of RDS [30], using a cut-off value of <15,000/30,000 count/μL [31,32].

Also, LBC can be done without any time-consuming sample preparation or sample dilution [33]. However, the assay is unfeasible for around 35% of samples due to blood contamination and the high viscosity of many samples [34], especially in the case of amniotic fluids collected vaginally at delivery [33], but also in gastric aspirates (around 23% of unsuitable samples) [35]. Unfortunately, LBC has a low reliability to predict CPAP failure caused by a lack of surfactant when testing gastric aspirates (AUC: 0.703, 95% CI: 0.696–0.710) [33]. This result is not so surprising, considering that although a simple count of LBPs shows a significant correlation with gestational age [35] and may suggest the insurgence of RDS, it is not always associated to the clinical evolution of the patient. The latter may be affected by other factors [8], including for instance, the influence of CPAP levels on alveoli recruitment and surfactant production [36], lung tissue inflammation, the dose of prenatal steroids [37] and extravascular water [38].

Moreover, LBC and the other quantitative tests detect the amount of lung surfactant, but do not provide direct information about its functional performance. This is critical since the activity of lung surfactant at a given lipid concentration may be influenced by multiple variables due to either low

proportion of lamellar bodies or alteration of their surface-active properties. In this regard, several conditions have been described that affect surfactant biophysical properties, including 1) levels of Surfactant Protein B (SP–B), Surfactant Protein C (SP–C) and/or SP-A [39–42], 2) the amount of anionic phospholipids, such as PG [43], 3) alteration of the chemical structure of surfactant lipids and proteins by reactive species of oxygen [44,45], 4) perturbation of the structural properties of surfactant membranes and, particularly for lamellar bodies, their hydration status [46], 5) the rate of secretory phospholipase A2 with respect to total phospholipids [47] and 6) the presence of substances known to inhibit surfactant activity that can be found in amniotic fluids [46,48].

Qualitative tests

As mentioned above, a qualitative test to assess the function of surfactant may be more informative and reliable than a quantitative assay, since it provides information about the status of surfactant under several conditions influencing its potential surface-active properties. As extensively revised, several methods can be employed to study lung surfactant activity [49,50]. However, only two tests can be considered technically quick and easy enough to be employed as point-of-care methods to predict CPAP failure: the Stable Microbubble Test (SMT) and the Surfactant Adsorption Test (SAT).

Stable Microbubble Test (SMT)

Once agitated, surfactant present in amniotic fluids or gastric aspirates contributes to the formation of numerous small stable microbubbles (<15 μm in diameter), which are much less abundant or absent in samples from RDS. This is the principle of an old and simple assay, the SMT [51], which is a rapid (5–7 min performance time) and effective method [52] to predict RDS, irrespectively of the sample matrix. Interestingly, a recent study demonstrated a high reliability for this method to predict RDS neonates who fail CPAP due to surfactant need (AUC: 0.8, 95% CI: 0.788–0.812) [52]. However, although SMT is very simple and quick, it is strongly influenced by the subjectivity of inter- and intra-observing variability under a microscope. Moreover, the method cannot discriminate the intrinsic dilution of the sample tested, since it lacks a pre-analytical quantification of the phospholipid content. The difference in phospholipid concentration may indeed influence results, affecting the activity and the apparent inactivation rate of surfactant [53–56], which affects the capability of the material to create a stable microbubble. Diluted surfactant seems to be characterised by unilamellar vesicles, while more concentrated material forms larger packed and complex surface-active structures [56]. Similarly, the presence of meconium may *in vitro* affect the stability of surfactant microbubbles tested by SMT [57]. With this in mind, a qualitative test that provides results quickly without any intrinsic sample dilution or inhibition of surfactant activity by contaminants should be considered a good choice.

Surfactant Adsorption Test (SAT)

Around ten years ago, Ravasio et al. developed a rapid, sensitive and high-throughput fluorescent method to test indirectly both adsorption and stable accumulation of surfactant at the air-liquid interface, defined as SAT [58]. This is possible by labelling surfactant with a fluorescent analogue of phosphatidylcholine at a final molar ratio of 1–4% (fluorescent phosphatidylcholine/surfactant ratio) and incubating the suspension at 37 °C with intermittent shaking. In this way, fluorescent species can be incorporated into surfactant membrane aggregates. After incubation, the fluorescent phosphatidylcholine/surfactant mixture is diluted with a saline buffer solution in a defined volume, avoiding the issues caused by sample dilution. This volume is then injected at the bottom of the wells of a microtiter plate filled with a quenching solution, typically the Brilliant Black dye. Since the fluorescent phosphatidylcholine is masked by the black dye-mediated light absorption in the bulk phase, the detection of fluorescence under shaking is strictly dependent on the adsorption of the labelled surfactant material at the air-liquid interface and its escape from the quenching subphase. With this in mind, SAT consists in two simple steps: a first step of incubation for labelling surfactant from a biological fluid, and the subsequent step of detection of both its capability to move up towards the air-liquid interface crossing the subphase volume and the kinetics of its interfacial accumulation overtime. Moreover, although the resulting data are not direct surface tension values, they are indirectly related to the adsorption properties and expressed as relative fluorescence units.

Due to its sensitivity and suitability, SAT has been widely used to test different types of materials. The method was employed to assess *in vitro* surfactant activity from animal or cellular sources [46,48,58,59], therapeutic surfactant preparations [60] and non-bronchoscopic bronchoalveolar lavages at different temperatures of asphyxiated neonates under therapeutic hypothermia [61]. Recently, Autilio et al. reported that SAT can be adjusted to perform the assay in 30–60 min of fluorimetric readings, reducing the timing for material labelling and demonstrating SAT accuracy to predict CPAP failure in RDS neonates by directly testing amniotic fluids (AUC: 0.84, 95% CI: 0.824–0.856) [62]. No data are available on gastric/tracheal aspirates to predict RDS or CPAP-failure. However, the same modified SAT technique could be employed to *ex vivo* test mice lung lavages [63] and non-bronchoscopic bronchoalveolar lavages from human preterm and term neonates [47,64]. This modified method can be also employed to describe the inhibition of surfactant activity in other pathological contexts such as neonatal Acute Respiratory Distress Syndrome (ARDS) due to meconium aspiration [64], as it has been previously demonstrated by using a more sophisticated biophysical technique [65,66]. At high amount, meconium, plasma/serum and albumin can *in vitro* affect the activity of purified porcine surfactant/clinical surfactants assayed by SAT [46,48,58,67]. However, the interference by contaminants present in amniotic fluids from neonates has not yet been investigated *ex vivo*.

Towards a precision medicine for RDS neonates

The ideal test to evaluate surfactant status should be reliable, use limited volume samples, as well as being quick and easy to perform at the bedside. Some of the tests described above have been abandoned because they did not have these characteristics and their added value to clinical decisions was low. Now that surfactant universal prophylaxis is no longer recommended, while early CPAP is provided immediately after birth, a personalised approach in the management of RDS in neonates is necessary to improve the characterisation of the respiratory failure and therefore reserve surfactant treatment for those patients who actually need it.

Exogenous surfactant has been given easily to preterm babies (and particularly those below 28–30 weeks' gestation [4]) on the understanding that this is a harmless therapy. However, this is not totally true as surfactant replacement may cause important, although generally transient, side effects and since its benefit can and should be optimised [68,69]. In fact, surfactant replacement is more efficacious when it occurs within 2–3 h after birth [4,70–72]. Thus, the turnaround time of an assay to measure the lung surfactant status becomes crucial. For a test to be ideal and respect the above-described characteristics, a clear development pathway should be designed and sample treatment should be investigated: gastric aspirate is probably the more suitable matrix and could provide more reliable results compared to amniotic fluid or tracheal aspirates. Large and high-quality studies in this context have not been performed so far, since the majority of studies have been based on a single center design and dedicated to predict RDS occurrence rather than CPAP failure [4]. Therefore, LBC along with SAT and/or SMT in early life in CPAP-treated preterm neonates deserve to be investigated in large and adequately designed studies with a clear pathway to support point-of-care devices in case of positive results.

Another candidate for future studies should be considered Surfactant Protein D (SP-D), the other collecting protein of pulmonary surfactant [73]. Genetic SP-D variations seem to be associated with severe RDS in very preterm birth infants [74,75]. Moreover, alveolar SP-D is very low immediately after birth in the presence of RDS [76,77]. However, up to now, no studies have been performed about SP-D levels in different sample matrix to predict CPAP failure in RDS neonates.

Lung inflammation, related to chorioamnionitis and/or fetal inflammatory response syndrome, is a common process in extremely preterm babies, which seems to reduce the incidence of RDS [78] and affect the composition of the pulmonary surfactant system. During intra-amniotic infections, LBC values are significantly higher before 34 weeks of gestation when compared to other clinical situations [79]. The chorioamnionitis-dependent inflammation status induces an increase in glycerophospholipids and sphingolipids with a decrease in sphingomyelin species in tracheal aspirates [80]. This may affect L/S ratio and PG levels. Moreover, animal models of chorioamnionitis also suggest changes in the levels of SP-A and SP-D upon this condition [81,82].

Overall, to heal the rift between the basic biophysics of lung surfactant and translational medicine in neonatology, a collaboration between the pharmaceutical industry, academics and clinical practitioners is required to make progress towards the development of a quick, highly reproducible, accurate, low-cost, bench-to-bedside, easy to use and minimally invasive tool to manage RDS.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

[1] Schmölzer GM, Kumar M, Pichler G, Aziz K, O'Reilly M, Cheung PY. Non-invasive versus invasive respiratory support in preterm infants at birth: systematic review and meta-analysis. BMJ 2013;347:f5980.

[2] Committee on Fetus and Newborn; American Academy of Pediatrics. Respiratory support in preterm infants at birth. Pediatrics 2014;133:171–4.

[3] Sweet DG, Carnielli V, Greisen G, Hallman M, Ozek E, Te Pas A, et al. European consensus guidelines on the management of respiratory distress syndrome - 2019 update. Neonatology 2019;115:432–50.

[4] Wiingreen R, Greisen G, Ebbesen F, Petersen JP, Zachariassen G, Henriksen TB, et al. Surfactant need by gestation for very preterm babies initiated on early nasal CPAP: a Danish observational multicentre study of 6,628 infants born 2000-2013. Neonatology 2017;111:331–6.

[5] Bahadue FL, Soll R. Early versus delayed selective surfactant treatment for neonatal respiratory distress syndrome. Cochrane Database Syst Rev 2012;11:Cd001456.

[6] Verder H, Albertsen P, Ebbesen F, Greisen G, Robertson B, Bertelsen A, et al. Nasal continuous positive airway pressure and early surfactant therapy for respiratory distress syndrome in newborns of less than 30 weeks' gestation. Pediatrics 1999;103:E24.

[7] Olmeda B, Martínez-Calle M, Pérez-Gil J. Pulmonary surfactant metabolism in the alveolar airspace: biogenesis, extracellular conversions, recycling. Ann Anat 2017;209:78–92.

[8] De Luca D, Autilio C, Pezza L, Shankar-Aguilera S, Tingay DG, Carnielli VP. Personalized medicine for the management of RDS in preterm neonates. Neonatology 2021;118:127–38.

[9] Roux JF, Nakamura J, Brown E, Sweet AY. The lecithin-sphingomyelin ratio of amniotic fluid: an index of fetal lung maturity? Pediatrics 1972;49:464–6.

[10] Kwak HS, Chung HJ, Choi YS, Min WK, Jung SY. Prediction of fetal lung maturity using the lecithin/sphingomyelin (L/S) ratio analysis with a simplified sample preparation, using a commercial microtip-column combined with mass spectrometric analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2015;993–994:81–5.

[11] Harker LC, Merritt TA, Edwards DK 3rd. Improving the prediction of surfactant deficiency in very low-birth weight infants with respiratory distress. J Perinatol 1992;12:129–33.

[12] Heiring C, Verder H, Schousboe P, Jessen TE, Bender L, Ebbesen F, et al. Predicting respiratory distress syndrome at birth using a fast test based on spectroscopy of gastric aspirates: 2. Clinical part. Acta Paediatr 2020;109:285–90.

[13] Verder H, Heiring C, Clark H, Sweet D, Jessen TE, Ebbesen F, et al. Rapid test for lung maturity, based on spectroscopy of gastric aspirate, predicted respiratory distress syndrome with high sensitivity. Acta Paediatr 2017;106:430–7.

[14] Schousboe P, Verder H, Jessen TE, Heiring C, Bender L, Ebbesen F, et al. Predicting respiratory distress syndrome at birth using fast test based on spectroscopy of gastric aspirates. 1. Biochemical part. Acta Paediatr 2020;109:280–4.
[15] Cañadas O, Olmeda B, Alonso A, Pérez-Gil J. Lipid-protein and protein-protein interactions in the pulmonary surfactant system and their role in lung homeostasis. Int J Mol Sci 2020;21:3708.
[16] Farquharson J, Jamieson EC, Berry E, Buchanan R, Logan RW. Assessment of the AmnioStat-FLM immunoagglutination test for phosphatidylglycerol in amniotic fluid. Clin Chim Acta 1986;156:271–7.
[17] Pastorek JG, Letellier RL, Gebbia K. Production of phosphatidylglycerol-like substance by genital flora bacteria. Am J Obstet Gynecol 1988;159:199–202.
[18] Halvorsen PR, Gross TL. Laboratory and clinical evaluation of a rapid slide agglutination test for phosphatidylglycerol. Am J Obstet Gynecol 1985;151:1061–6.
[19] Benoit J, Merrill S, Rundell C, Meeker CI. Amniostat-FLM: an initial clinical trial with both vaginal pool and amniocentesis samples. Am J Obstet Gynecol 1986;154:65–8.
[20] Smith AJ. The role of phosphatidyl-glycerol in the determination of fetal lung maturity. S Afr Med J 1983;63:45–7.
[21] Hallman M. Lung surfactant in respiratory distress syndrome. Acta Anaesthesiol Scand Suppl 1991;95:15–20. discussion 21.
[22] Sozo F, Ishak N, Bhatia R, Davis PG, Harding R. Surfactant phospholipid composition of gastric aspirate samples differs between male and female very preterm infants. Pediatr Res 2017;82:839–49.
[23] Hallman M, Arjomaa P, Mizumoto M, Akino T. Surfactant proteins in the diagnosis of fetal lung maturity. I. Predictive accuracy of the 35 kD protein, the lecithin/sphingomyelin ratio, and phosphatidylglycerol. Am J Obstet Gynecol 1988;158:531–5.
[24] Kuroki Y, Takahashi H, Fukada Y, Mikawa M, Inagawa A, Fujimoto S, et al. Two-site simultaneous" immunoassay with monoclonal antibodies for the determination of surfactant apoproteins in human amniotic fluid. Pediatr Res 1985;19:1017–20.
[25] Satoh K, Sakata H, Nishijima S, Fujimoto S, Koga Y, Nakano H, et al. [Assessment of fetal lung maturity using newly developed immunological measurement of fetal pulmonary surfactant apoprotein-A in amniotic fluid]. Nippon Sanka Fujinka Gakkai Zasshi 1992;44:1269–76. Japanese.
[26] Kumazawa K, Hiramatsu Y, Masuyama H, Mizutani Y, Nakata T, Kudo T. Prediction markers for respiratory distress syndrome: evaluation of the stable microbubble test, surfactant protein-A and hepatocyte growth factor levels in amniotic fluid. Acta Med Okayama 2003;57:25–32.
[27] Stevens PA, Schadow B, Bartholain S, Segerer H, Obladen M. Surfactant protein A in the course of respiratory distress syndrome. Eur J Pediatr 1992;151:596–600.
[28] Casals C, Miguel E, Perez-Gil J. Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles. Biochem J 1993;296:585–93.
[29] Szallasi A, Gronowski AM, Eby CS. Lamellar body count in amniotic fluid: a comparative study of four different hematology analyzers. Clin Chem 2003;49:994–7.
[30] Neerhof MG, Haney EI, Silver RK, Ashwood ER, Lee IS, Piazze JJ. Lamellar body counts compared with traditional phospholipid analysis as an assay for evaluating fetal lung maturity. Obstet Gynecol 2001;97:305–9.
[31] Besnard AE, Wirjosoekarto SA, Broeze KA, Opmeer BC, Mol BW. Lecithin/sphingomyelin ratio and lamellar body count for fetal lung maturity: a meta-analysis. Eur J Obstet Gynecol Reprod Biol 2013;169:177–83.
[32] Abdou AM, Badr MS, Helal KF, Rafeek ME, Abdelrhman AA, Kotb M. Diagnostic accuracy of lamellar body count as a predictor of fetal lung maturity: a systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol X 2019;5:100059.
[33] Neerhof MG, Dohnal JC, Ashwood ER, Lee IS, Anceschi MM. Lamellar body counts: a consensus on protocol. Obstet Gynecol 2001;97:318–20.
[34] Raschetti R, Centorrino R, Letamendia E, Benachi A, Marfaing-Koka A, De Luca D. Estimation of early life endogenous surfactant pool and CPAP failure in preterm neonates with RDS. Respir Res 2019;20:75.
[35] Verder H, Ebbesen F, Fenger-Grøn J, Henriksen TB, Andreasson B, Bender L, et al. Early surfactant guided by lamellar body counts on gastric aspirate in very preterm infants. Neonatology 2013;104:116–22.
[36] Cavicchioli P, Zimmermann LJ, Cogo PE, Badon T, Giordano G, Torresin M, et al. Endogenous surfactant turnover in preterm infants with respiratory distress syndrome studied with stable isotope lipids. Am J Respir Crit Care Med 2001;163:55–60.
[37] Costa S, Zecca E, De Luca D, De Carolis MP, Romagnoli C. Efficacy of a single dose of antenatal corticosteroids on morbidity and mortality of preterm infants. Eur J Obstet Gynecol Reprod Biol 2007;131:154–7.
[38] Jobe A, Jacobs H, Ikegami M. Lack of correlation of severity of lung disease with the phosphatidylcholine concentration in fetal lung fluid from premature lambs at 133–136 days gestational age. J Dev Physiol 1984;6:417–21.
[39] Lopez-Rodriguez E, Pascual A, Arroyo R, Floros J, Perez-Gil J. Human pulmonary surfactant protein SP-A1 provides maximal efficiency of lung interfacial films. Biophys J 2016;111:524–36.
[40] Ross M, Krol S, Janshoff A, Galla HJ. Kinetics of phospholipid insertion into monolayers containing the lung surfactant proteins SP-B or SP-C. Eur Biophys J 2002;31:52–61.
[41] Walters RW, Jenq RR, Hall SB. Distinct steps in the adsorption of pulmonary surfactant to an air-liquid interface. Biophys J 2000;78:257–66.
[42] Autilio C, Echaide M, Cruz A, García-Mouton C, Hidalgo A, Da Silva E, et al. Molecular and biophysical mechanisms behind the enhancement of lung surfactant function during controlled therapeutic hypothermia. Sci Rep 2021;11:728.
[43] Chavarha M, Loney RW, Rananavare SB, Hall SB. An anionic phospholipid enables the hydrophobic surfactant proteins to alter spontaneous curvature. Biophys J 2013;104:594–603.
[44] Rodríguez-Capote K, Manzanares D, Haines T, Possmayer F. Reactive oxygen species inactivation of surfactant involves structural and functional alterations to surfactant proteins SP-B and SP-C. Biophys J 2006;90:2808–21.
[45] Stadtman E, Levine R. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 2003;25:207–18.
[46] Cerrada A, Haller T, Cruz A, Pérez-Gil J. Pneumocytes assemble lung surfactant as highly packed/dehydrated states with optimal surface activity. Biophys J 2015;109:2295–306.
[47] De Luca D, Shankar-Aguilera S, Autilio C, Raschetti R, Vedovelli L, Fitting C, et al. Surfactant-secreted phospholipase A(2) interplay and respiratory outcome in preterm neonates. Am J Physiol Lung Cell Mol Physiol 2020;319:L95–104.
[48] Lopez-Rodriguez E, Echaide M, Cruz A, Taeusch HW, Perez-Gil J. Meconium impairs pulmonary surfactant by a combined action of cholesterol and bile acids. Biophys J 2011;100:646–55.
[49] Autilio C, Pérez-Gil J. Understanding the principle biophysics concepts of pulmonary surfactant in health

and disease. Arch Dis Child Fetal Neonatal Ed 2019;104:F443–51.
[50] Parra E, Pérez-Gil J. Composition, structure and mechanical properties define performance of pulmonary surfactant membranes and films. Chem Phys Lipids 2014;185:153–75.
[51] Pattle R, Kratzing C, Parkinson C, Graves L, Robertson R, Robards G, et al. Maturity of fetal lungs tested by production of stable microbubbles in amniotic fluid. Br J Obstet Gynaecol 1979;86:615–22.
[52] Bhatia R, Morley CJ, Argus B, Tingay DG, Donath S, Davis PG. The stable microbubble test for determining continuous positive airway pressure (CPAP) success in very preterm infants receiving nasal CPAP from birth. Neonatology 2013;104:188–93.
[53] Eastoe J, Dalton JS. Dynamic surface tension and adsorption mechanisms of surfactants at the air–water interface. Adv Colloid Interface Sci 2000;85:103–44.
[54] Holm BA, Wang Z, Notter RH. Multiple mechanisms of lung surfactant inhibition. Pediatr Res 1999;46:85–93.
[55] Zuo YY, Veldhuizen RA, Neumann AW, Petersen NO, Possmayer F. Current perspectives in pulmonary surfactant—inhibition, enhancement and evaluation. Biochim Biophys Acta 2008;1778:1947–77.
[56] Gunasekara L, Schoel WM, Schürch S, Amrein MW. A comparative study of mechanisms of surfactant inhibition. Biochim Biophys Acta Biomembr 2008;1778:433–44.
[57] Oh MH, Bae CW. Inhibitory effect of meconium on pulmonary surfactant function tested in vitro using the stable microbubble test. Eur J Pediatr 2000;159:770–4.
[58] Ravasio A, Cruz A, Pérez-Gil J, Haller T. High-throughput evaluation of pulmonary surfactant adsorption and surface film formation. J Lipid Res 2008;49:2479–88.
[59] Hobi N, Siber G, Bouzas V, Ravasio A, Pérez-Gil J, Haller T. Physiological variables affecting surface film formation by native lamellar body-like pulmonary surfactant particles. Biochim Biophys Acta 2014;1838:1842–50.
[60] Danhaive O, Chapin C, Horneman H, Cogo PE, Ballard PL. Surface film formation in vitro by infant and therapeutic surfactants: role of surfactant protein B. Pediatr Res 2015;77:340–6.
[61] De Luca D, Vázquez-Sánchez S, Minucci A, Echaide M, Piastra M, Conti G, et al. Effect of whole body hypothermia on inflammation and surfactant function in asphyxiated neonates. Eur Respir J 2014;44:1708–10.
[62] Autilio C, Echaide M, Benachi A, Marfaing-Koka A, Capoluongo ED, Pérez-Gil J, et al. A noninvasive surfactant adsorption test predicting the need for surfactant therapy in preterm infants treated with continuous positive airway pressure. J Pediatr 2017;182:66–73.e1.
[63] Schipke J, Jütte D, Brandenberger C, Autilio C, Perez-Gil J, Bernhard W, et al. Dietary carbohydrates and fat induce distinct surfactant alterations in mice. Am J Respir Cell Mol Biol 2021;64:379–90.
[64] Autilio C, Echaide M, Shankar-Aguilera S, Bragado R, Amidani D, Salomone F, et al. Surfactant injury in the early phase of severe meconium aspiration syndrome. Am J Respir Cell Mol Biol 2020;63:327–37.
[65] Autilio C, Echaide M, Dell'Orto V, Perez-Gil J, De Luca D. Effect of whole body hypothermia on surfactant function when amniotic fluid is meconium stained. Ther Hypothermia Temp Manag 2020;10:186–9.
[66] Autilio C, Echaide M, De Luca D, Perez Gil J. Controlled hypothermia may improve surfactant function in asphyxiated neonates with or without meconium aspiration syndrome. PLoS One 2018;13:e0192295.
[67] Echaide M, Autilio C, López-Rodríguez E, Cruz A, Pérez-Gil J. In vitro functional and structural characterization of A synthetic clinical pulmonary surfactant with enhanced resistance to inhibition. Sci Rep 2020;10:1385.
[68] De Luca D, Shankar-Aguilera S, Bancalari E. LISA/MIST: complex clinical problems almost never have easy solutions. Semin Fetal Neonatal Med 2021 Apr;26:101230.
[69] De Luca D, Shankar-Aguilera S, Centorrino R, Fortas F, Yousef N, Carnielli VP. Less invasive surfactant administration: a word of caution. Lancet Child Adolesc Health 2020;4:331–40.
[70] Dargaville PA. CPAP, surfactant, or both for the preterm infant: resolving the dilemma. JAMA Pediatr 2015;169:715–7.
[71] Dargaville PA, Kamlin CO, De Paoli AG, Carlin JB, Orsini F, Soll RF, et al. The OPTIMIST-A trial: evaluation of minimally-invasive surfactant therapy in preterm infants 25-28 weeks gestation. BMC Pediatr 2014;14:213.
[72] Dargaville PA, Aiyappan A, De Paoli AG, Kuschel CA, Kamlin CO, Carlin JB, et al. Minimally-invasive surfactant therapy in preterm infants on continuous positive airway pressure. Arch Dis Child Fetal Neonatal Ed 2013;98:F122–6.
[73] Arroyo R, Kingma PS. Surfactant protein D and bronchopulmonary dysplasia: a new way to approach an old problem. Respir Res 2021;22:141.
[74] Sorensen GL, Dahl M, Tan Q, Bendixen C, Holmskov U, Husby S. Surfactant protein-D-encoding gene variant polymorphisms are linked to respiratory outcome in premature infants. J Pediatr 2014;165:683–9.
[75] Hilgendorff A, Heidinger K, Bohnert A, Kleinsteiber A, König IR, Ziegler A, et al. Association of polymorphisms in the human surfactant protein-D (SFTPD) gene and postnatal pulmonary adaptation in the preterm infant. Acta Paediatr 2009;98:112–7.
[76] Bae YM, Bae CW, Oh MH, Lee SH, Woo KM, Jung KB. Effect of exogenous surfactant therapy on levels of pulmonary surfactant proteins A and D in preterm infants with respiratory distress syndrome. J Perinat Med 2009;37:561–4.
[77] Beresford MW, Shaw NJ. Bronchoalveolar lavage surfactant protein a, B, and d concentrations in preterm infants ventilated for respiratory distress syndrome receiving natural and synthetic surfactants. Pediatr Res 2003;53:663–70.
[78] Sarno L, Della Corte L, Saccone G, Sirico A, Raimondi F, Zullo F, et al. Histological chorioamnionitis and risk of pulmonary complications in preterm births: a systematic review and Meta-analysis. J Matern Fetal Neonatal Med 2021;34:3803–12.
[79] Tsuda H, Takahashi Y, Iwagaki S, Kawabata I, Hayakawa H, Kotani T, et al. Intra-amniotic infection increases amniotic lamellar body count before 34 weeks of gestation. J Matern Fetal Neonatal Med 2010;23:1230–6.
[80] Giambelluca S, Verlato G, Simonato M, Vedovelli L, Bonadies L, Najdekr L, et al. Chorioamnionitis alters lung surfactant lipidome in newborns with respiratory distress syndrome. Pediatr Res 2021;90:1039–43.
[81] Salminen A, Paananen R, Vuolteenaho R, Metsola J, Ojaniemi M, Autio-Harmainen H, et al. Maternal endotoxin-induced preterm birth in mice: fetal responses in toll-like receptors, collections, and cytokines. Pediatr Res 2008;63:280–6.
[82] Kuypers E, Collins JJ, Kramer BW, Ofman G, Nitsos I, Pillow JJ, et al. Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs. Am J Physiol Lung Cell Mol Physiol 2012;302:L380–9.