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

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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 FiO2. 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 FiO2 exceeds 0.30 for all babies with a clinical diagnosis of neonatal respiratory distress syndrome (RDS), the latest European guidelines suggest starting surfactant...
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 multilamellar vesicles containing 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 phosphatidylycholine, 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 spectrometry 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

| 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 | - technically complex |
| | | | - sensitive | - does not test surfactant quality |
| | | | - assays major surfactant lipid | - interference by blood and meconium |
| | | | - quick | - technically complex/coarse, depending on the assay |
| PG | 100 | 50 | - simple | - does not test surfactant quality |
| | | | - major surfactant protein | - does not test surfactant quality |
| SP-A | 88–100 | 83–93 | - | - |

| **to predict CPAP-failure in RDS neonates** | **Sensitivity (%)** | **Specificity (%)** | **Advantages** | **Drawbacks** |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| LBC | 70 | 67 | - quick | - does not test surfactant quality |
| | | | - simple | - interference by blood and meconium |
| SMT | 71 | 75 | - quick | - dilution problem |
| | | | - simple | - inter/intra-observing variability |
| | | | - tests surfactant quality | - possible interference by contaminants |
| SAT | 95 | 70 | - quick | - needing a fluorimeter |
| | | | - simple | - possible interference by contaminants |
| | | | - tests surfactant quality | - |

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.
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 contaminant 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 cutoff 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 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).

**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 [8]) 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.

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