Observational Study

Surfactant proteins analysis in perinatal deceased preterm twins among the Romanian population

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Abstract
The molecular basis of the evaluation of children suspected of having disorders of surfactant proteins is still under discussion. In this study, we aimed to describe the morphological characteristics and to evaluate the immunohistochemical expression of surfactant proteins (surfactant protein A [SPA], surfactant protein B, and pro-surfactant protein C) in the preterm twins that deceased due to unexplained respiratory distress syndrome (n = 12). Results showed statistically significant positive correlations between surfactant protein B expressions and pulmonary hemorrhage (ρ = 0.678; P < .05), SPA levels, and Apgar score (ρ = 0.605; P < .05) and also expressions of SPA and broncho pneumonia (ρ = 0.695; P < .05). The fetuses and neonates of the same gestational age showed differences among surfactant proteins regarding the immunostaining expression. Our data evidence a marked interindividual variability in the expression of all 3 surfactant proteins among the cases analyzed (n = 12), suggesting the intervention of some individual and epigenetic factors during gestation that might influence surfactant protein production and consequently survival rate.

Abbreviations: ELBW = extremely low birth weight, GA = gestational age, PI = ponderal index, RDS = respiratory distress syndrome, SPA = surfactant protein A, SPB = surfactant protein B, SPC = surfactant protein C, SPD = surfactant protein D.

Keywords: hemorrhage, preterm twins, respiratory distress syndrome, stillborn, surfactant proteins

1. Introduction
According to the literature, multiple pregnancies are associated with an increased risk of mortality compared to single fetal pregnancies.[1,2] In this sense, multifetal pregnancies represent 3% of pregnancies, of which 98% are twins, more frequently exposed to the phenomenon of premature birth, and 10% of them are stillbirths.[1] Identifying the causes of neonatal morbidity and mortality is essential for planning its reduction since respiratory diseases are the biggest contributor to morbidity and mortality in premature and low-birth-weight infants.[3]

At the same time, studies on twins have shown the common involvement of genetic and environmental risk factors in the etiopathogenesis of these diseases.[4] The physiologic transition from fetal to neonatal period requires the production of pulmonary surfactant, a complex mixture of 10% proteins, 70% to 80% phospholipids, and 10% neutral lipids, which are mostly produced by the type II alveolar cells.[5] The main role of surfactants is to reduce the surface tension within the alveoli to avoid alveolar collapse, contribute significantly to pulmonary immunity processes, being involved in viral neutralization, clearance of bacteria, fungi, apoptotic cells, and regulation of inflammation. The inability of premature neonates to produce surfactants and the immaturity of their lungs represent the primary etiologies of respiratory distress syndrome (RDS). In the alveoli, the surfactant is arranged in the form of myelin tubes. There are 4 specific surfactant proteins, 2 hydrophilic proteins with high molecular weight (surfactant protein A [SPA] and surfactant protein D [SPD]) and 2 lipophilic proteins with low molecular weight (surfactant protein B [SPB] and surfactant protein C [SPC]), which are highly interconnected in their functions.[6]
2. Materials and Methods

2.1. Case selection

Our study used premature (<37 weeks of gestation) infants born in Constanta, Romania, from multiple gestations during the last 5 years (2017–2021), being chosen from the patients’ bases of the Neonatology Department and the Clinical Service of Pathology, “Sf. Apostol Andrei” Emergency County Hospital.

Among the total number of newborn cases from twin pregnancies (n = 12), 6 were stillborn and the other 6 were liveborn. All cases were chosen without other excluding criteria, besides genetic syndromes, and other associated pathologies.

The study was approved by the Ethics Committees of the “Sf. Apostol Andrei” Emergency County Hospital, from Constanta. Gestational age (GA), gender, mode of delivery, birth order, and maternal and neonatal clinical history were obtained from their medical records. All newborns did not receive the exogenous surfactants at their birth.

2.2. Morphological and clinical characteristics of cases

An autopsy was performed for each case, to determine the cause of death. The stillborn underwent external examination, with the performance of characteristic measurements (birth weight, length, cranial, thoracic, and abdominal perimeter) and internal examination, by analyzing the cranial cavity, thymus, lungs, heart, and large vessels, organs of the abdominal cavity, kidneys, and adrenal glands. The cases were named in capital letters, in alphabetical order, marking with the numbers 1 and 2 the pairs of fetuses and with a single number the child who died from that pair.

Preterm birth is defined as birth before 37 complete weeks of gestation. Preterm birth is classified according to the GA as late preterm (between 32 and 35 weeks of gestation), very preterm (between 26 and 32 weeks of gestation), extremely preterm (<26 weeks of gestation).

Birth weight among children born preterm can be classified as extremely low birth weight (ELBW; <1000 g), very low birth weight (<1500 g), low birth weight (1500–2500 g), and normal birth weight (>2500 g).

Infants born small for gestational age were identified based on World Health Organization–specific references on fetal birth weight being less than the 10th centile for GA. The weight-length ratio was calculated using the Rohrer ponderal index (PI), by the formula 100 x weight in grams/length in centimeters, which has normal values in newborns between 2.2 and 3, irrespective of weeks of gestation, child’s sex, and maternal parity.

The gravidity of a woman is defined as the number of pregnancies that the woman had, regardless of the stage of the pregnancies, so primigravida refers to a woman in her first pregnancy, while multigravida is a woman who has been pregnant more than once. The parity refers to the number of pregnancies that a woman had with GAs of over 24 weeks, which led to childbirth, even though the child was stillborn or was born alive, so a primiparous woman is a woman who has given birth once, while a multiparous woman has given birth more than once.

The Apgar score is a method used to assess the status of a newborn immediately after birth. The Apgar score is composed of 5 elements: heart rate, respiratory effort, muscle tone, reflex irritability, and skin color. It has values between 0 and 10, the normal values being between 7 and 10; the score is reported at 1 and 5 minutes after birth and then every 5 minutes, for the first 20 minutes for infants with a score of <7 at birth. A stillborn is defined as a baby born beginning with 28 weeks of gestation or with a birth weight of at least 1000 g, without any sign of life.

2.3. SPA, SBP, and pro-SPC determinations by immunohistochemical techniques

Fragments of organs with representative lesions taken from the autopsy were fixed, paraffin embedded, and subsequently stained with hematoxylin-eosin. The sampled lung tissue fragments were subjected to immunohistochemical examination, using polyclonal antibodies such as the SPA antibody, SPB antibody, and pro-SPC antibody.

Immunohistochemical examination was performed in compliance with the indications recommended by the manufacturer (Novus Biologicals). For immunohistochemical tests, we used formalin-fixed, paraffin-embedded tissue, being sectioned at 4 μm thickness. The staining protocol, as recommended by the producer, included deparaffinized using xylene and decreasing grades of alcohol, then rehydrated with hematoxylin, and subsequently with a phosphate buffered solution.

For detection, we used a polymer detection kit (peroxidase, 2.5% normal horse serum-HRP and DAB reagent; Vector Laboratories) and finally counterstained with hematoxylin and mounted.

Immunoreaction of SPA is considered positive in alveolar type II cells, non-ciliated bronchial cells, and a subset of alveolar macrophages. SPB positivity and pro-SPC reactivity are considered in alveoli. The expressions for each marker, SPA, SBP, and pro-SPC, were evaluated as absent (−), weakly positive (+), and intense positive (++), compared to the histopathological normal area without inflammation or a proliferative lesion in the same specimens. For the evaluation of the expression of the proteins, the histopathological normal area of the lungs was used as a control, especially type II alveolar cells (positive for SPA, SBP, and pro-SPC) and the bronchiolar epithelial cells (weak positive for SPA and pro-SPC and intense positive for SPB).

2.4. Statistical analysis

Obtained results were presented as mean values with standard errors for maternal age, GA, weight at birth, length of the fetuses, and being using the SPSS Statistics software package (version 17.01; IBM). Because of the interindividual variability of the parameters, the Shapiro-Wilk test was applied for testing the normality distribution.

To analyze the correlations between morphological characteristics and expression of surfactant proteins by immunohistochemical tests (SPA, SBP, and pro-SPC), we used the Spearman’s rank correlation coefficient, and P < .05 was considered statistically significant.

3. Results

During the study period, from all cases of necropsies on children (n = 315), were analyzed the autopsy records of premature newborns from twin pregnancies (n = 12). The data from the quantification of the parameters are presented in Table 1. The mean age of the mothers was 27.25 ± 1.69 years (range, 21–38 years). The mean GA was 28.17 ± 1.06 weeks (range, 23–36 weeks), being shorter in boy-boy pairs than in boy-girl and girl-girl pairs.

In the studied group, stillborn (n = 6) and liveborn (n = 6) cases were identified. The liveborn presented a 5-minute Apgar score between 1 and 6. The weight at birth varied from 600 to
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2900 (mean, 1132.5 ± 189.03) g. The mean length of the fetuses was 33.75 ± 1.22 cm. The fetus length at birth was smaller in the population of preterm twins born from multiparous multigravida mothers compared to the ones born from nulliparous primigravida. A single fetus associated a congenital malformation, represented by giant encephalocele, with anal imperforation and facial dimorphism. Four of 6 stillborn fetuses came from multiparous mothers, with an age under 30 years.

Histopathological examination of the lung tissue showed inflammatory, hemorrhagic, and pulmonary atelectasis changes. Meningeal and renal hemorrhage, circumscribed purulent collections (in liveborn), and congenital malformations were observed (Table 2). All cases showed pulmonary lesions, and 8 cases were associated with extrapulmonary lesions. The most common lesions were pulmonary hemorrhage in 58.33% of cases and meningeal and renal hemorrhage in 50% of cases.

Table 3 presents the expression of surfactant proteins by immunostaining technique at the pulmonary level on samples recovered from the studied group. Immunohistochemical examination (Fig. 1) shows a negative response to SPA in 6

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**Table 1**

The morphological characteristics of the cases from the studied group.

| Cases | Sex | Weight at birth (g) | Fetus length (cm) | PI (g/cm³) | GA (wk) | 5-min Apgar score | Maternal age (y) | G and P | Prematurity grade |
|-------|-----|---------------------|-------------------|---------|--------|-------------------|-----------------|-------|------------------|
| A1    | M   | 640                 | 30                | 2.37     | 23     | 1                 | 27              | G3P2  | ELBW             |
| A2    | M   | 600                 | 29                | 2.46     | 23     | Stillborn         | 27              | G3P2  | ELBW             |
| B1    | M   | 1150                | 34                | 2.9      | 30     | 6                 | 25              | G1P1  | ELBW             |
| B2*   | F   | 900                 | 36                | 1.93     | 30     | Stillborn         | 25              | G1P1  | ELBW             |
| C1    | F   | 1300                | 30                | 4.8      | 27     | Stillborn         | 23              | G3P3  | VLBW             |
| C2    | F   | 1050                | 33                | 2.9      | 27     | Stillborn         | 23              | G3P3  | VLBW             |
| D1*   | F   | 700                 | 33                | 1.95     | 26     | Stillborn         | 38              | G4P2  | ELBW             |
| D2*   | F   | 650                 | 32                | 2        | 26     | 2                 | 38              | G4P2  | ELBW             |
| E1    | F   | 2900                | 45                | 3.2      | 36     | 1                 | 32              | G1P1  | NBW              |
| F1    | M   | 1800                | 32                | 5.5      | 32     | Stillborn         | 27              | G1P1  | LBW              |
| G1    | F   | 1100                | 36                | 2.36     | 29     | 5                 | 21              | G1P1  | ELBW             |
| G2    | F   | 800                 | 35                | 1.87     | 29     | 5                 | 21              | G1P1  | ELBW             |

*B2*: cross-sectional presentation.

**Table 2**

Spectrum of pulmonary and extrapulmonary lesions on necropsy macroscopic examination of the studied group.

| Cases | Lung injuries | Extrapulmonary lesions |
|-------|---------------|------------------------|
| A1 liveborn | Bilateral pulmonary atelectasis, pulmonary hemorrhage | Meningeal hemorrhage, renal hemorrhage |
| A2 stillborn | Bilateral pulmonary atelectasis | Meningeal hemorrhage and edema, renal and adrenal hemorrhage |
| B1 liveborn | Bilateral bronchopneumonia, left pleurotomy under the drain | Purulent peritonitis |
| B2 stillborn | Acute bilateral bronchopneumonia | Acute enterocolitis |
| C1 stillborn | Bilateral pneumonia, total bilateral atelectasis | - |
| C2 stillborn | Pulmonary hemorrhage | Edema and meningeal hemorrhage, adrenal hemorrhage |
| D1 stillborn | Atelectasis, bilateral pulmonary hemorrhage | Pulmonary hemorrhage, pericardial effusion, congenital malformation: giant encephalocele, anal imperforation, rectovaginal fistula, facial dimorphism |
| D2 liveborn | Bilateral pneumonia | - |
| E1 liveborn | Pulmonary hemorrhage | - |
| F1 stillborn | Pulmonary hemorrhage | Pulmonary hemorrhage, cerebral edema |
| G1 liveborn | Pneumopathy and pulmonary hemorrhage | Pulmonary hemorrhage, left occipital hematoma, hemorrhagic gastritis |
| G2 liveborn | Pulmonary hemorrhage | - |

**Table 3**

Expression of surfactant proteins by immunostaining technique at the pulmonary level on samples recovered from the studied group.

| Cases | Weight at birth (g) | GA (wk) | SPA | SPB | Pro-SPC |
|-------|---------------------|--------|-----|-----|---------|
| A1 liveborn | 640 | 23 | Negative (−) | Weak positive (+) | Negative (−) |
| A2 stillborn | 600 | 23 | Negative (−) | Weak positive (+) | Negative (−) |
| B1 liveborn | 1150 | 30 | Weak positive (+) | Negative (−) | Negative (−) |
| B2 stillborn | 900 | 30 | Weak positive (+) | Negative (−) | Negative (−) |
| C1 stillborn | 1300 | 27 | Weak positive (+) | Negative (−) | Negative (−) |
| C2 stillborn | 1050 | 27 | Negative (−) | Weak positive (+) | Negative (−) |
| D1 stillborn | 700 | 26 | Negative (−) | Weak positive (+) | Weak positive (+) |
| D2 liveborn | 650 | 26 | Weak positive (+) | Negative (−) | Weak positive (+) |
| E1 liveborn | 2900 | 36 | Negative (−) | Intense positive* (++) | Weak positive (+) |
| F1 stillborn | 1800 | 32 | Negative (−) | Intense positive (++) | Weak positive (+) |
| G1 liveborn | 1100 | 29 | Intense positive (++) | Intense positive (++) | Weak positive (+) |
| G2 liveborn | 800 | 29 | Intense positive (++) | Intense positive (++) | Weak positive (+) |

GA = gestational age, SPA = surfactant protein A, SPB = surfactant protein B, SPC = surfactant protein C.

*Samples are intense positive (++) in the areas of alveolitis and pulmonary atelectasis.
cases (50%), a weak positive response in 4 cases (33.33%), and an intense positive response in 2 cases (16.67%). Regarding immunoreactivity to SPB, 4 cases (33.33%) had a negative response, while 3 cases (25%) were intense positive in the areas of leukocyte alveolitis and pulmonary atelectasis, while the others were weakly positive (41.67%). Evaluation of pulmonary tissue cases with pro-SPC showed negativity in 7 cases (58.33%), weak positivity in 5 cases (41.67%), and no case of intense focal positivity.

The correlations between SPA, SPB, and pro-SPC expressions at the pulmonary level, GA, and birth weight are shown in Table 4. The expression of surfactant proteins was not correlated with GA and birth weight of cases from the studied group.

Immunohistochemical expression of surfactant proteins in correlation with the type of pulmonary lesions and 5-minute Apgar score are shown in Table 5. SPA was positively correlated with bronchopneumonia and 5-minute Apgar score ($\rho = 0.695; P < 0.05$) and 5-minute Apgar score ($\rho = 0.605; P < 0.05$). Immunohistochemical expression of SPB was positively correlated with pulmonary hemorrhage ($\rho = 0.678; P < 0.05$).

In Table 6, statistical analyses evidenced a significant positive correlation ($\rho = 0.850; P < 0.01$) between the expression of the birth weight and GA. Also, a significant positive correlation ($\rho = 0.723; P < 0.01$) is between the GA and fetus length.

### 4. Discussion

The present study examined the protein variants of the pulmonary surfactant in a population consisting of 12 preterm twins, perinatally deceased due to acute RDS. The sampled lung tissue fragments from the autopsies were subjected to immunohistochemical examination, using the polyclonal antibodies SPA, SPB, and pro-SPC.

The major glycoprotein is SPA, which is released into the alveolar lumen from cytoplasmic lamellar bodies from the type II alveolar epithelial cells, although SPA can be found in small quantities in the pseudostratified epithelium of the airways, in the non-ciliated bronchiolar cells, and the submucosal glands of the trachea. SPA has a role in the tubular myelin formation, the formation of the surface's film, and contributes to the inflammatory response by binding with the microbial pathogens that invade the lungs. SPA is also found in small quantities in extrapulmonary sites such as small and large intestines, mesentery, epithelium, salivary glands, prostate, thymus, amniotic fluid, and the placenta.

### Table 4

| Spearman rank correlation coefficient | SPA | SPB | Pro-SPC |
|-------------------------------------|-----|-----|---------|
| Birth weight (g)                    | $\rho$ = 0.076 | $-0.074$ | $-0.024$ |
| GA (wk)                             | $\rho$ = 0.200 | $-0.023$ | 0.074 |

$\rho$ = Spearman rank correlation coefficient, GA = gestational age, SPA = surfactant protein A, SPB = surfactant protein B, SPC = surfactant protein C, Sig. (2 tailed) = $P$ values.
Table 5

Correlations between immunohistochemical expression of surfactant proteins and the clinicomorphological characteristics on cases from the studied group.

| Spearman rank correlation coefficient | SPA | SPB | Pro-SPC |
|--------------------------------------|-----|-----|---------|
| Atelectasis                           | ρ  | −0.391 | −0.027 | −0.239 |
| Sig. (2 tailed)                       | 0.208 | 0.933 | 0.454 |
| Pulmonary hemorrhage                  | ρ  | −0.267 | 0.678* | 0.371 |
| Sig. (2 tailed)                       | 0.401 | 0.015 | 0.235 |
| Bronchopneumonia                      | ρ  | 0.095* | −0.496 | −0.029 |
| Sig. (2 tailed)                       | 0.012 | 0.101 | 0.930 |
| 5-min Apgar score                     | ρ  | 0.605* | 0.287 | 0.446 |
| Sig. (2 tailed)                       | 0.037 | 0.365 | 0.146 |

ρ = Spearman rank correlation coefficient. SPA = surfactant protein A, SPB = surfactant protein B, SPC = surfactant protein C.

*Correlation is significant at the 0.05 level (2 tailed).

Table 6

Correlations between GA, birth weight, and fetus length on cases from the studied group.

| Spearman rank correlation coefficient | Birth weight (g) | Fetus length (cm) |
|--------------------------------------|------------------|------------------|
| GA (wk)                              | ρ  | 0.850* | 0.723* |
| Sig. (2 tailed)                      | 0.000 | 0.008 |

ρ = Spearman rank correlation coefficient. GA = gestational age. Sig. (2 tailed) = P values.

*Correlation is significant at the 0.01 level (2 tailed).

fluid, and lacrimal apparatus. SPA formation is stimulated by interleukin-1, adenosine monophosphate, and a low concentration of corticosteroids, while a high concentration of corticosteroids inhibits the SPA secretion.[21–23] SPB is produced in Clara cells as pro-SPB and the type II alveolar cells as mature SPB. The processing of pro-SPB by the Clara cells into active mature SPB consists of a complex process of proteolytic cleavage.[24] SPB has an essential role in the absorption and surface distribution of phospholipids, in the fusion of multivesicular bodies into lamellar structures, and it helps the organization of phospholipids into myelin tubes and is involved in their transport at the air-liquid interface. In case of SPB deficiency, the structure of the lamellar body will be disorganized. SPB is also involved in the posttranslational modification of SPC. Because mature SPC is formed from pro-SPC after cleavage in the lamellar bodies, in case of SPB deficiency, this process will be disrupted, which will lead to the accumulation of pro-SPC and a deficiency of mature SPC. There is a hypothesis that suggests that SPB deficiency also affects the recycling of SPA, which will lead to increased SPA in the airspaces and decreased levels in the type II alveolar cells. Exogenous surfactant provides just a transitory improvement in gas exchange in case of SPB deficit and does not normalize the surfactant composition and function, so the patients require lung transplantation usually in the first year of life.[25]

SPC is produced exclusively in the lungs by the type II alveolar cells. SPC has a role in film formation and stabilization, decreasing the risk of alveolar collapse. SPC does not interact with SPA and is not essential for the formation of myelin tubes. SPC deficiency has multiple manifestations, from RDS developed in the first hour after birth to interstitial lung disease developed in childhood and adulthood. One study performed on mice with negative expression of SPC showed that they developed enlargement of the airspaces and chronic pneumonitis until the age of 6 months, so SPC is not essential for the perinatal transition to breathing, as individuals lacking SPC survive but develop a variety of interstitial or fibrotic lung disease outcomes. Corticosteroids are the main treatment for SPC deficiency, sometimes in association with azithromycin and hydroxychloroquine.[26] SPD is a molecule with pulmonary and extrapulmonary localization on the apical luminal surface of the respiratory epithelium, included in the collectin family, like SPA, whose distribution and properties partially overlap with SPD, which is why the expression of this protein was not determined in this article. The most important function of SPD in the lungs is to regulate pulmonary surfactant lipid levels and is also considered a potential role in involvement in phospholipid homeostasis at extrapulmonary sites, although this has not been fully demonstrated.[21–23]

Studies of explants from the human lung have shown that SPA is expressed in the epithelial cells of the main and segmental bronchi from the 21st week of gestation, while in the alveolar cells, SPA is expressed from the 29th week of gestation. SPB and SPC are expressed in the epithelial cells of the terminal airways from the 15th week of gestation and in the alveolar cell from the 25th week of gestation.[24] Thus, it is important to determine whether these surfactant disorders also include protein deficiencies, especially for the differentiated postpartum therapeutic approach. It is also imperative to find out, in the case of twin pregnancies, if only one or both children experience a qualitative protein disorder to establish the causal sequence, which may open the door in the future to other studies in the field, useful in increasing survival.

All subjects included in this study were born preterm, with a preponderance of extremely preterm newborns (A1/A2, C1/C2, and D1/D2), followed by 2 pairs of very preterm infants (B1/B2 and G1/G2), while 2 cases were late preterm (E1 and F1). RDS represents the first cause of morbidity and mortality in the first year of life of preterm neonates, due to insufficient secretion, inadequate production, or inactivation of the surfactant caused by lung immaturity.[20] Data from literature, supported also by the results of the present study, showed that the incidence of RDS is inversely proportional to the GA.[26–27]

The immunohistochemical examination showed a negative or a week positive response to SPA, SPB, and pro-SPC in the majority of cases of the studied group (10 of 12; 83.33%). One premature pair of neonates (G1/G2) with ELBW, born at 29 weeks of gestation from a young primiparous primigravida (of 21 years old), presented intense positive reactions for both SPA and SPB and weak reactivity for pro-SPC. The only case (E1) with intense positive expression for SPB, exclusively in the areas of alveolitis, was born at 36 weeks of gestation, with normal birth weight, from a 32-year-old mother, at the first pregnancy. The reactivity of the surfactant proteins was not correlated with GA and birth weight, suggesting an interindivudal variability. As the study by Cau et al.[8] showed, other factors may affect surfactant production and influence the survival rate of preterm infants.

In this study, 11 of 12 (91.66%) preterm twins were born with low birth weight, of which 7 had ELBW (<1000 g). Birth weight was not correlated with a specific deficit of surfactant proteins but was in direct relationship with mortality. These results are in concordance with previous studies that had shown that in infants with birth weight <2500 g, the risk of death has been around 200x higher compared to normal-birth-weight infants.[28,29]

Regarding the length at birth, 10 of 12 (83.33%) preterm twins presented lower fetus length at birth, according to their gestation age, and low PI. Similar results were reported by authors, which showed that infants with small fetus length at birth and low PI have a high risk of morbidity and mortality.[30]

From the population of preterm twins that we analyzed, 8 of 12 (75%) were female. Other studies performed on singleton term neonates have shown that the masculine sex has a higher risk for developing RDS.[31,32] It was believed that female lungs produce surfactants earlier during gestation because of the different hormonal profiles. It is known that estrogens have a...
positive effect on surfactant synthesis and the secretion of SPA and SPB, by increasing the number of alveolar type II cells. A possible explanation for the female predominance observed in our study may be the fact that all the newborns were from twin pregnancies.

In this study, all the infants who were born alive had a 5-minute Apgar score <7, and a significant positive association between negative expression of SPA and a 5-minute Apgar score was found. These results were also previously observed in the study by Chambless et al., who showed that a 3-minute Apgar score of ≤7 is a risk factor for developing RDS.

Half of the babies included in this study were stillborn, of whom 4 were extremely preterm and 2 were very preterm. The maternal age was <28 years in 5 cases, one mother of 38 years of age who also had an HIV infection associated, while 4 mothers were multiparous and multigravida. Four stillborns presented negative SPA, 2 negative SPB, and 5 negative pro-SPC. The main risk factors for stillbirth were previously identified in a systematic review of 96 studies, which found the advanced maternal age, primiparity, small fetus length at birth, maternal smoking, maternal obesity, diabetes, and hypertension as the most frequent risk factors associated with this condition, but further studies will be necessary to identify whether there is an association between a certain protein deficit and stillbirth.

In our study, the infants born from multigravida and multiparous mothers (6 of 12) were extremely preterm, 4 of them were stillborn, while the other 2 had low 5-minute Apgar score (of 1, respectively 2). We noticed that 4 of these 6 fetuses presented negative SPA and 4 negative SPB. Multiple gestations have been previously suggested to be associated with RDS in preterm infants, especially in the case of advanced maternal age. In another study performed on singleton infants, parity was associated with respiratory complications and increased risk of developing RDS. The underlying mechanism and the possible association between the surfactant protein deficiency and the parity of the mothers are still unclear, and further studies need to be performed in this area.

In the present study, a statistically significant positive correlation between SPA expression and pulmonary hemorrhage was noticed. From the 7 cases with pulmonary hemorrhage from our study, 5 of them presented negative expression of SPA. Multiple other studies showed that SPA deficiency increases apoptosis and decreases cell viability, induces an inflammatory reaction, and causes acute lung injury. Pulmonary hemorrhage may be associated with RDS, possibly due to high capillary pressure caused by hypoxia, heart failure, and volume overload. Following the endothelial damage, neutrophils will be released, which will lead to increased values of proteases, cytokines, and oxygen-free radicals. These components will damage the type II alveolar cells that produce SPs, resulting in lower levels of SPs.

SPA plays a major role in protecting the lungs against infections, by enhancing the attachment of phagocytic cells with pathogens and the clearances. The 4 twins with bronchopneumonia from this study showed nonintense positive SPA and negative SPB, similar to other studies from literature that showed bacterial infection is associated with low levels of SPA and SPB.

In our study, 4 newborns also presented pulmonary atelectasis, without any specific correlation with a certain surfactant protein. Because surfactant prevents lung collapse at low volumes by reducing the surface tension, surfactant deficiency can lead to atelectasis, which is clinically evident in the case of neonatal RDS, congenital SPB deficiency, and pulmonary alveolar proteinosis. In the presence of protein surfactant deficiencies, children are more likely to develop atelectasis compared with adults, due to the lack of Kohn pores and Lamber canals, which allow collateral ventilation of the obstructed alveol.

The infants from our study presented numerous extrapulmonary lesions. Half of them presented at least one extrapulmonary hemorrhage: 5 (41.66%) with renal and adrenal, 6 (50%) with meningeal, and 3 (25%) with plurivisceral hemorrhage. From the 3 surfactant proteins analyzed, SPA is the only one that was proven to be found in extrapulmonary sites, although no protein expression was found in the corresponding tissues by previous immunohistochemistry studies. An association between SPA deficit and renal and adrenal hemorrhage was found. New studies on mice have shown that SPA is also expressed in the renal tubular epithelial cells. SPA has a role in inflammation modulation and apoptosis in case of acute renal injury induced by sepsis. Another surfactant protein, SPD, which was not analyzed in our study, was found to be produced in many extrapulmonary sites, such as the brain, salivary gland, lachrymal glands heart, prostate gland, kidney, all bladder and intrahepatic bile ducts, and pancreas. This raises the question of whether SPD deficit may be associated with extrapulmonary lesions.

In summary, we found that premature twin newborns who died from RDS presented deficits of surfactant proteins SPA, SPB, and pro-SPC in fragments of organs with representative lesions taken from the autopsy. The pulmonary lesions identified at the autopsy were pulmonary hemorrhage, bronchopneumonia, and atelectasis. We also found numerous extrapulmonary lesions such as renal and adrenal hemorrhage, meningeal edema, and cerebral hemorrhage.

Although imaging and lung histopathology findings may strongly support a diagnosis of surfactant dysfunction, identifying additional genetic mechanisms is essential, as the inheritance patterns (and hence recurrence risk) and prognoses vary, depending upon the causative gene. Therefore, a multidisciplinary approach of clinical, genetic, epidemiologic, and histopathological considerations is necessary for an in-depth understanding of the pathophysiology of pulmonary diseases determined by protein surfactant deficiencies.

In this study, limitations to developing the utility of these observed surfactant proteins were a small number of cases from the studied group, because the newborns from twin pregnancies are not often met, marked heterogeneity in the clinical phenotype of cases, and no matching of cases for birth weight. There are more necessary cases to determine the association between surfactant deficits and pulmonary lesions, as well as determining the precise causes and pathogenesis of lung disease in preterm infants with RDS, to increase the survival rate.

5. Conclusions

Our analysis performed on deceased preterm twins suggests that surfactant protein deficiency is an important cause of mortality and morbidity. Low birth weight, fetal size at birth, GA, low 5-minute Apgar score, multiparity, and female gender of the fetuses were identified as risk factors for RDS caused by surfactant protein deficiency. We also identified numerous extrapulmonary lesions, preponderant hemorrhagic lesions with renal, adrenal, cerebral, and gastric localizations. Pulmonary hemorrhage was positively correlated with SPB expressions. Bronchopneumonia and 5-minute Apgar score were positively associated with the levels of SPA.

Future directions in this research area will be to study the genotype of surfactant proteins and the molecular functions of these genetic variants, which could contribute toward more effective, individualized prevention of respiratory failure and its serious consequences.

Author contributions

S.-A.G., M.A., and G.C.C designed the research; M.E., A.-N.N., and G.-I.B. performed the experiments; N.D. and E.M. performed the statistical analysis and made the tables and figures;
N.D. is a certified specialist in biostatistics; E.M. is a certified specialist in biostatistics and computational uncertainty measurement and performance indicators in the laboratory; R.E.C., M.E., A.P.F., and G.C.C. wrote the manuscript.

References
[1] Santana DS, Silveira C, Costa ML, et al. Perinatal outcomes in twin pregnancies complicated by maternal morbidity: evidence from the WHO Multicountry Survey on Maternal and Newborn Health. BMC Pregnancy Childbirth. 2018;18:449.
[2] Gao L, Luu SF, Zhao XR, et al. Systematic management of twin pregnancies to reduce pregnancy complications. Chin Med J (Engl). 2020;133:1355–7.
[3] Ananth CV, Chauhan SP. Epidemiology of twinning in developed countries. Semin Perinatol. 2012;36:156–61.
[4] Moss TJ. Respiratory consequences of preterm birth. Clin Exp Pharmacol Physiol. 2006;33:280–40.
[5] Levit O, Jiang Y, Bizzarro MJ, et al. The genetic susceptibility to respiratory distress syndrome. Pediatr Res. 2009;66:693–7.
[6] Somaschini M, Presi S, Ferrari M, et al. Surfactant proteins gene variants in premature newborn infants with severe respiratory distress syndrome. J Perinatol. 2018;38:337–44.
[7] Howson CP, Kinney MV, Lawn J. March of dimes, PMNCH, save the children. WHO; 2018:649.
[8] Verlato G, Cogo P, Balzani M, et al. Surfactant status in preterm neonates recovering from respiratory distress syndrome. Pediatrics. 2008;122:102–8.
[9] Pinheiro Ribeiro LP, de Albuquerque D. The importance of surfactant on the development of neonatal pulmonary diseases. Clinics. 2007;62:181–90.
[10] American Academy of Pediatrics and American Heart Association. Textbook of Neonatal Resuscitation. 6th edition. Elk Grove Village, IL: American Academy of Pediatrics and American Heart Association; 2011; Neonatal resuscitation.pdf (moscm.org).
[11] Yadav S, Lee B, Kamity R. Neonatal Respiratory Distress Syndrome. In: StatPearls Treasure Island (FL): StatPearls Publishing; 2021; Neonatal Respiratory Distress Syndrome - StatPearls - NCBI Bookshelf (nih.gov).
[12] Donn SM, Sinha SK. Respiratory Distress Syndrome. Manual of Neonatal Respiratory Care (Third Edition), 2017; p. 301–4.
[13] Sun J, Qu S, Zhang C, et al. Neonatal mortality rate and risk factors in the extreme south of Brazil. Popul Health Metrics. 2019;17:15.
[14] Condo V, Cipriani S, Colnaghi M, et al. Neonatal respiratory distress syndrome: are risk factors the same in preterm and term infants? J Matera Fetal Neonatal Med. 2015;40:1267–72.
[15] Liu J, Yang N, Liu Y. High-risk factors of respiratory distress syndrome in term neonates: a retrospective case-control study. Balkan Med J. 2014;31:64–8.
[16] Chambliss Linda R, Bay Curtis R. The predictive value of a 5-minute apgar for developing respiratory distress syndrome. Obstet Gynecol. 2005;4:158.
[17] Flenady V, Koopmans L, Middleton P, et al. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. Lancet. 2011;377:1331–40.
[18] Wang S, Yang L, Shang L, et al. Changing trends of birth weight with maternal age: a cross-sectional study in Xi’an city of Northwestern China. BMC Pregnancy Childbirth. 2020;20:744.
[19] Shiels MS, Kirk GD, Drummond MB, et al. HIV Infection and Circulating Levels Of Prosurfactant Protein B and surfactant Protein D. J Infectious Diseases. 2018;314:313–7.
[20] Epasr R, Iekami M, Whitsett JA, et al. Akinbi HT: surfactant protein B inhibits endotoxin-induced lung inflammation. Am J Respir Cell Mol Biol. 2003;28:377–85.
[21] Nikaido PO, Merritt TA, Pillers DA. An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. Mol Genet Metab. 2009;97:95–101.
[22] Kishore U, Greenhough T, Waters P, et al. Surfactant proteins SP-A, and SP-D: structure, function and receptors. Mol Immunol. 2006;43:1293–315.
[23] D’Aronco S, Simonato M, Vedovelli L, et al. Surfactant Protein B and A concentrations are increased in neonatal pneumonia. Pediatr Res. 2015;78:401–6.
[24] Kadits AG, Motoyama EK, Zin W, et al. The effect of lung expansion and positive end-expiratory pressure on respiratory mechanics in anesthetized children. Anesth Analg. 2008;106:775–85, table of contents.
[25] Madsen J, Torneoe I, Nielsen O, et al. Expression and localization of lung surfactant protein A in human tissues. Am J Respir Cell Mol Biol. 2003;29:591–7.
[26] Liu J, Abdel-Razek O, Liu Z, et al. Role of surfactant proteins A and D in sepsis-induced acute kidney injury. Shock. 2015;43:31–8.
[27] Madsen J, Klem A, Torneo I, et al. Localization of lung surfactant protein D on mucosal surfaces in human tissues. J Immunol. 2000;164:5866–70.