Introduction

In the process of fetal maturity, the lungs are the last vital organ that needs to develop in order to enable life in the extraterrestrial environment. Because of the importance of the lung function for survival, perinatal research and care are directed to the examination of its maturity. Unfortunately, until recently, lung immaturity resulted in abrupt deaths of prematurely born babies. Management in perinatology, contemporary maternal/fetal medicine and neonatology is aimed at achieving the optimal conditions for the delivery. The research encompasses the attempt to postpone the birth term, either by applying corticosteroid therapy and improving the neonatal aeration technique, or by the surfactant treatment (1). During early pregnancy there are none or just a few particle-shaped materials in the amniotic fluid. In the 16th week of gestation, the amniotic fluid is full of cells that fell off the amnion surface, from the skin or tracheobronchial tree. They are very important in the antenatal diagnostics. In the process of maturation, the lungs start producing the detergent like material 'surfactant' that forms a film on the alveolar surface.

APPLICATION OF BIOMARKERS IN EVALUATION OF FETAL LUNG MATURITY

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Summary: In the process of maturation, alveolar epithelial cells produce surface-active material (surfactant) which consists mainly of phospholipids and, although to a significantly lesser degree, neutral lipids, proteins and carbohydrate. Pulmonary surfactant is synthesized in alveolar type-II granular pneumocytes and "packed" as lamellar bodies. Before delivery, the lamellar bodies diffuse through the bronchial tree into the amniotic fluid. Parameters which indicate the maturity of lungs, except the concentration of lamellar bodies, are also observed in the ratio against the quantity of surfactant with other components of the amniotic fluid (sphingomyelin and albumin). The study concerns the determination of the surfactant-to-albumin ratio and the lamellar body count in the amniotic fluid as predictors of fetal lung maturity. The research involved 90 pregnant women with gestational age from 32 to 40 weeks. The samples of the amniotic fluid were obtained by amniocentesis and were filtrated through special filters. The surfactant-to-albumin ratio was measured by the Fetal Lung Maturity II (FLM) test, on a TDX instrument (ABBOTT). The concentration of lamellar body count was measured on a hematology cell counter by the platelet channel, on the Cell Dyn 3700 instrument (ABBOTT). The average value of S/A ratio was 55.11 mg/g (min=6.96 mg/g, max=112.00 mg/g). The surfactant-to-albumin ratio of less than 39.00 mg/g predicts respiratory distress syndrome with the probability of 92%. The concentration of lamellar body count was measured on a hematology cell counter by the platelet channel, on the Cell Dyn 3700 instrument (ABBOTT). The average value of LB was 69.4 × 10^9/L (min=4.2 × 10^9/L, max=199.0 × 10^9/L). Lamellar body concentration of 34.0 × 10^9/L or less predicts respiratory distress syndrome with the probability of 93%. The two tests correlated with each other, r=0.341, p<0.005. In 90 patients delivered within 72 hours the surfactant-to-albumin ratio and concentration of the lamellar body correctly predicted six cases of the respiratory distress syndrome. There was a high degree of positive correlation between gestational age and surfactant-to-albumin ratio, as to lamellar body concentration.

Key words: fetal lung maturity, lamellar bodies, surfactant/albumin ratio, respiratory distress syndrome

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face tension in the alveoli so that, at the end of the expirium, the walls of the alveoli do not collapse or fix. When there is not enough surfactant, the alveoli collapse, and each breath should have the higher negative tension in order to make the alveoli open. Depending on the level of difficulty, it may happen that the premature baby cannot open the alveoli again, or can do, it but becomes exhausted trying to breathe. In that case, it is necessary to provide additional oxygen and mechanical ventilation in order to keep regular oxygenation (2).

Pulmonary surfactant consists of phospholipids and protein with the surface active properties, and it covers the alveolar surface and terminal airways. Alveolar surface, consists of the alveolar wall, the film that covers the wall, and the alveolar pores at the contact surface, in contact with the air. The alveolar wall is made up of a one-layer epithelium which contains the pores, called the Kohn’s pores. Regarding the epithelial cells, 85% belongs to type-I cells, while the rest of 15% are type-II cells (pneumocytes) with cytoplasmic granular bodies (lamellar bodies) and microvilli on the surface. Type-II cells produce and store the pulmonary surfactant and react as a precursor of cells type-I which also have a phagocyte activity. The liquid phase of the limiting lining is called hypophase, and its surface is the surface film or air-liquid interface. The molecular surfactants are concentrated on the air-liquid contiguous surface with the polar side turned to the liquid phase and the non-polar side turned to the air phase. These molecules reduce the surface tension on the air-liquid contiguous surface, and that provides the stability of the alveoli at the end of the expirium, preventing their collapse. Other functions of the surfactant are: prevention of edema by the effect of the transepithelial fluid activation and the bacteria phagocytosis stimulation with the help of the macrophage, which gives the surfactant an important role in the ultimate line of the lungs’ defense.

The surfactant contains 70–80% of phospholipids, about 10% of proteins, about 10% of neutral lipids (especially cholesterol) and about 5% of carbohydrates. 80% of phospholipids make the phosphatidylcholine. About 50% of phosphatidylcholine is saturated, and saturation on both C-atoms is carried out by the palmitic acid (3, 4). Most of the other phosphatidylcholine has unsaturated fatty acids on the second C-atom. The saturated phosphatidylcholine is the main surface active component of the surfactant. The acid phospholipids phosphatidylglycerol (PG) is present in a small but relatively stable quantity, which is between 4 and 15% of phospholipids (of various types). The composition of phospholipids in the lipoprotein surfactant structure is changed in the course of pregnancy. Phospholipids in the immature fetus or the newborn baby contain relatively great amounts of phosphatidylinositol, but its concentration is reduced with lung maturation, along with the emerging of phosphatidylglycerol (5, 6). The substitution of phosphatidylinositol with phosphatidylglycerol is probably an outcome of the reduced concentration of free inositol in circulation in the lung tissue during the later gestation (7). Phosphatidylglycerol constitutes 11% of the surface active molecules. There is an opinion that claims that the premature infants whose surfactant lacks phosphatidylglycerol have a bigger percentage of RDS, but some researches proved that PG is not necessary for the functioning of the surfactant, i.e. absence of PG will not cause RDS (7, 8).

The presence of the specific surfactant proteins was noted for the first time in 1973. Recently, 4 specific surfactant proteins were described, and their function was explained as well. In line with the nomenclature introduced by Possmayer in 1988, proteins called SP-A, SP-B, SP-C, and SP-D are hydrosoluble, while SP-B and SP-C are small hydrophobe proteins that come out from the precursor of the bigger proteins. Proteins in the composition of the surfactant have many functions. SP-A and SP-B are essential for forming the tubular myelin and grouping of lamellar bodies, which is important for the preserving and secretion of surfactant. SP-A is a metabolic regulator which controls the secretion and the repeated input of other surfactant components using the specific membrane receptors on the type-II cells, and it represents a non-immune protein of the ultimate defense. SP-D has only been isolated a short time ago and has many structural and functional similarities with SP-A, but the precise role of SP-D is still to be identified. SP-D does not associate with surfactant lipids or participate in surfactant function. Surfactants from which the SP-D is removed retain the features of the surface active molecules, but it is still assumed that SP-D has the role of the ultimate defense protein (9). SP-B grows along with the progression of the gestational age, and it is localized in respiratory epithelial cells only in the distal lung. This protein is the extreme simulator of the lipid surfactant adsorption and formation of the surface film (10). The lack of SP-B in premature infants manifests in the strong form of RDS that is not likely to be cured with the surfactant treatment. About 30% of newborn infants who die of unexplained acute RDS lack the SP-B protein. The lack of SP-B in humans and mice is related to the lack of lamellar bodies in type-II cells, as well as to the lack of the conversion of proSP-C into SP-C. That is the reason why these newborn infants do not have SP-B or active SP-C. They also do not have the organelle to recycle the surfactant, and that explains for their weak response to the surfactant therapy. SP-C is a very hydrophobe protein. It is associated with phospholipids in the alveolar surfactant and facilitates surface adsorption, although SP-C-based surfactants may not reach very low surface tension on surface compression. Protein nonspecifically facilitates the lipid input by means of the cells. The states caused by the deficiency of the SP-C have not been detected in the human organism.
Although the procedure of the external surfactant synthesis is known, details about the condensation of components into the lipoprotein surfactant complex which contains phospholipids within the lamellar bodies, SP-B and SP-C, are still pretty vague. Hydrophobe surfactant proteins are essential in the process of forming lamellar bodies, as lamellar bodies are not present in the type-II cells that lack SP-B (11).

Type-II cells have beta receptors and respond to beta agonists by increased surfactant secretion. Theoretically, if maternal administration of beta agonists to stop preterm labor caused the release of surfactant stores that were then lost into the amniotic fluid, the fetus may be made surfactant-deficient. However, the release of stored surfactant to the airways may facilitate the initiation of air breathing and have a beneficial effect. The clinical importance of the impact of beta agonists on surfactant metabolism is not presented. Purines, such as ATP, are more potent stimulators of surfactant secretion than beta agonists and may be important for surfactant secretion at birth. Surfactant secretion also occurs with mechanical stimuli, such as lung distention and hyperventilation. The surfactant secretion that occurs with the initiation of ventilation following birth probably results from multiple stimuli, such as the combined effects of elevated catecholamine and lung expansion (1).

The surfactant synthesis starts in relatively late pregnancy, between the 20–25th week, and after the 33rd week it even becomes capable of making the alveoli stable. The surfactant synthesis occurs at the smooth and the rough endoplasmatic reticulum of the type-II cells that begin to differentiate between the 20th and 24th gestational week. Differentiation of the type-II cells requires close contact with the lung fibroblasts, which alternately produce the peptides assisting the synthesis of the surface active material.

From the previously said, it can be concluded that the lipid and the protein surfactant fraction is synthesized by the type-II pneumocytes and stored in the intercellular organelles called «lamellar bodies» (LB). The secretion of these structures is enabled by the exocytose, the fusion of their external membrane and the apical cell membrane, and later by the diffusion of their contents into the alveolar space. The surfactant dispersed into the alveolar space has three different cycles: recycling, degradation and elimination. During recycling, the surfactant components are reused by the type-II cells and incorporated again in the lamellar bodies. Alternatively, the surfactant can be disintegrated and its components reused for the synthesis of new lipids and proteins in type-II cells, or eliminated from the system in the form of an intact molecule or a degradational product such as fatty acids (12).

Tests for lung maturity analyses

The need for testing fetal lung maturity is most common in cases when general and gynecologic indications point to preterm birth. The results that prove that the fetal lungs are not mature may result in postponing the delivery, or fast and active intervention in order to prevent preterm delivery. There are many tests for lung maturity analyses by using the amniotic fluid. For example, colored preparations of the amniotic fluid cells (they analyzed skin maturation), amniotic creatinine or osmolarity (possibly test kidney maturation), measuring the optic density on 450 and 650 nm may correlate with the gestational age, but they either do not have a specific use in defining lung maturity, or cannot be used if there is blood or meconium (13).

Antenatal laboratory tests used for fetal lung maturity (FLM) consist of biochemical and biophysical analyses of the amniotic fluid in the presence of surfactant components that are formed in the process of lung maturation. These tests are designed in order to define the specific component in the surfactant connected with the phospholipids (biochemical approach), or to measure the surface active effects of these pulmonary surfactant components on the sample of the amniotic fluid (biophysical tests) (12). In the last few years, a huge number of FLM methods have been developed, although most of them are not accepted as methods for routine work.

Determination of the lecithin/sphingomyelin ratio (LSR)

Gluck and Kulovich (6) were the first to correlate the relative concentrations of lecithin and sphingomyelin in amniotic fluid to the functional status of the fetal lung. The LSR was the first laboratory test designed to assess fetal lung maturity directly, and, largely because of this historical fact, it has come to be considered by many as the standard test for fetal lung maturity (12, 14–16).

The concentration of phosphatidylycholine in the amniotic fluid can be measured directly and correlated with fetal lung maturity, or its concentration can be connected to another lipid – sphingomyelin, and this relation correlates to lung maturity. Sphingomyelin has surface active properties and was found in lungs, but, taking into account its wide ubiquity, it is distributed into the cell membrane and plasma. It is believed that sphingomyelin is not important as a component of surfactant in the lungs; however, it is used as a proper marker in regard to which lecithin is measured (2).

Before 34 weeks of gestation, lecithin and sphingomyelin are present in the amniotic fluid in approximately equal amounts, but at about 34 weeks the concentration of lecithin starts to increase rapidly
in comparison with sphingomyelin. When the concentration of lecithin in the amniotic fluid reaches at least a twice higher value when compared to the value of sphingomyelin, the probability that respiratory distress will appear after delivery is minimal. During recent years, many modifications of the original procedure have been proposed in order to eliminate a number of observed practical and analytical deficiencies. But, these activities caused the development of many unusual chromatographic methods for determining the ratio of lecithin-to-sphingomyelin in the amniotic fluid, and each of these methods may give significantly different LSR values. So far, there is no standard method for the LSR, and many problems still exist, including poor interlaboratory and intralaboratory reproducibility and excessive analysis time (12).

Due to the belief that the LSR method is less successful in showing fetal lung maturity in diabetic pregnancies, tests for other surface active lipids or surfactant proteins have been developed to be used together with the LSR, or as independent tests (16, 17).

**Determination of phosphatidylglycerol (PG)**

Due to the contribution of phosphatidylglycerol (PG) to the functional properties of the surfactant, tests for this phospholipid became popular as an adjunct to the LSR. Functional lung maturity is clearly associated with measurable quantities of PG, however, the lack of PG does not necessarily mean that RDS is inevitable. Common experience with the PG test indicates that the predictive value of the presence of PG is nearly 100% for lung maturity, whereas the predictive value of the absence of PG in predicting RDS may be so low as to be really insignificant. Because of the appearance of very small concentrations of PG in the blood, measurement of PG is particularly valuable at times when fetal lung status must be determined from a sample of the amniotic fluid contaminated either by blood or meconium. The measurement of PG is also important in the evaluation of the maturity of fetuses of diabetic mothers, because the LSR and other FLM tests are less reliable in these cases (8, 18, 20).

**Fluorescence polarization assays**

The level of proteins in amniotic fluid does not depend on the function of lungs and remains relatively constant during later months of pregnancy. Parameters that explain lung maturity are observed as the ratio of the amount of surfactant compared to other components of the amniotic fluid. TDx Fetal Lung Maturity Assay uses the technology of fluorescence polarization and measures the ratio of the surfactant compared to albumin, and the result is shown as a surfactant/albumin ratio. The FLM II test implies fluorescent dye being added to the sample. This dye mediates between albumin and the aggregates formed by the surfactant. Dyed molecules linked to albumin are restricted in movement and are exposed to a polar environment. Consequently, the fluorescence lifetime is decreased and a high level of polarization is achieved. When linked to the surfactant, the dye is in a much lower polarity in the middle, fluorescence lasts much longer and, therefore, polarization is lower. The total fluorescence polarization measured on a sample reflects the distribution of the dye between the protein and surfactant components of the amniotic fluid, and is used as a way to determine the ratio of surfactant/albumin in the sample. The precise relationship between the surfactant/albumin ratio and measured fluorescence polarization is founded on the basis of the standard curve. Values of the surfactant/albumin ratio highly correlate with lung maturity.

The expected values obtained by the FLM II test are: for immature lungs – lower or equal to 33 mg/g, and for mature lungs – 55 mg/g or higher. Results in the range between 40 mg/g and 54 mg/g cannot be specified either as mature or immature, and must be interpreted separately (20–22).

**Concentration of lamellar bodies**

Pulmonary surfactant is mainly composed of surface active phospholipids. It is synthesized in the alveolar type-II pneumocyte and packed as lamellar bodies (also called tubular myelin figures) that reach 1–5 μm in diameter. Lamellar bodies (structural form of pulmonary surfactant) first occur in the cytoplasm of fetal pneumocytes between 20 and 24 weeks of gestation. Before delivery, surfactant is diffused through the bronchial tree into the amniotic fluid, where it can be measured. Phospholipidic content and the laminated structure of these particles change with fetal lung maturity. By determining the number and size of lamellar bodies distributed in the amniotic fluid, fetal lung maturity can be predicted.

Many standard hematologic instruments can measure lamellar bodies on the platelet channel. Most platelet channels measure all particles between 2 and 20 fl in volume (MPV), and most lamellar bodies are in this range (2, 19–21).

The expected values of lamellar bodies for mature lungs are $50 \times 10^9/L$ or higher, and for immature lungs lower than $14 \times 10^9/L$. Results in the range between $15 \times 10^9/L$ and $50 \times 10^9/L$ denote transitory lungs (2, 23–25).

**Material and Methods**

Examination of fetal lung maturity was made in specimens of the amniotic fluid in 90 pregnant wo-
men with the gestation age from 32 to 40 weeks. Multiple pregnancies were not included in this examination. Average age of the pregnant women was 28 years. Pregnant women were hospitalized in the Clinic of Gynecology and Obstetrics, Clinical Center of Montenegro in Podgorica. As indicators of fetal lung maturity, we determined the lamellar body count and surfactant-to-albumin ratio (S/A). The LB count was determined by a hematological counter Cell Dyn 3700 (Abbott), on the platelet channel. We used centrifuged samples of the amniotic fluid, without blood, meconium, and increased number of leukocytes or vaginal secretions. Surfactant/albumin ratio was determined by the Fetal Lung Maturity II (FLM) test. The measurement was performed on the TDX instrument (Abbott). Samples of the amniotic fluid were filtered by special filters during the sampling of amniotic fluid.

The following statistic methods were performed: average value, standard deviation, variation coefficient, correlation between LB and gestation age, correlation between S/A and gestation age, correlation between two dependencies, specificity and sensitivity of methods, predictive values for LB and S/A.

**Results**

The results of examining LB and S/A are presented in Table I and Table II.

It was determined that there is a high degree of positive correlation between LB and gestational age $r_1=0.934$, as well as between S/A and gestational age $r_2=0.373$, with high statistic significance in both cases ($p<0.001$, $p<0.005$, respectively). Ratio of LB count is higher when compared to gestational age, which is confirmed by the statistically significant difference between these relations $r_1-r_2=0.934-0.373=0.561$, $t=6.45$, $p<0.001$. The two tests correlated with each other $r=0.341$, $p<0.005$.

LB count greater than $34.0 \times 10^9$/L predicts the absence of respiratory distress with a probability of 86% (specificity). LB count of less than $34.0 \times 10^9$/L predicts respiratory distress with a probability of 93% (sensitivity). Six of 90 babies (6.7%) developed respiratory distress and had LB counts from 6.2 to 33.4 $\times 10^9$/L. Six of 90 babies (6.7%) did not develop RDS and had LB counts of 4.2 to 24.2 $\times 10^9$/L. One baby that developed RDS had an LB count of 65.1 $\times 10^9$/L.

Surfactant/albumin ratio greater than 39.00 mg/g predicts the absence of respiratory distress with a probability of 86% (specificity). Surfactant/albumin ratio of less than 39.00 mg/g predicts respiratory distress with a probability of 92% (sensitivity). Six of 90 babies (6.7%) developed respiratory distress and had surfactant/albumin ratio from 6.96 to 29.39 mg/g. Seven of 90 babies (7.8%) did not develop RDS and had surfactant/albumin ratio from 23.94 to 36.55 mg/g. One baby that developed RDS had surfactant/albumin ratio of 46.43 mg/g.

**Discussion**

Biochemical or biophysical procedures for FLM evaluation can provide important clinical information. The L/S ratio and other tests of lung maturation are based on the fact that the amniotic fluid exactly reflects the degree of differentiation of the type-II cells in the development of alveoli of the fetal lung. When complexities of the pathway from fetal surfactant synthesis to its appearance in the amniotic fluid are taken into account, as well as the factors influencing it, it is surprising that these tests of lung maturation detect complications during pregnancy (26). Synthesis of amniotic fluid phospholipids in type-II cell differs in both time and metabolic pathway.

Many tests used for examination of surfactant components in amniotic fluid have been proposed to both simplify and improve the predictability of the tests (13). The commercially available Abbott FLM test measures the amount of surfactant via a fluorescent probe that changes properties in interaction with the lipids. The change in fluorescence depolarization is measured in relation to the albumin content of the amniotic fluid. This test is simple for the clinical laboratory to perform using either a kit or commercially available reagents, and the measurement is reproducible (25). The assay is also comparable to the L/S ratio in predictive value based on relative operating characteristic curves (27). A problem with comparative studies of lung maturity tests is the low number

<table>
<thead>
<tr>
<th>Value</th>
<th>Lamellar bodies ($n \times 10^9$/L)</th>
<th>Surfactant/albumin (mg/g)</th>
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</thead>
<tbody>
<tr>
<td>Medium</td>
<td>69.4</td>
<td>55.11</td>
</tr>
<tr>
<td>Lowest</td>
<td>4.2</td>
<td>6.96</td>
</tr>
<tr>
<td>Highest</td>
<td>199.0</td>
<td>112.00</td>
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<tr>
<td>CV%</td>
<td>65%</td>
<td>80%</td>
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<tr>
<th>Value</th>
<th>Lamellar bodies</th>
<th>Surfactant/albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>86%</td>
<td>86%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93%</td>
<td>92%</td>
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<tr>
<td>PV (+)</td>
<td>99%</td>
<td>99%</td>
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<tr>
<td>PV (-)</td>
<td>50%</td>
<td>47%</td>
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of infants who went on to have RDS, making it difficult to estimate the positive predictive value of the tests (21).

A recent, rapid test that does not require any reagents is the determination of the number of lamellar bodies in amniotic fluid using a hematological counter. Lamellar bodies can be measured on almost all hematological counters on the platelet channel. Lamellar body counts correlate well with other tests used for fetal lung maturity (28).

Our results demonstrate a high level of positive correlation between LB count and the gestational age (r=0.934), as well as surfactant/albumin ratio and gestation age (r=0.373), showing high statistic importance.

A group of scientists also obtained similar results concerning the correlation of LB counts, lecithin/sphingomyelin ratio and PG value with gestation age (29–35). There is no need to make phospholipid analyses if LB count is higher than $30 \times 10^9/L$ or lower than $10 \times 10^9/L$ (36), i.e. if it is higher than $32 \times 10^9/L$ and lower than $8 \times 10^9/L$ (36).

Similar to most FLM tests, the clinical predictability of LSR varies widely. Sensitivity and specificity for these tests range between 80% and 85% (37). This variability is likely to result from poor analytical standardization, differences in study populations, lack of essential facts in RDS diagnosis and the use of different reference values. Our research demonstrates that tests for the determination of LB and S/A have specificity of 86% and sensitivity of 93% for LB and 92% for S/A, which is in accordance with the literature (30, 34, 36, 37).

Our results, and comparable studies of fetal lung maturity, force upon us the conclusion that although lecithin/sphingomyelin ratio is considered the gold standard, rapid tests, such as TDX/FLM or lamellar body counts in amniotic fluid, show high levels of comparability with L/S and are simple to use in routine work.
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