“CAROL DAVILA” UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST
DOCTORAL SCHOOL - FIELD OF MEDICINE

DOCTORAL THESIS - SUMMARY

Scientific adviser:
PROF. UNIV. DR. PELTECU GHEORGHE

PhD student:
OPRICAN (CĂS CHIRCULESCU) RALUCA

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Morphopathological and immunohistochemical evaluation of the pulmonary system in preterm neonates - a comprehensive exploration of the degree of pulmonary maturation.

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I. The central issue

Premature birth refers to the delivery of an infant prior to the completion of 37 weeks of gestation and stands as the foremost contributor to infant mortality on a global scale [1]. Although a variety of strategies have been employed over time to mitigate the incidence of premature births, minimal progress has been achieved in this area, and the prevalence of such births has remained relatively stable over the years. In the United States, the preterm birth rate for 2023 was registered at 10.41%, a percentage that is almost identical to the 10.38% noted in 2022 [2] It is estimated that approximately 15 million premature births occur worldwide each year [1]. This incidence is markedly elevated in socioeconomically disadvantaged regions and among specific ethnic groups, such as Black and Asian populations, with the incidence reported at 8.6 per 100 births within these demographics [3]. Furthermore, numerous countries have documented a rise in the incidence of premature births. This phenomenon can be attributed, on one hand, to the increasing prevalence of multiple pregnancies resulting from fertility treatments, and on the other hand, to the growing number of women opting to bear children at advanced ages [4].

Premature delivery can occur either spontaneously or be induced through elective/iatrogenic methods, carried out at the discretion of the obstetrician in the interest of maternal or fetal health [5, 6]. Iatrogenic preterm birth may be achieved either through cesarean section or the induction of labor. A significant proportion (30-40%) of all preterm deliveries is attributed to iatrogenic causes, primarily arising from conditions such as preeclampsia/eclampsia and significant intrauterine growth restriction (IUGR) [7]. The prevalence of iatrogenic preterm births has escalated in recent decades, and in certain countries, it has become the foremost contributor to preterm deliveries, constituting almost 50% of all instances [8]. Among spontaneous preterm deliveries, a significant percentage (40-45%) results from unknown, idiopathic causes, whereas 25-30% correlates with cervical dilation and premature rupture of membranes (PROM) [9]. Premature onset of labor may arise from either infectious or inflammatory processes, or it may stem from uteroplacental ischemia or hemorrhage [5].

The World Health Organization (WHO) categorizes premature infants based on their gestational age at birth as follows [1, 10]:

- extremely preterm - less than 28 weeks of gestation;

- very preterm - between 28 0/7 and 31 6/7 weeks of gestation;
- moderately and late preterm - between 32 0/7 and 36 6/7 weeks of gestation.

In Romania, in accordance with Order No. 1241 issued by the Ministry of Health on 09.08.2019 and published in the Official Monitor of Romania, Part I, no. 738 on 10 September 2019, the threshold for viability at birth has been established at 24 weeks of gestation [11]. In recent years, the survival rate of preterm neonates has significantly improved [12]; however, neonatal morbidity and mortality, primarily attributable to pulmonary immaturity, cerebral intraventricular hemorrhage, and infections, have become increasingly pronounced as the gestational age at birth diminishes [13].

Considering the unique trait of term newborns' lungs to undergo postnatal maturation extending into the young adult period, this research is particularly crucial, as it aims to investigate whether this maturation similarly occurs in premature infants. Most studies in the literature have been conducted on animal models such as murine specimens, lambs, baboons, or even various cell cultures, with investigations involving living or deceased human subjects being exceedingly rare and restricted to a limited cohort of individuals.

Premature delivery, notably when it occurs at an exceedingly early gestational age (below 28 weeks of gestation), is invariably associated with the onset of respiratory distress syndrome (RDS) attributable to surfactant deficiency. A proportion of these premature infants will develop chronic lung disease, with bronchopulmonary dysplasia representing the condition that exerts the most significant socioeconomic impact. This affliction was delineated in the literature over five decades ago, and despite notable advancements in medical care for these vulnerable infants in recent years, its prevalence continues to remain alarmingly high.

This study initially assessed the morphological development of pulmonary tissue in relation to gestational age at birth, followed by the assessment of histopathological modifications that occurred with an increased lifespan or duration of oxygen supplementation. A variety of antenatal factors acknowledged for their role in promoting lung maturation were also thoroughly investigated. As postnatal lung maturation signifies not solely the escalation in the count of alveolar structures, but also the development and quantitative expansion of septal capillaries, the subsequent segment of our study scrutinized the complexities of pulmonary microvascularization concerning antenatal and postnatal risk factors associated with the emergence of bronchopulmonary dysplasia. Since the progression of lung development cannot be fully conveyed solely through a structural

examination of lung tissue, the third component of this research sought to analyze the functional growth of lung tissue by objectively measuring the presence of surfactant and Napsin A within type II pneumocytes.

All these objectives were tested on lung tissue obtained from necropsy of 67 premature newborns with gestational ages ranging from 23 to 35 weeks of gestation, who survived between 1 and 149 days. Microscopic examination of lung tissue was performed utilizing standard hematoxylin-eosin staining in conjunction with a suite of immunohistochemical markers, which elucidated the presence of endothelial cells (CD34), the media of pulmonary arterioles (SMA), as well as surfactant and Napsin A from type II pneumocytes. The results were interpreted through a careful examination of micrographs at magnifications of 200x and 400x.

Morphological assessment of pulmonary tissue revealed that prolonged oxygen therapy precipitates a decline in the density of alveoli per square millimeter, irrespective of the gestational age at birth; a similar observation was noted in neonates exhibiting lobar structural anomalies. Infants born with extreme prematurity demonstrated a reduced alveolar diameter in comparison to their counterparts born at more advanced gestational ages. Furthermore, neonates who developed bronchopulmonary dysplasia exhibited a significant thickening of the alveolar septa relative to other premature infants.

Following the assessment of pulmonary microvascularization, a negative correlation was discerned between the quantity of CD34 positive capillaries and the duration of oxygen therapy administered. Furthermore, it was noted that all premature neonates, irrespective of gestational age or chronological age, exhibited the immature morphology characteristic of a dual capillary network pertinent to the canalicular and saccular stages of pulmonary development. Premature infants who subsequently developed pulmonary fibrosis also demonstrated a diminished presence of septal capillaries.

Assessment of pulmonary functional maturity revealed the presence of surfactant within type II pneumocytes, irrespective of gestational age or lifespan. The intensity of Napsin A expression demonstrated a significant correlation with gestational age, duration of mechanical ventilation, administration of antenatal corticosteroids, and the occurrence of maternal antenatal infections.

To undertake this study, it was essential to integrate maternal obstetric clinical data, neonatal clinical data, and the morphopathological and immunohistochemical features of the lung specimens collected during the necropsy examination.

II. The essential aims of the study and the primary objectives of the research.

Fetal lung development is a sophisticated process encompassing a series of interconnected phases and stages, ultimately resulting in the formation of a fully operational respiratory system by the end of the gestational period. Although the term newborn possesses the capacity to adapt to the extrauterine environment, the lungs represent a remarkably complex organ that continues to evolve postnatally. This postnatal progression is characterized by an increase in the number of alveoli, alongside a comprehensive reorganization and maturation of the pulmonary vascular architecture [14, 15].During fetal life, the lungs do not fulfill their vital role as organs responsible for gas exchange; this critically important function is instead assumed by the placenta [16]. Due to the fact that the lungs do not necessitate increased blood flow until the onset of birth, the fetal organism possesses the remarkable capacity to reroute blood circulation through the foramen ovale, the ductus arteriosus, and the ductus venosus, thereby preventing an excessive influx of blood to the lungs. This distinctive adaptation results in the fetal pulmonary vascular network being characterized by diminished blood flow and heightened vascular resistance [17, 18]. With the clamping of the umbilical cord, systemic arterial pressure initiates its ascent, while the initially elevated pulmonary arterial pressure begins to diminish, concurrently accompanied by an increase in blood flow to the lungs [18]. This phenomenon occurs as a result of air infiltrating the neonate's lungs, which triggers pulmonary vasodilation and a decline in pulmonary vascular resistance [17]. This transition from intrauterine to extrauterine existence, as instinctual as it may be for the term newborn, becomes markedly more challenging for the premature infant due to physiological and metabolic immaturity. This circumstance precipitates an enhanced requirement for neonatal resuscitation interventions [19].

As advancements in neonatal medicine have progressed, the life expectancy for infants born at extremely premature gestational ages of 24 to 28 weeks has significantly improved. Nevertheless, despite this increase in survival rates among these vulnerable patients, a considerable proportion will develop chronic lung disease, a condition precipitated by inadequate lung maturation and exacerbated by the necessity for invasive medical interventions to sustain respiratory function [20, 21].

1.1 Assumptions

As a result, the present research endeavors to:

- assess the impact of the external environment on the underdeveloped pulmonary tissue of premature neonates.
- investigate the trajectory of pulmonary microvascularization and the extent to which antenatal and postnatal factors may influence this phenomenon.
- examine the degree of pulmonary functional maturation, aiming to determine whether the underdeveloped lung continues its evolution postnatally.
- analyze the presence of pulmonary inflammation.

1.2 Aims

- Determination of the alveolar density (number of alveoli/mm^2) within the pulmonary tissue of premature neonates, utilizing standard hematoxylin-eosin staining, and its assessment in relation to various antenatal and postnatal factors.
- Assessment of the maximal thickness of the alveolar septum, employing standard hematoxylin-eosin staining, and its appraisal concerning diverse antenatal and postnatal factors.
- Appraisal of the minimal thickness of the alveolar septum, utilizing standard hematoxylin-eosin staining, and its evaluation in relation to a range of antenatal and postnatal factors.
- Examination of the maximum alveolar diameter, using standard hematoxylin-eosin staining, alongside its evaluation with respect to various antenatal and postnatal factors.
- Examination of the minimum alveolar diameter, employing standard hematoxylin-eosin staining, and its assessment in relation to various antenatal and postnatal factors.
- Evaluation of arteriolar wall thickness, utilizing standard hematoxylin-eosin staining, and its analysis concerning various antenatal and postnatal factors.
- Evaluation of venular wall thickness, employing standard hematoxylin-eosin staining, and its assessment in relation to various antenatal and postnatal factors.
- Evaluation of the minimal quantity of alveolar capillaries through the implementation of the immunohistochemical marker for endothelial cells, CD34, alongside its examination in relation to various antenatal and postnatal determinants.
- Evaluation of the maximal quantity of alveolar capillaries employing the immunohistochemical marker for endothelial cells, CD34, in conjunction with its scrutiny concerning various antenatal and postnatal determinants.

- Evaluation of the mean quantity of alveolar capillaries—calculated as the average of five measurements at the level of alveolar structures with diameters ranging from 60.0 μm to 120.0 μm (+/- 5 μm)—through the utilization of the immunohistochemical marker for endothelial cells, CD34, along with its analysis in relation to various antenatal and postnatal determinants.
- Evaluation of the thickness of the arteriolar media through its delineation via the immunohistochemical marker for smooth muscle, SMA (Smooth Muscle Actin), coupled with its examination in relation to various antenatal and postnatal determinants.
- Assessment of pulmonary functionality through the examination of the immunohistochemical marker surfactant, localized within type II pneumocytes, and its analysis in relation to various antenatal and postnatal determinants.
- Assessment of pulmonary functionality through the examination of the immunohistochemical marker Napsin A, situated within type II pneumocytes, and its analysis in relation to various antenatal and postnatal determinants.

III. General research methodology

Subjects

The research encompassed a cohort of 67 deceased premature neonates, selected from the database of the Pathology Laboratory at two distinguished maternity hospitals in Bucharest. Within the entire study population, 39 cases were sourced from the National Institute for Maternal and Child Health "Alessandrescu-Rusescu" during the interval from January 2018 to December 2020, while the remaining 28 cases were procured from the Filantropia Clinical Hospital between January 2017 and December 2024. This investigation was conducted subsequent to the acquisition of favorable approvals from the Ethics Committees of both medical institutions, bearing the registration numbers 21875/02.12.2020 and 12407/19.12.2024, respectively.

The selection of subjects was based on specified inclusion and exclusion criteria. Recognizing the well-established premise that neonatal lung development continues postnatally until the individual attains young adulthood, we sought to determine whether this paradigm is equally applicable to premature infants. Consequently, we established two principal parameters as inclusion criteria: a gestational age at birth of less than 37 weeks, indicative of pulmonary immaturity, and a minimum lifespan of 24 hours, thereby allowing external environmental factors to exert their influence on the morphological characteristics of lung tissue.

Subjects characterized by:

- a gestational age of 37 weeks or greater;
- lifespan less than 24 hours;
- pulmonary hypoplasia;
- diaphragmatic hernia leading to pulmonary hypoplasia were excluded from the study.

Given that the subject selection period coincided with the COVID-19 pandemic, it is crucial to emphasize that none of the newborns included in the study, nor their mothers, exhibited any clinical or laboratory indicators of COVID-19 infection.

All medical information was obtained from digital archives, neonatal and maternal medical records, and necropsy reports from both medical institutions. Of all the parameters delineated in this research, the Apgar score necessitates further elucidation. This score serves as an immediate evaluation of the neonate, assessing five critical parameters: respiration, heart rate, skin color, muscle tone, and reflexes. Each parameter is assigned a

score ranging from 0 to 2 points. The assessment is conducted at one and five minutes post-delivery, and should the score fall below 7, the evaluation must be repeated after an additional five minutes [22]. Given that the determination of the 10-minute Apgar score was not accessible for all study participants, we were unable to conduct an assessment of this score as well.

Another crucial parameter for this study, which could not be documented in the database due to the absence of pertinent information in the medical records, was maternal smoking; thus, this study could not provide an assessment of the intrauterine exposure of the fetus to cigarette smoke.

2.2 Characteristics of the examined cohort

After conducting a comprehensive analysis of the 67 cases encompassed in the study, we discerned that 55.2% of the cases involved male preterm infants, while 44.8% pertained to female preterm infants, with a median gestational age at birth of 28 weeks (ranging from 23 to 35 weeks). Upon stratifying the subjects into four distinct age cohorts based on gender, we noted a predominance of females in the 24-27 weeks gestational age group, contrasted with a male predominance in the 28-31 weeks gestational age group. In the 32-33 weeks gestational age group, the distribution between genders was relatively balanced. Notably, the 32-33 weeks and 34-35 weeks gestational age groups exhibited the smallest number of subjects, with only five individuals represented in each cohort.

After evaluating birth and death weights, we discerned that the mean birth weight was 1116.34 grams (ranging from 250 to 3850 grams), whereas the mean death weight was 1409.91 grams. A meticulous analysis of the extreme birth weights reveals that both the minimum and maximum weights are atypical for a premature newborn, necessitating a thorough individual assessment. The premature infant weighing 250 grams was the second fetus in a twin gestation at 25 weeks of gestation, born to a mother with gestational hypertension and thrombophilia, and exhibited severe intrauterine growth restriction (IUGR), attributable to pregnancy-induced hypertension. Conversely, the premature newborn with a birth weight of 3650 grams originated from a singleton pregnancy at 34 weeks of gestation and was diagnosed with non-immune fetal hydrops, thereby elucidating the anomalously high weight for a premature infant.

Of the entire cohort examined, 20.9% (n = 14) of newborns exhibited intrauterine growth restriction. Among these cases, 11 (78.57%) were born to mothers who

experienced pregnancy-induced hypertension, with 4 of these (28.57%) subsequently developing preeclampsia.

A closer examination of the mode of delivery reveals that a significant majority of subjects (62.7%) were delivered via cesarean section, while cephalic presentation emerged as the predominant fetal position at birth (68.65%), in stark contrast to the least common presentation, which was transverse (8.95%).

When we meticulously examined survival rates across diverse gestational age cohorts at birth, it became apparent that the highest proportion of preterm infants survived for a duration of 1 to 3 days (n = 22), predominantly within the 24 to 27 weeks gestational age range. Preterm infants who endured for 4 to 10 days, as well as those who persisted for 11 to 20 days, demonstrated relatively comparable case numbers (n = 13 vs. n = 15).

If we scrutinize the gestational age categories at birth and assess the maternal characteristics associated with gestational hypertension and antenatal maternal infections, we will discern that within the 24-27 weeks gestation cohort, 15.15% (n = 5) of the mothers of these neonates exhibited pregnancy-induced hypertension, with 40% (n = 2) of these cases advancing to preeclampsia. Conversely, in the 28-31 weeks gestational age group, 33.33% (n = 8) of the mothers of these infants developed pregnancy-induced hypertension, and 37.5% (n = 3) of these progressed to preeclampsia.

When assessing the prevalence of maternal infections within the two most representative gestational age cohorts of our study, it becomes apparent that maternal infections were observed with greater frequency in the 24-27 weeks gestational age group, which corresponds to the second trimester of pregnancy. Within this cohort, 42.42% (n = 14) of the mothers of these neonates experienced infections during gestation, with a significant 87.71% (n = 12) of these cases involving premature rupture of membranes, occurring with a mean interval of 140 hours prior to delivery. Conversely, in the 28-31 weeks gestational age group, indicative of the third trimester of pregnancy, only 29.16% (n = 7) of the mothers encountered infections during their pregnancies. Furthermore, 71.42% (n = 5) of these mothers experienced premature rupture of membranes, with an average duration of 44.6 hours preceding birth.

When we evaluated the subjects encompassed in the study regarding life expectancy and Apgar scores at birth, which we stratified into three distinct categories (Apgar score less than 3, Apgar score between 4-6, and Apgar score exceeding 7), we discerned that a significant proportion of these individuals (n = 35) exhibited an Apgar

score of less than 3 at one minute, with nearly half succumbing within the initial three days of life.

The assessment of the Apgar score at five minutes revealed a notable upward trajectory, as the majority of newborns were classified within the Apgar score category of 4-6 points after this interval (n = 29). Furthermore, we observed that 25.37% (n = 17) of the newborns in the entire study cohort survived beyond 21 days.

Due to the lack of a documented 10-minute Apgar score assessment in the medical records of all study participants, we were unable to carry out this evaluation.

If we evaluate one of the most significant interventions administered to these neonates, specifically oxygen therapy, it becomes apparent that all premature infants included in the study (100%) benefitted from some form of oxygen treatment. Furthermore, an overwhelming 97% (n = 65) of these preterm neonates necessitated invasive modalities of oxygen administration, such as intermittent positive pressure ventilation (IPPV), synchronized intermittent mandatory ventilation (SIMV), and high-frequency oscillatory ventilation (HFOV), while merely 3% (n = 2) required non-invasive oxygen treatment methods, including continuous positive airway pressure (CPAP), nasal high-flow oxygen ventilation (NHFOV), and heated, humidified high-flow nasal cannula (HHHFNC). The duration of oxygen therapy exhibited considerable variability, ranging from 1 to 131 days, and was intrinsically correlated with the survival duration of these infants, which spanned from 1 to 149 days.

Upon analyzing the maternal characteristics of the premature infants included in this study, it is apparent that 17.91% (n = 12) of them belong to age extremes, with 3 born to mothers under the age of 18, and 9 born to mothers over 40 years old. The majority of these pregnancies were singleton, whereas 22.38% (n = 15) involved multiple births, with 2 cases resulting from in vitro fertilization.

In analyzing the incidence of previous pregnancies, it becomes apparent that a significant fraction of these mothers were primiparous (53.73%), while only 22.38% had experienced three or more pregnancies. It is important to note that among the group of multiparous mothers, two had a history of stillbirth, and one mother disclosed an extraordinary total of 20 pregnancies, with 19 resulting in miscarriage. Within the entire sample investigated, 15 mothers displayed pregnancy-induced hypertension, and fewer than half of this subset (40%) proceeded to develop preeclampsia.

An essential determinant of fetal lung maturation is the antenatal administration of corticosteroids. This intervention is conducted at the discretion of the obstetrician for

pregnant women at a gestational age ranging from 24 weeks to 33 weeks and 6 days, in whom the impending birth is anticipated within the next 7 days. Notably, among the entire cohort examined, only 5 mothers delivered at a gestational age exceeding 34 completed weeks, and they had no indication for antenatal corticosteroid therapy; nevertheless, over half of them (56.71%) did not receive antenatal steroid treatment.

IV. Special Section - Synopsis of Chapters

Chapter 7 is devoted to the inaugural retrospective cross-sectional study entitled "*The impact of external environment on pulmonary development - A morphological evaluation of pulmonary tissue in preterm infants*" [63]. This chapter aims to evaluate the morphological characteristics of immature lung tissue subsequent to its exposure to environmental influences and neonatal therapeutic interventions. In *subchapter 7.1*, we clarify the decisive moment when the survival of a premature newborn becomes attainable, while also addressing a multitude of questions that we sought to explore through this study: What happens to lung development when it is disrupted by premature birth? Is there a possibility for it to resume its natural pathway? In what ways do external factors impact the eventual growth and maturation of the pulmonary system?

Subchapter 7.3 elucidates the findings derived from the statistical analysis, revealing the detrimental impact of oxygen therapy on the morphological characteristics of lung tissue. Specifically, a significant negative correlation was identified between the duration of oxygen treatment and the density of alveoli per square millimeter ($p<0.001$; $r = 0.92$) (Figure 1). Furthermore, a notable reduction in the number of alveoli per square millimeter was observed in the cohort of neonates exhibiting lobar lung anomalies, in contrast to their counterparts who did not present such malformative alterations (mean difference of -4.60; 95% CI: -7.56 to -1.65; $p=0.003$).

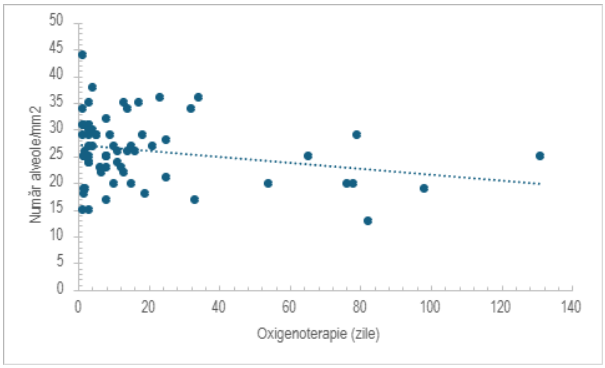

Figure 1. Prolonged effect of oxygen therapy on the density of alveoli per square millimeter.

Also in this subchapter, we elucidated that extremely preterm neonates (those delivered prior to 28 weeks of gestation) exhibited a significantly diminished alveolar diameter ($p = 0.006$) in comparison to their more mature premature counterparts (Figure 2). Additionally, it was noted that premature neonates who later developed pulmonary fibrosis exhibited a

remarkable increase in alveolar septal thickness (p = 0.019) when compared to other premature infants.

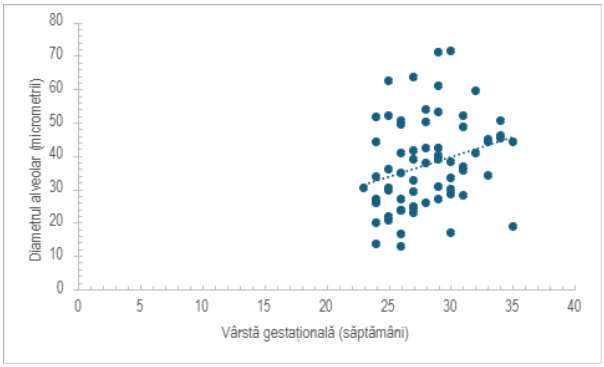

Figure 2. Alveolar diameter in relation to gestational age at birth.

In *subchapter 7.4*, we diligently analyzed the identified findings and compared them with the established data available in the literature.

Chapter 8 is devoted to the second retrospective cross-sectional study entitled "*The Impact of Antenatal and Postnatal Factors on the Development of the Pulmonary Microvasculature in Preterm Infants*" [24]. The primary aim of this chapter is to investigate the effects of premature birth on septal capillary density, alongside assessing various risk factors connected to the emergence of bronchopulmonary dysplasia, which may influence the later development of pulmonary microvascularization. A secondary aspect of this study involved measuring the media thickness of pulmonary arterioles to determine if these newborns demonstrate heightened vascular tone and an increased susceptibility to developing pulmonary hypertension. In *subchapter 8.3*, the statistical findings elucidating the impact of antenatal factors—such as pregnancy-induced hypertension, administration of antenatal corticosteroids, and maternal infections—alongside postnatal risk factors, including incomplete pulmonary development, oxygen therapy, and various neonatal interventions, on pulmonary vascular remodeling in premature neonates are presented in meticulous detail.

A histopathological change that readily captures attention is the dual network of septal capillaries, observable in both premature infants afflicted by pulmonary fibrosis and those experiencing alveolar simplification. Furthermore, within the cohort of premature infants who developed pulmonary fibrosis, there was a notable reduction of 5.43 CD34-positive septal capillaries when contrasted with infants whose primary morphological alteration was alveolar simplification (95% CI: 3.40-7.44; $p<0.001$).

Along with this change, the present study elucidated the detrimental influence on the mean quantity of CD34 positive septal capillaries and the duration of oxygen therapy (r = -0.31; p<0.001) (Figure 3).

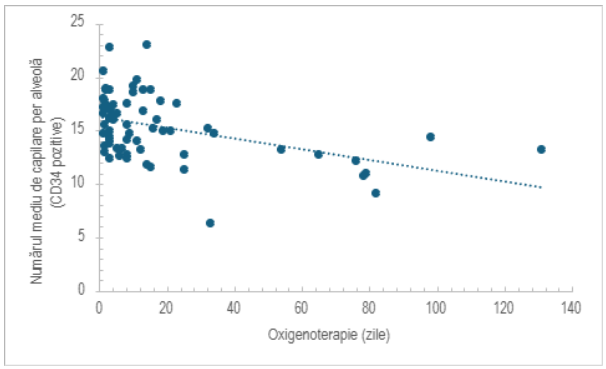

Figure 3. Impact of oxygen therapy on the mean capillary density per alveolus.

The assessment of the average number of CD34-positive septal capillaries in premature infants born to mothers with pregnancy-induced hypertension revealed no statistically significant correlation between these variables (p = 0.70). Conversely, when we examined infants from mothers who experienced preeclampsia, we noted a mean reduction of 2.82 capillaries in this cohort, in contrast to infants born to mothers without such a pregnancy complication (95% CI: 0.32-5.32; p = 0.027).

Also in this study, we elucidated a positive correlation between the duration of oxygen therapy and the media thickness of the pulmonary arterioles (r = 0.20; p = 0.017). Additionally, we observed a similar relationship between the lifespan of these neonates and the media thickness of the pulmonary arterioles (r = 0.22; p = 0.008). A final aspect we underscored in this investigation was a reduction of -5.31 units in the media thickness of the pulmonary arterioles in the cohort of premature newborns exhibiting predominant morphopathological changes characterized by alveolar simplification, in contrast to the group of premature newborns displaying histopathological evidence of pulmonary fibrosis (95%CI: -9.84 — -0.79; p = 0.022).

In *subchapter 8.4*, we thoroughly explored these modifications in detail and compared them with the existing literature, much of which stems from studies conducted on laboratory animals.

In *Chapter 9*, entitled "*Distal pulmonary epithelial maturation in preterm infants: Does the lung of the preterm infant continue its functional pulmonary development postnatally*?" [25], a comprehensive retrospective cross-sectional study was undertaken, with the primary objective of evaluating the presence of surfactant and Napsin A within the

distal lung epithelium. The study aimed to interpret the expression of these markers in relation to various antenatal and postnatal factors, thereby assessing the degree of postnatal functional lung maturation in preterm newborns. A secondary objective of this investigation was to analyze the pulmonary microenvironment through immunohistochemical assessment of B- and T-lineage lymphocyte markers.

Subchapter 9.3 delineated the findings of this investigation, underscoring that all cases encompassed within the study (100%), irrespective of gestational age at birth, exhibited granular cytoplasmic expression of surfactant, with an intensity ranging from 2 to 3, at the level of type II pneumocytes (Figure 4).

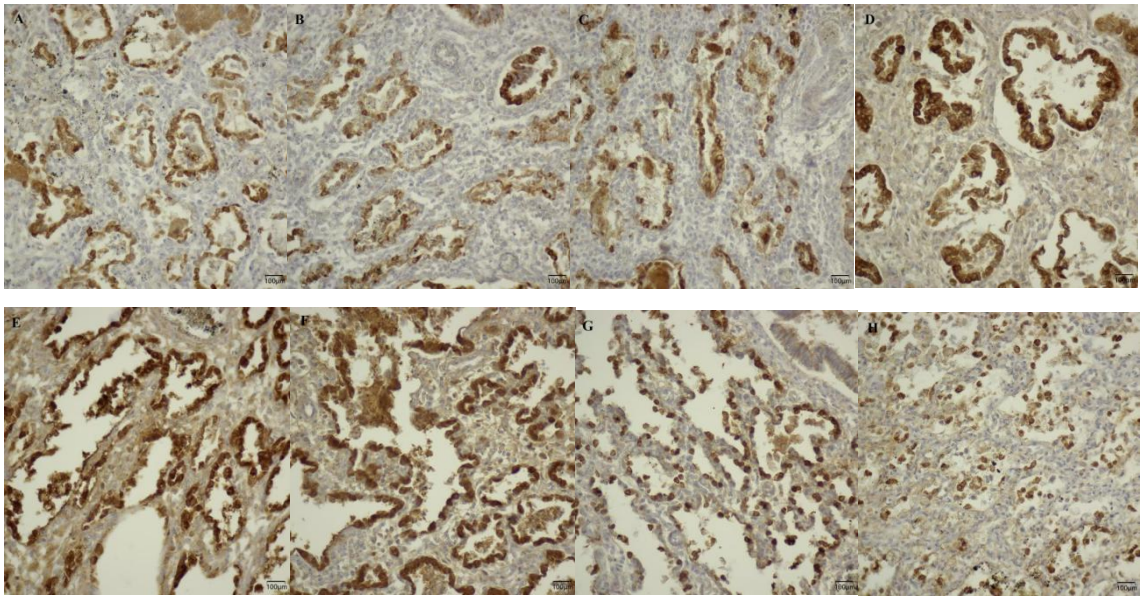

Figure 4. Immunohistochemical illustrations of surfactant, 400x. Lung tissues obtained from neonates with gestational ages ranging from 24 to 29 weeks of gestation.

This study demonstrated that the intensity of Napsin A expression in alveolar macrophages among neonates born to mothers who did not receive antenatal corticosteroid treatment exhibited a mean reduction of -0.466 units, in contrast to the cohort of neonates born to mothers who did receive such intervention (95% CI: -0.91 — -0.14; p = 0.022). Another noteworthy observation concerns the cohort of premature infants delivered by mothers with a prior history of antenatal infections during pregnancy. Within this population, a statistically significant variation of 0.28 units was identified in the intensity of Napsin A expression at the level of the alveolar epithelium, when juxtaposed with the population of infants born to mothers with no recorded history of infections throughout gestation (95% CI: 0.01 - 0.54; p = 0.038). If we consider the primary intervention administered to these

premature neonates, specifically oxygen therapy, an impressive 97% underwent mechanical ventilation throughout their hospitalization. Furthermore, the statistical analysis underscored a significant correlation between the duration of mechanical ventilation and the intensity of Napsin A expression within the alveolar epithelium ($r = 0.30$; $p = 0.002$).

When we assessed the prevalence of lymphocytes within the pulmonary tissue, we discerned a statistically significant disparity of 10 CD20-positive lymphocytes between the cohort of newborns born to mothers who did not experience preeclampsia and the cohort of newborns born to mothers who did develop preeclampsia (95% CI: 0.53-19.42; $p = 0.040$). Furthermore, we identified a positive correlation between the quantity of CD8-positive T lymphocytes in the lung tissue (cytotoxic T lymphocytes) and the duration of oxygen therapy ($r = 0.59$; $p < 0.001$) (Figure 5).

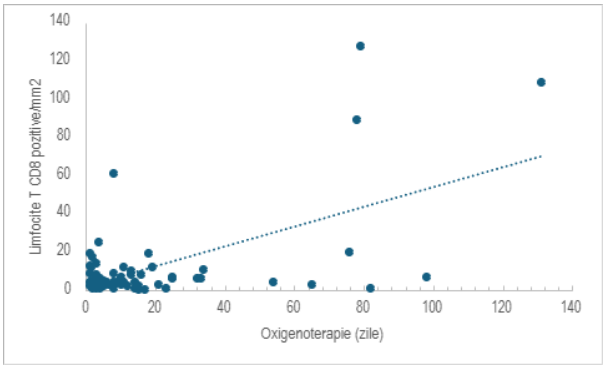

Figure 5. The impact of oxygen therapy on the number of CD8 positive T lymphocytes.

In *subchapter 9.4*, we meticulously examined all these findings, juxtaposing them with extant data in the literature, much of which is derived from investigations conducted on laboratory animals or from research involving cell cultures.

V. Conclusions and Personal Contributions

The manuscript "Morphopathological and immunohistochemical evaluation of the pulmonary system in preterm neonates - a comprehensive exploration of the degree of pulmonary maturation" primarily aims to assess the degree of pulmonary development from three distinct perspectives:

1. The initial approach of this work is elucidated in the first study, wherein the examination and comprehensive analysis were conducted exclusively based on the morphological characteristics of lung tissues derived from deceased premature neonates, underscoring:

- a pronounced negative correlation between the duration of oxygen therapy and the quantity of alveoli reported per square millimeter ($r = 0.92$; $p<0.001$).
- a statistically significant diminution in the number of alveoli per square millimeter within the cohort of premature neonates exhibiting lobar lung anomalies, in contrast to their counterparts devoid of such associated morphological deviations - the mean difference between the two groups was -4.60 alveoli per square millimeter (95% CI: -7.56－-1.65; $p = 0.003$).
- a significantly diminished alveolar diameter in the population of extremely preterm infants, compared to premature neonates who were born at more advanced gestational periods ($p = 0.006$);
- increased thickness of the alveolar septa in the cohort of premature infants who developed pulmonary fibrosis, in contrast to those whose primary histopathological alteration was characterized by alveolar simplification ($p = 0.019$).

2. The subsequent approach delineated in this work is elucidated in the second study, which pertains to the assessment of pulmonary vasculature, with a particular emphasis on the pulmonary microvasculature of premature neonates.

- a negative correlation is evident between the mean quantity of CD34-positive septal capillaries and the duration of oxygen therapy ($r = -0.31$; $p<0.001$).
- a significant reduction of 5.43 CD34-positive septal capillaries was observed in the cohort of premature infants who developed pulmonary fibrosis, in contrast to their counterparts who did not experience this complication (95%CI: 3.40－7.44; $p<0.001$).
- a mean reduction of 2.82 CD34-positive septal capillaries in premature infants born to mothers who experienced preeclampsia, in contrast to their counterparts born to mothers

who did not encounter such a complication during gestation (95% CI: 0.32 — 5.32; p = 0.027).

- a positive correlation between lifespan and the thickness of media in pulmonary arterioles (r = 0.22; p = 0.008).

- a positive correlation between the duration of oxygen therapy and the thickness of media in pulmonary arterioles (r = 0.20; p = 0.017).

- a difference of 5.31 units in the media thickness of pulmonary arterioles in the cohort of neonates who developed pulmonary fibrosis compared to those who did not manifest such a complication (95% CI: -9.84—-0.79; p = 0.022).

3. The third approach in this analysis is centered on the assessment of the degree of pulmonary functional maturation.

- a positive correlation exists between the intensity of Napsin A expression at the level of alveolar macrophages and gestational age at birth (r = 0.58; p<0.001), as well as between the intensity of Napsin A expression at the level of alveolar epithelium and gestational age at birth (r = 0.31; p).

- a statistically significant mean difference of -0.466 units in the intensity of Napsin A expression was observed between newborns whose mothers did not receive antenatal corticosteroid treatment and those whose mothers did receive such treatment (95%CI: -0.91—-0.14; p = 0.022).

- A statistically significant mean difference of 0.28 units in the expression of Napsin A intensity was observed between neonates born to mothers with a history of antenatal infections and those born to mothers without such a history (95% CI: 0.01 — 0.54; p = 0.038).

- A statistically significant positive correlation was identified between the intensity of Napsin A expression at the level of the alveolar epithelium and the duration of mechanical ventilation (r = 0.30; p = 0.002).

- A notable difference of 10 CD20 positive T lymphocytes (B lymphocytes) was detected in the lung tissue of neonates born to mothers who experienced preeclampsia compared to those born to mothers who did not encounter this pregnancy complication (95% CI: 0.53—19.42; p = 0.040).

- a significant positive correlation exists between the quantity of positive CD8 T lymphocytes (cytotoxic T lymphocytes) and the duration of oxygen therapy - (r = 0.59; p<0.001).

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