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Neonatal Disease

From Pathophysiology to Current and Emerging Therapeutic Approaches (2nd Edition)

Edited by
Fumihiko Namba

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Neonatal Disease: From Pathophysiology to Current and Emerging Therapeutic Approaches (2nd Edition)

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Guest Editor

Fumihiko Namba

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About the Editor

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Fumihiko Namba is a neonatal intensive care physician–scientist working in one of the largest NICUs in Japan. Dr. Namba is a Professor of Pediatrics at Saitama Medical Center, Saitama Medical University. He is currently involved in research on prematurity-related diseases, such as bronchopulmonary dysplasia, patent ductus arteriosus, and retinopathy of prematurity, using in vitro/in vivo models, cohort studies, clinical trials, national databases, and systematic review and meta-analysis. He is also a board member of the Japan Society for Neonatal Health and Development and chair of its International Liaison Committee.

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Article

Early Transpyloric Tube Feeding in Preventing Adverse Respiratory Events in Extremely Low Birth Weight Infants

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Abstract: Background: It has been demonstrated that aspiration during endotracheal intubation in preterm infants with gastroesophageal reflux is a contributing factor in the worsening of lung diseases and the development of bronchopulmonary dysplasia (BPD). This study aims to compare the safety and efficacy of early transpyloric (TP) tube feeding with that of nasogastric (NG) tube feeding in relation to BPD. Methods: The study population consisted of 39 extremely low birth weight infants (ELBWIs) with mechanical ventilation and an enteral feeding volume of 50 mL/kg/day, which were randomly assigned to different groups based on the method of tube feeding. The primary outcome was the incidence of adverse events. Results: The hazard ratio for primary adverse events was significantly lower in the TP group. The TP group had a median time of 34 days (range 24–85) and the NG group 24 days (range 13–70). In general, neither group exhibited severe intestinal complications or poor growth. Conclusions: Early TP tube feeding may be a safer alternative method of NG tube feeding for intubated ELBWIs and has been shown to reduce the frequency of adverse respiratory events.

Keywords: transpyloric tube feeding; nasogastric tube feeding; bronchopulmonary dysplasia; extremely low birth weight infant; adverse events

1. Introduction

The recent advancements in perinatal medicine have led to significant improvements in the protective care of the lungs of extremely low birth weight infants (ELBWIs). However, despite these advances, the incidence of bronchopulmonary dysplasia (BPD) remains a significant concern in this population [1–3]. The pathophysiological characteristics of BPD are complicated by multiple factors, including the immaturity of the lungs, antenatal inflammation, and the use of oxygen and mechanical ventilation [4]. Various modalities have been used clinically to prevent and/or treat BPD, including surfactant replacement therapy, high-frequency oscillatory ventilation, and administrations of antenatal, postnatal systemic and inhaled steroids, vitamins, and low-dose antibiotics. However, these treatments have not shown to reduce the incidence of BPD, contrary to expectation [3].

Gastroesophageal reflux (GER) is a common occurrence in preterm infants, with the potential to precipitate respiratory complications and inadequate weight gain. The etiology

of GER is complex and influenced by multiple factors, including the supine position, immaturity of the lower esophageal sphincter, limited stomach capacity, delayed gastric emptying, and the presence of an NG tube in preterm infants [5,6]. In ELBWIs, it has been reported that tracheal silent aspiration and small amounts of refluxed gastric contents without clear symptoms do not significantly influence the development of respiratory failure [7]. The prevalence of tracheal silent aspiration in tracheally-intubated premature infants has been estimated to be as high as 80% [8]. Symptomatic GER is considered a risk factor for silent aspiration in intubated infants. Therefore, it can be suggested that good control of GER reduces the risk of silent aspiration [9–11]. Moreover, aspiration during endotracheal intubation in preterm infants with GER has been shown to contribute to the worsening of lung diseases and the development of BPD [12,13].

To prevent the occurrence of aspiration and reduce the incidence of respiratory failure, transpyloric (TP) tubes are commonly used for enteral feeding in preterm infants. A Cochrane review published in 2013 concluded that the available data do not provide evidence of any beneficial effect of TP tube feeding for preterm infants. Conversely, there is some evidence of harm, including a higher risk of gastrointestinal disturbance and mortality [14]. However, a retrospective study of 59 ELBWIs revealed that the duration of supplemental oxygen use was significantly shorter in the TP tube feeding group than in the nasogastric (NG) tube feeding group [15]. Moreover, the incidence of adverse events associated with TP tube feeding was only 8%, with bile-stained fluid regurgitation occurring without any severe complications.

These findings led us to investigate the relationship between the nutritional route of the TP tube and adverse effects, particularly those affecting respiratory functions, in intubated ELBWIs. We hypothesized that early TP tube feeding would reduce the incidence of BPD, which is one of the most serious complications. We then re-evaluated TP tube feeding in the context of lower mortality rates and a high incidence of BPD in ELBWIs.

2. Materials and Methods

2.1. Setting and Design

This study was conducted in the neonatal intensive care unit at Osaka Medical Center and Research Institute for Maternal and Child Health. Prior to the study, approval was obtained from the human research ethics committee (trial ID: UMIN000001728), and the study was conducted in accordance with the principles of the Declaration of Helsinki [16]. Before enrollment, informed parental consent was obtained. Once the feeding volume reached 50 mL/kg/day, which was identified as a point at which the risk of aspiration increased while the risk of intestinal disorders decreased [17], the patients were randomly divided into two groups using sealed, opaque envelopes. The patients were stratified by the presence of intrauterine infection/inflammation (high neonatal serum IgM levels, chorioamnionitis, and a negative gastric stable microbubble test [18]) and gestational age (three blocks: 22–23, 24–26, and 27 weeks). The patients were monitored until they reached a postmenstrual age of 36 weeks.

2.2. Participants

The study population comprised infants born prematurely between July 2001 and April 2003 who met the following criteria: birth weight of less than 1000 g, gestational age of less than 28 weeks, mechanical ventilation, enteral feeding volume of 50 mL/kg/day, and parental consent. Gestational age was determined by combining maternal dates with early prenatal ultrasound assessment. Infants with intestinal disorders, including necrotizing enterocolitis (NEC), and those with congenital anomalies, including congenital heart disease, chromosomal anomalies, and hydrops, were excluded from the study. Furthermore, all infants were administered a probiotic supplementation (*Bifidobacterium breve*) on a daily basis from the first day of life in order to prevent NEC [19,20].

2.3. Intervention

The infants were fed via an NG tube until the total volume of feeding reached 50 mL/kg/day. A commercially available TP tube (5 Fr, New Enteral Feeding Tube, Nippon Sherwood Medical Industries Ltd., Tokyo, Japan) was used in the TP group. The distance from the oral cavity to the pylorus along the greater curvature of the stomach was measured. Subsequently, the TP tube was passed through the mouth and the tip was successfully guided into the pylorus by gentle manual manipulation over the abdomen at the bedside. Subsequently, the tube was advanced until the bile-stained fluid could be aspirated or, if that proved impossible, until resistance was encountered. The infants were positioned in a semi-prone position on the right side, and the TP tube was advanced gradually via peristaltic movement. To confirm the position of the TP tube within the duodenum, abdominal X-rays were conducted. The optimal location for the tip of the TP tube was considered to be the third or fourth portion of the duodenum. In the event that an infant was unable to continue feeding in the allocated group, an infant in the NG group was promptly transitioned to TP tube feeding, or an infant in the TP group was similarly switched to NG tube feeding. Moreover, the follow-up process was continued.

A safe technique was implemented for the handling of the TP tube in infants with intestinal functional problems, including meconium diseases or severe acute infection from birth. The tube was carefully inserted into the duodenum to avoid injury to the intestinal mucosa in ELBWIs, which can be easily damaged by the tip of the tube during the process. The NG tube was also meticulously positioned within the nasal cavity and advanced into the gastric region, subsequent to the determination of the requisite length of tube necessary to achieve gastric access in the infant. Both groups were fed intermittently with only human milk for more than one hour.

2.4. Measurements

The baseline data set included the following variables: gestational age, birth weight, birth height, birth head circumference, sex, Apgar score, prolonged rupture of the membranes (>24 h), the administration of antenatal corticosteroids, the use of surfactants and diuretics, and the presence of chorioamnionitis of the placenta.

The primary outcome was the incidence of adverse events as follows: (1) BPD at 36 weeks postmenstrual age (Shennan's definition [21]); (2) respiratory deterioration (as indicated by a ventilatory index (VI) of >0.1 or fraction of inspired oxygen (FSp95) of >0.6 for more than eight hours); (3) intestinal disorder, including cases of more than stage two of NEC; and (4) death.

The secondary outcome variables included growth variables, such as the days when feeding commenced and when the volume of feeding reached 50 and 100 mL/kg/day, daily feeding intake, body weight (measured weekly), head circumference (measured weekly), the rate of maximal weight loss, and the day of return to birth weight. The respiratory variables included the ventilatory index, FiO_2, FSp95, and room air saturation (RAS, %). These data were recorded on a daily basis until the postmenstrual age of 36 weeks. Furthermore, the times of surfactant replacement therapy, durations of supplemental oxygen, mechanical ventilation, and hospital stay, as well as the presence of home oxygen therapy, were recorded.

2.5. Statistical Analysis

All analyses were performed in accordance with the intention-to-treat principle. The baseline characteristics and outcomes of the patients were expressed as mean (standard deviation), median (range: minimum–maximum), and frequency (percentage) for the comparison between the two groups. The binary data were analyzed using the chi-square test or Fisher's exact test, whereas the continuous data were analyzed using Student's *t*-test. The Kaplan–Meier curves were used to illustrate the event rates of the primary outcome, with the log-rank test utilized for analysis. The hazard ratios were calculated using Cox regression to compare the incidence rates of the primary outcome between the two groups.

Statistical significance was set at a p value of <0.05. SPSS version 11.01 and JMP version 6.0 were used for statistical analysis.

3. Results

3.1. Study Participants

The flow diagram depicts the screening process for infants enrolled in the study, which entailed a random assignment to receive either TP tube feeding or NG tube feeding (Figure 1). Of the 100 infants initially recruited for the study, 51 did not meet the inclusion criteria, while 10 were excluded due to mortality before randomization. The remaining 39 infants were randomly assigned to either the TP or NG group, with 20 infants assigned to the TP group and 19 to the NG group.

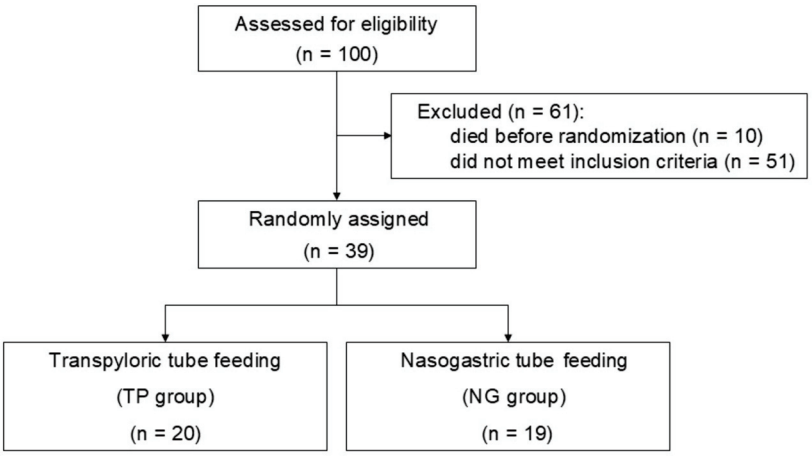


Figure 1. Flow diagram of the study participants.

3.2. Demographic and Baseline Characteristics

The demographic and clinical characteristics of the mothers and infants did not differ between the two groups (Table 1). The mean entry day of the study was Day 11.8 ± 3.0 from birth in the TP group and Day 10.1 ± 2.7 from birth in the NG group. However, no significant difference was observed.

Table 1. Demographic and baseline characteristics.

	TP Group (n = 20)	**NG Group (n = 19)**	p
PROM, n (%)	9 (45)	5 (26)	0.23
Chorioamnionitis, n (%)	10 (50)	7 (37)	0.42
Prenatal steroid treatment, n (%)	14 (70)	12 (63)	0.66
Gestational age, mean (SD), wk	25.6 (1.4)	25.6 (1.2)	0.98
Birth weight, mean (SD), g	762 (157)	716 (126)	0.31
Birth head circumference, mean (SD), cm	23.2 (1.7)	22.6 (1.4)	0.78
Male, n (%)	7 (35)	8 (42)	0.66
Small for gestational age, n (%)	2 (10)	2 (11)	0.96
Multiplicity of birth, n (%)	5 (25)	3 (16)	0.49
Caesarean section, n (%)	16 (80)	13 (68)	0.42
Apgar score < 6 at 5min, n (%)	3 (15)	2 (11)	0.69

TP, transpyloric tube feeding; NG, nasogastric tube feeding; PROM, premature rupture of membranes; SD, standard deviation.

3.3. Incidence of Adverse Events

The hazard ratio for primary adverse events, including BPD at 36 weeks, was significantly lower in the TP group than in the NG group (0.27, 95% CI 0.08–0.75; likelihood ratio test (p = 0.012), log rank test (p = 0.01)). The median time to event was 34 days (range:

24–85 days) with an incidence of 5/20 in the TP group. In contrast, the median time to event was 24 days (range: 13–70 days) with an incidence of 11/19 in the NG group (Figure 2). Table 2 shows the details of the primary outcomes observed in the study. At 36 weeks, three infants (15%) in the TP group and seven (37%) in the NG group have developed BPD. None of the infants in the TP group showed respiratory deterioration, whereas in the NG group, eight infants (42%) showed respiratory deterioration, and one infant died of respiratory failure on Day 10.2 ± 5.1. Only three infants (15%) in the TP group showed mild intestinal disorders on Day 18.3 ± 4.9, whereas one infant (5%) in the NG group showed a similar condition.

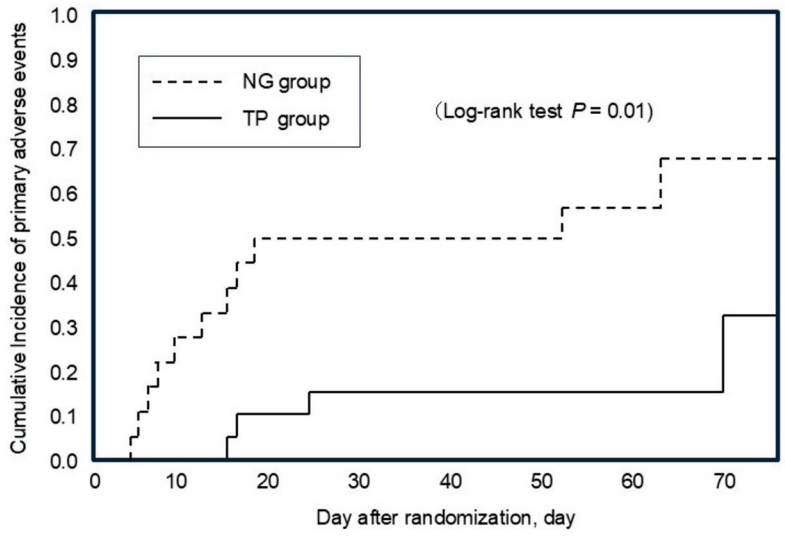


Figure 2. Kaplan–Meier estimates of the cumulative incidence of primary adverse events.

Table 2. Contents of primary outcome.

	TP Group (n = 20)	NG Group (n = 19)	*p*
Incidence of primary adverse events	5 (25)	11 (58)	0.05
BPD at 36 wk, n (%)	3 (15)	7 (37)	0.16
Adverse events			
Death, n (%)	0 (0)	1 (5) *	0.49
Respiratory deterioration, n (%)	0 (0)	8 (42) *	0.00
Intestinal disorder			
NEC stage 2≤, n (%)	0 (0)	0 (0)	1.00
NEC stage 1, n (%)	3 (15)	1 (5)	0.61

* Died of respiratory deterioration; TP, transpyloric tube feeding; NG, nasogastric tube feeding; BPD, bronchopulmonary dysplasia; NEC, necrotizing enterocolitis.

3.4. Incidence of Prematurity-Related Complications

Neither group presented with severe intestinal complications. Table 3 shows the incidence of prematurity-related complications. No differences were observed between the groups in relation to prematurity-related complications. However, the number of days of supplemental oxygen in the NG group was longer than that in the TP group.

3.5. Impacts of Tube Feedings on Growth and Respiratory Variables

No significant differences in the growth variables were observed between the two groups of infants, including body weight; head circumference at 4, 8, 12, and 16 weeks; and feeding intake (Table 4). The respiratory variables of the subgroup that were switched from NG tube feeding to TP tube feeding were found to be inferior to those of the other subgroups (Table 5). The day of switching between the two feeding methods due to adverse

events occurred on Day 31.0 ± 6.1 after birth in the TP group and Day 21.2 ± 5.5 in the NG group (the difference was not statistically significant).

Table 3. Prematurity-related complications.

	TP Group (n = 20)	**NG Group (n = 19)**	*p*
Pneumonia, n (%)	2 (10)	2 (11)	0.96
Pulmonary hemorrhage, n (%)	1 (5)	2 (11)	0.96
Pneumothorax/PIE, n (%)	0 (0)	1 (5)	0.33
Times of surfactant administration, times median (min–max)	1 (1–4)	1 (1–5)	0.45
Days of supplemental oxygen, days median (min–max)	50.5 (26–344)	66 (20–476)	0.38
Days of mechanical ventilation, days median (min–max)	48 (19–145)	52 (20–101)	0.78
Home oxygen therapy, n (%)	3 (15)	4 (21)	0.64
IVH ≥ grade 3, n (%)	2 (10)	1 (5)	0.59

Two cases of IVH in the TP group were onset on day 0-1 (before randomization). PIE, pulmonary interstitial emphysema; IVH, intraventricular hemorrhage.

Table 4. Growth variables.

	TP Group (n = 20)	**NG Group (n = 19)**	*p*
Day of start to feeding (SD), day	2.0 (1.3)	1.7 (1.2)	0.51
Day of feeding			
at 50 mL/kg/day (SD), day	11.6 (3.0)	10.1 (2.8)	0.11
at 100 mL/kg/day (SD), day	15.4 (3.6)	17.6 (8.5)	0.30
Body weight			
at 4 wk (SD), g	799 (148)	763 (158)	0.48
at 8 wk (SD), g	1099 (289)	1057 (206)	0.60
at 12 wk (SD), g	1692 (478)	1649 (423)	0.77
at 16 wk (SD), g	2495 (599)	2212 (602)	0.23
Head circumference			
at 4 wk (SD), cm	24.3 (1.5)	24.5 (1.5)	0.67
at 8 wk (SD), cm	27.7 (2.3)	27.5 (1.7)	0.73
at 12 wk (SD), cm	29.9 (2.6)	30.8 (2.4)	0.33
at 16 wk (SD), cm	33.6 (2.0)	33.3 (1.9)	0.71
Rate of maximal weight loss (SD), %	13.4 (5.9)	15.0 (5.7)	0.41
Day of return to birth weight (SD), day	20.5 (9.5)	22.6 (10.1)	0.49

Table 5. Respiratory variables.

Allocated Groups	**TP Tube (n = 20)**		**NG Tube (n = 19)**	
Subgroups	**TP Tube Only (n = 17)**	**Switch to NG Tube (n = 3)**	**NG Tube Only (n = 10)**	**Switch to TP Tube (n = 9)**
Times of surfactant administration, times median (min–max)	1.0 (1–4)	1.0 (1–3)	1.0 (1–2)	3.0 (1–5)
Days of supplemental oxygen, days median (min–max)	48 (26–344)	84 (26–129)	44 (31–114)	97 (20–476)
Days of mechanical ventilation, days median (min–max)	43 (19–145)	50 (49–63)	35.5 (23–101)	58 (20–78)
Home oxygen therapy, n (%)	2 (12)	1 (33)	0 (0)	4 (44)

3.6. Impacts of Tube Feedings on Oxygenation Factors

Nine infants in the NG group showed significantly poorer oxygenation factors, including FiO_2 on Days 7 and 14, FSp95 on Day 14, and RAS on Day 7, compared with infants in the TP group. Following the switch to TP feeding on Day 10.2 ± 5.2, the infants showed an immediate improvement in oxygenation factors (Figure 3).

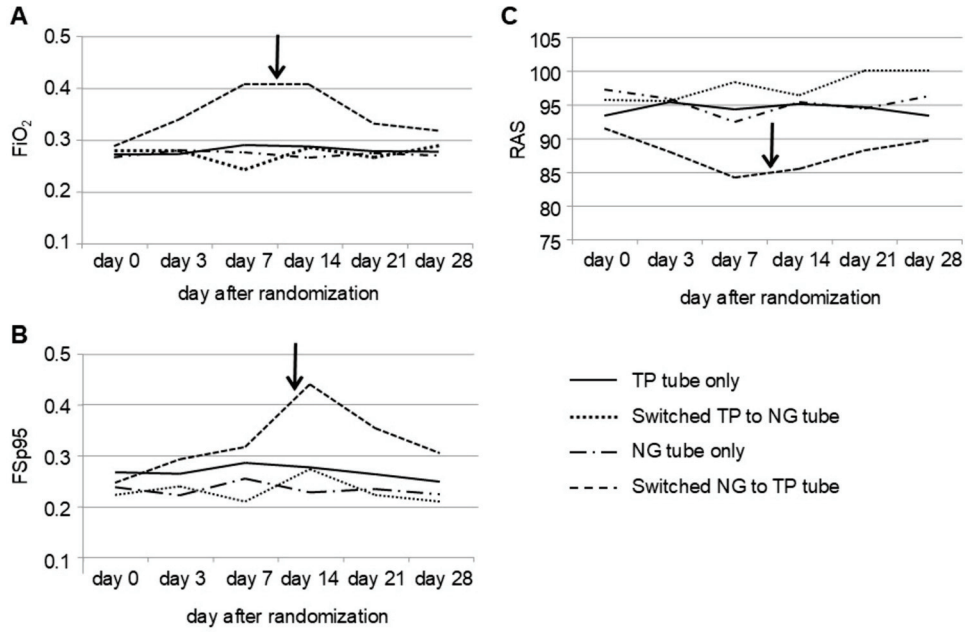


Figure 3. Course of oxygenation factors. (**A**), FiO_2; (**B**), FSp95; and (**C**), RAS. The NG group showed significantly poorer oxygenation factors, including FiO_2, FSp95, and RAS, compared with the TP group. However, after these infants were switched to TP feeding, they showed an immediate improvement in oxygenation factors. NG, nasogastric tube feeding; FSp95, FiO_2 to keep the SpO_2 at ≥95% at rest; RAS, room air saturation; TP, transpyloric. The arrow indicates the date on which the NG tube was replaced with the TP tube.

4. Discussion

The findings of our study suggested that TP tube feeding is a safer alternative method of NG tube feeding for intubated ELBWIs. Our results revealed that TP tube feeding resulted in a reduction in adverse events, including intestinal disorders and respiratory deterioration, including BPD at 36 weeks postmenstrual age in ELBWIs.

A Cochrane review of nine eligible trials involving a total of 359 preterm infants revealed an absence of evidence indicating a beneficial effect of TP tube feeding in comparison with NG tube feeding among preterm infants. The meta-analysis also revealed that infants who underwent TP tube feeding showed a higher risk of gastrointestinal disturbance, including abdominal distention, gastric bleeding, diarrhea, and all-cause mortality. However, no statistically significant differences were observed in the incidence of other adverse events, including NEC, intestinal perforation, and aspiration pneumonia. Moreover, BPD was not reported in any of the trials [14].

However, it should be noted that the studies included in the review were not entirely free from bias. First, all trials were of a small scale and lacked a power or sample size calculation, which may have introduced a degree of bias. Second, the allocation was not concealed, and this may have affected the outcome independent of the intervention. Third, the largest study revealed differences in the baseline characteristics of the feeding groups, which may have influenced the results [22]. The exclusion of this study from the sensitivity analysis would result in a reduction in the relative risk of the incidence of gastrointestinal disturbance and mortality rates, from 1.5 to 1.4 and from 2.5 to 2.2, respectively. Fourthly, the validity of the data in the review was constrained by the absence of a comprehensive

follow-up in the included trials. The included trials did not adhere to the intention-to-treat principle. In comparison with this systematic review, our study was not of a large scale. It was a pilot randomized controlled trial conducted at a single center. Furthermore, all cases included in the study underwent the allocated tube procedure and were followed up until the end of the study.

The objective of managing ELBWIs has undergone a significant transformation. Initially focused on survival, the current emphasis is on "intact survival" without complications. This shift was largely due to advancements in perinatal technology. Consequently, the mortality rate in ELBWIs, which represents a significant concern in the systematic review, has declined. However, the incidence of BPD has increased and now represents a significant challenge in perinatal care [23–27]. A nationwide cohort study using data from the affiliated hospitals of the Neonatal Research Network of Japan revealed a significant decline in the mortality rate among ELBWIs, from 19.0% in 2003 to 8.0% in 2016. In contrast, among the 17,126 survivors, 7792 (45.5%) infants have developed BPD, representing a significant increase from 41.4% in 2003 to 52.0% in 2016 [23]. Various treatments for BPD, including antenatal corticosteroids, surfactant, diuretics, inhaled bronchodilators, Phosphodiesterase-5 inhibitors, inhaled pulmonary vasodilators, endothelin-1 receptor antagonists, and macrolide antibiotics have been used in clinical settings but have not been effective in reducing the incidence of BPD [28]. In light of this, we undertook a reassessment of TP tube feeding as a potential strategy for BPD prevention.

GER is considered a major factor in tracheal silent aspiration in intubated preterm infants. In these infants, gastric contents can easily flow backward into the oral cavity through the lower esophageal sphincter, subsequently entering the trachea around the endotracheal tube [9]. The occurrence of a minimal amount of tube-mediated aspiration is considered a mechanism of deterioration in premature infants with BPD due to silent aspiration, which is a distinct phenomenon from acute aspiration syndrome that is caused by massive aspiration [8,10,29]. In the context of treating silent aspiration, it is important to control GER in order to reduce the incidence of apnea and the necessity for supplemental oxygen and to improve the appearance of chest X-rays [10]. In our study, we observed an immediate improvement in oxygenation factors in infants who were switched from NG tube feeding to TP tube feeding to control GER.

TP tube feeding has been used as a method of enteral feeding for over three decades. In view of the advances in perinatal technology and the enhanced quality of TP tube devices, several studies have reported the beneficial impact of TP tube feeding. Dryburgh documented the positive outcomes of TP tube feeding in 49 infants, including eight ELBWIs who exhibited good weight gain and were generally secure with this method [17]. Similarly, Malcolm et al. have observed that TP tube feeding, particularly when restricted to human milk, may effectively reduce the occurrence of apnea and bradycardia in preterm infants with suspected GER [30]. In a recent retrospective study, Srivatsa et al. investigated the impact of TP feed initiation on short-term oxygenation and manual oxygen blender titration among ELBWIs, with a sample size of 56 infants. The findings indicated that no significant differences were observed in any oxygenation measures during TP tube feeding vs. NG tube feeding among the 14 intubated infants. However, among the 42 nonintubated patients, significant improvements were observed in the SpO_2/FiO_2 ratios, frequency of manual oxygen titrations, number of hypoxemic episodes, and severity of hypoxemic episodes after TP tube placement. It was concluded that the transition from NG tube feeding to TP tube feeding was associated with acute improvement in oxygenation for nonintubated infants (n = 42) but not for intubated infants (n = 14) [31]. In contrast, Shimokaze et al. also evaluated the short-term effects of TP tube feeding on respiratory status in 33 preterm infants during mechanical ventilation and concluded that TP tube feeding during mechanical ventilation could prevent the deterioration of oxygenation without major complications at the stage of respiratory exacerbation in preterm infants [32]. The discrepancy in the efficacy of TP tube feeding on oxygenation in intubated preterm infants between the two studies may be attributed to several factors, including

differences in data collection methods, ventilation modes (such as conventional ventilation and high-frequency ventilation), and the number of enrolled ventilated infants (14 vs. 33). Our findings align with the acute respiratory effects of TP tube feeding for respiratory exacerbation in preterm infants, as previously reported by Shimokaze and colleagues.

This study is limited in several ways. First, it is a single-center pilot study with a small sample size. Second, the data collected during the study period (2001–2003) may not be representative of current neonatal care practices and populations. However, there are few studies investigating the efficacy of early TP tube feeding in preventing adverse respiratory events in ELBWIs. Therefore, this study could contribute to strengthening the evidence base by providing data for a systematic review and meta-analysis. Furthermore, the long-term outcomes of included patients, such as neurodevelopmental and respiratory outcomes, will be incorporated in the future.

5. Conclusions

We concluded that early TP tube feeding may be a more effective alternative method of NG tube feeding. This is evidenced by a reduction in adverse respiratory events, including BPD, which may be attributed to the potential for silent aspiration in ELBWIs. However, due to the limited sample size in our pilot study, further confirmation of these findings is necessary. To this end, a large-scale multicenter randomized controlled trial is required to assess the clinical safety and efficacy of early TP tube feeding in ELBWIs.

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Structural Disruption of Cilia and Increased Cytoplasmic Tubulin in Biliary Atresia—An Exploratory Study Focusing on Early Postoperative Prognosis Following Portoenterostomy

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Abstract: Introduction: Biliary atresia (BA) is a progressive hepatobiliary disease in infants, leading to liver failure and the need for transplantation. While its etiopathogenesis remains unclear, recent studies suggest primary cilia (PC) disruption plays a role. This study investigates correlations between PC and cytoplasmic tubulin (TUBA4A) alterations with hypoxia in patients with the isolated form of BA, focusing on native liver survival. **Methods:** Using qualitative and quantitative digital image analysis of immunofluorescence-stained liver samples, we assessed PC and TUBA4A features correlating these findings with HIF-1α nuclear positivity, clinical–laboratory data, and early native liver survival. Liver samples from fourteen BA patients and six controls with another liver disease were analyzed by digital image analysis, with data evaluated using Spearman's correlation and independent *t*-tests. **Results:** HIF-1α positivity in cholangiocytes was observed in 42.8% of BA patients. While the PC ratio per biliary structure (cilia ratio status, CRs) was similar between BA patients and controls, PC length was decreased in BA patients. Cytoplasmic TUBA4A levels were elevated in BA patients. CRs positively correlated with lower cytoplasmic TUBA4A expression and was higher in patients without HIF-1α nuclear positivity. Reduced cilia length correlated with higher bilirubin levels at portoenterostomy. Predictors of early poor prognosis (death or need for transplantation until 1 year of life) included HIF-1α positivity, elevated direct bilirubin levels, decreased cilia length, PC bending, and increased TUBA4A expression. **Conclusions:** Reduced PC length, PC bending, and increased intensity of cytoplasmic TUBA4A expression occur in the isolated BA clinical type and negatively impact the early prognosis after post-portoenterostomy. These findings suggest the existence of a disruption in the tubulin transport between cytoplasm and PC. The detrimental effect of HIF-1alpha pathway activation over early native liver survival was confirmed, although independently from PC or cytoplasmic tubulin features.

Keywords: biliary atresia; primary cilia; cytoplasmic tubulin; hypoxia; native liver survival

1. Introduction

Biliary atresia (BA) is a rare, life-threatening disease that starts in infancy and comprises a progressive obstructive cholangiopathy expanding from the extrahepatic biliary tract to the intrahepatic biliary tree [1,2]. In BA, there is an early extensive ductular reaction and associated fibrosis, which unleashes cirrhosis and chronic liver failure, leading to the need for liver transplantation (LTx), or death, until the second year of life. Presently, the only treatment for BA is a portoenterostomy developed by Morio Kasai. If timely performed, Kasai portoenterostomy enables 5- and 10-year survival rates of around 75% and 67%, respectively [3,4]. Whenever portoenterostomy is ineffective, postoperative native liver survival (NLS) does not surpass 2 years of life. Around 22% of BA patients reach 30 years of life without LTx given the increased NLS rates after portoenterostomy presently obtained [5]. The occurrence of different clinical forms of BA, including the most frequent variant, called isolated BA, suggests the existence of diverse etiopathogenetic mechanisms for the development of the disease [6,7]. The etiology of BA seems to result from viral infection, toxins, immunogenetic factors, and defects in embryogenesis. Genetic and epigenetic predispositions, associated with environmental factors affecting the mother, are potential triggers for BA [8,9]. Concerning the effects of environmental factors on the mother, a recent large case-control study indicates an association between prenatal maternal intestinal or genitourinary tract infection and the occurrence of BA in the offspring [10].

Our group, after confirming a progressive medial layer thickening of hepatic arterial branches—suggestive of vascular remodeling in patients with isolated BA [11,12]—investigates the role of hypoxia-ischemia affecting the biliary structures as a putative cause of the cholangiopathy. We detected histopathologic alterations and specific gene expression profiles in *VEGF*, Angiopoietins, their receptors, and HIF-1alpha (HIF-1α) pathways [13–15]. Later, we uncovered through immunohistochemistry a strong HIF-1α positivity in intrahepatic biliary structures of at least 35.7% of patients with isolated BA, involving the portal tracts, portal–parenchymal interfaces, and fibrovascular septal and subcapsular areas [16]. Since the HIF-1α pathway was found to be activated in cholangiocytes within the portal–parenchymal interface, where the hepatobiliary progenitor cells' niche is located, it is conceivable that hypoxia and other processes involved with HIF-1α activation affect liver regeneration and fibrogenesis [17]. These consecutive studies suggest that at least a subset of BA patients present a cholangiopathy attributable to hypoxia [6,18]. A relevant investigation involving systems analysis integrating high-throughput biological data confirmed a central role of hypoxia and HIF-1α pathway activation in the development of BA [19]. One recent study confirmed the presence of hepatic artery medial layer thickening in BA and its detrimental effects on the liver, attributable to hypoxia, relating the vascular disruption with a disorder of the Notch3/Hey1 pathway [12]. Another study, using hypoxic injury over fetal/neonatal extrahepatic bile ducts (EHBDs) in sheep, revealed that hypoxia causes luminal narrowing and formation of biliary mucous plugs, a process which, after completion of the fetal/neonatal period, transitions into the adult scarring/fibrosing process, characteristic of BA [20]. Thus, hypoxic injury represents a putative mechanism for the pathophysiology of BA.

Primary cilia (PC) are solitary luminal projections present in the cholangiocyte apical membrane that function as tunable sensing organelles and whose permanent loss leads to disease. The structural base of PC, as that of cytoplasmic microtubules, consists of doublets of alpha- and beta-tubulin dimers assembled as closed cylinders that collectively are referred to as axoneme [21,22]. Sensory and signaling functions of PC are essential for embryonic development, and gene variants that cause the loss of PC result in ciliopathies. Primary cilia act as chemoreceptors and mechanoreceptors in the biliary system, likely involved in cell proliferation, senescence, activation of the progenitor cell compartment, regeneration,

and embryonic developing mechanisms [22,23]. Concerning BA, PC abnormalities seem to occur in BA patients with or without associated extrahepatic anomalies [24,25]. A reduced amount of PC in intrahepatic biliary structures in the livers of patients with BA seems to worsen the postoperative prognosis after portoenterostomy [26]. However, as far as we know, alterations in the behavior of the cytoplasmic microtubule Tubulin-alpha 4A (TUBA4A)—which are known to be associated with PC disorders [27,28]—have not yet been analyzed in BA.

Given the described alterations of cholangiocyte PC in biliary atresia patients and their effects on the disease prognosis in the scarce references from the literature [26–29], we performed this study using adequate quantitative image analysis of fluorescent stained samples to (1) confirm specific cholangiocyte PC characteristics, including deciliation, PC length, and bending in patients with the isolated form of BA; (2) investigate the effects of ciliary alterations over the 1-year native liver survival after portoenterostomy. In addition, considering the recognized effects of hypoxia over PC dysfunction and the previous evidence of HIF-1alpha activation in the biliary epithelium of a subset of BA patients, we aimed to correlate the HIF-1α pathway activation in cholangiocytes of BA patients with the primary cilia features and the 1-year native liver survival. Finally, since the intensity of cytoplasmic TUBA4A expression has not been evaluated in BA patients, we intended to correlate this variable with PC features and the 1-year native liver survival.

2. Materials and Methods

This histopathological study analyzed selected microanatomic structures in the liver of each patient included in a convenience sample of the isolated BA group, compared to controls with another disease, to identify specific histopathological (immunofluorescence and morphometric) behavior patterns involving the variables of interest (Tables 1 and 2, and Figure S2 from Supplementary Materials).

Table 1. Compared variables in the present study.

• **Diagnostic (groups)**	
	Isolated biliary atresia Intrahepatic neonatal cholestasis
• **Clinical–laboratory**	
	Age at portoenterostomy (days of life) Total and direct-reacting bilirubin serum levels at exploratory laparotomy
• **Prognostic outcome evaluation**	
	Age at liver transplantation (days of life) Age at death (days of life) Cause of liver transplantation Cause of death 1-year native liver survival
• **Immunofluorescence features**	
	Subjective (qualitative) method HIF-1alpha nuclear positivity in intrahepatic cholangiocytes ***Objective (quantitative, morphometric) methods (portal biliary structures; cells of the portal, or septal/parenchymal interface; and areas of ductular reaction distributed externally to parenchymal zone 1)***
○ HIF-1alpha nuclear positivity (according to the positivity threshold of the control specimen)	
○ TUBA4A positivity in primary cilia (according to the positivity threshold of the control specimen): • Cilia ratio status (ratio between cilia count/number of bile ducts and ductules analyzed per patient) • Cilia length • Cilia bending	
○ Cytoplasmic tubulin TUBA4A positivity (according to the positivity threshold of the control specimen)	
○ DAPI-positive nuclei	

Table 2. Demographic and clinical characteristics of patients and controls.

Patient ID	Diagnosis	Age PE (Days)	Age LTx (Days)	Age Death (Days)	Cause of LTx	Cause of Death	Total Bilirubin (mg/dL)	Direct Bilirubin (mg/dL)
BA1	Isolated Biliary Atresia	110	118	*Alive*	Liver Failure	*Alive*	9.5	7.5
BA2		56	*No LTx*	208	*No LTx*	Cirrhosis; liver failure; hemorrhage	7	5.4
BA3		48	*No LTx*	*Alive*	*No LTx*	*Alive*	7.8	3.5
BA4		54	*No LTx*	285	*No LTx*	Cirrhosis; liver failure	6.7	5.3
BA5		59	*No LTx*	283	*No LTx*	Cirrhosis; liver failure	5.8	4.2
BA6		93	*No LTx*	165	*No LTx*	Cirrhosis; liver failure	10	6.7
BA7		59	*No LTx*	*Alive*	*No LTx*	*Alive*	12	8.8
BA8		92	2559	*Alive*	Cirrhosis; liver failure	*Alive*	12.9	9.6
BA9		69	*No LTx*	*Alive*	*No LTx*	*Alive*	7.5	6
BA10		65	114	122	Liver failure	Cirrhosis; liver failure	19.1	14.3
BA11		52	212	*Alive*	Cirrhosis; liver failure	*Alive*	11.6	8.6
BA12		56	1409	*Alive*	Recurrent cholangitis; cirrhosis; liver failure	*Alive*	16.6	11.8
BA13		32	*No LTx*	*Alive*	*No LTx*	*Alive*	4.7	3.5
BA14		43	*No LTx*	*Alive*	*No LTx*	*Alive*	4.6	4
C1	Idiopathic Neonatal Cholestasis	35	*No LTx*	*Alive*	*No LTx*	*Alive*	14.6	10
C2		78	*No LTx*	*Alive*	*No LTx*	*Alive*	19.2	14.9
C3		62	*No LTx*	*Alive*	*No LTx*	*Alive*	12.1	8.8
C6	Parenteral Nutrition-Associated Cholestasis	62	*No LTx*	*Alive*	*No LTx*	*Alive*	14.6	10
C4	A1ATD	40	*No LTx*	*Alive*	*No LTx*	*Alive*	9.2	6.4
C5	CMV Hepatitis	35	*No LTx*	*Alive*	*No LTx*	*Alive*	*Not Available*	*Not Available*

2.1. Patients and Samples

All the patients enrolled in this study underwent exploratory laparotomy between 2005 and 2018 as part of the diagnostic workup for neonatal cholestasis at Hospital de Clínicas de Porto Alegre, Brazil. The inclusion criteria for patients in this study comprised infants with isolated BA and controls with intrahepatic neonatal cholestasis of comparable age—submitted to exploratory laparotomy with trans-operative cholangiogram in the process of diagnostic investigation—and patients with BA that maintained postoperative follow-up until the end of the study or died. Exclusion criteria comprised infants with clinical types of BA different from the isolated form, absence of histopathological confirmation of BA through the analysis of biliary remnants in the porta hepatis, and BA patients that did not fulfill the follow-up until the completion of the study.

The study group consisted of fourteen patients with BA for whom exploratory laparotomy preceded portoenterostomy, and the control group included six patients with intrahepatic cholestasis (IHC) for whom this invasive diagnostic procedure was necessary to rule out BA (Table 2). The diagnosis of BA was confirmed by both exploratory laparotomy with intraoperative cholangiogram and postoperative bile duct evaluation in porta hepatis. Isolated BA was characterized by the absence of biliary atresia splenic malformation (BASM), extrahepatic bile duct cysts, or positive IgM serology for cytomegalovirus. Clinical–laboratory, molecular, and histological criteria defined the IHC group. The final diagnoses of IHC controls included idiopathic neonatal cholestasis (n = 3), alpha-1

antitrypsin deficiency (n = 1), cytomegalovirus hepatitis (n = 1), and parenteral nutrition-associated cholestasis (n = 1). During the exploratory laparotomy, a wedge liver biopsy was excised from the hepatic segment IV in each patient. In those patients with image evidence of choledochal anatomical blockade, the porta hepatis was extracted. Liver samples were paraffin-embedded and used for immunofluorescence analysis. Preoperative laboratory tests were conducted for all the patients, and the surgery was performed if serum hemoglobin levels were adequate for a safe procedure. The duration and type of anesthesia did not differ between patients and controls, and there was no evidence of intraoperative hypoxia in any of the infants studied.

2.2. Clinical–Laboratory Data Collection

Clinical and laboratory data were prospectively collected and stored in a secure databank (Table 2). Concerning the laboratory tests for comparisons between groups, the serum bilirubin level was selected as an indicator of NLS after portoenterostomy.

2.3. Immunofluorescence Staining

Liver paraffin-embedded samples were microtome-sectioned into 5 µm slices, deparaffinized with xylene, and rehydrated in decreasing ethanol concentrations (100%, 95%, and 70%) and distilled water. Since the samples were fixed in formalin, and formaldehyde residues are autofluorescent in the green spectrum, a treatment with 0.02% Methanol–Peroxidase was performed for 30 min at room temperature under constant agitation. Antigen retrievals were executed using Sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker for 6 min after boiling.

Samples were permeabilized with PBS 1% Tween for 15 min at room temperature and blocked with PBS 10% Fetal Bovine Serum for 1 h at room temperature. Immunostaining with recombinant HIF-1α (Novus Biological, AF1935; 1:100) and TUBA4A (Santa Cruz Biotechnologies, Sc-23950; 1:100) antibodies was carried out at room temperature for 3 h. Secondary antibodies (Alexa-568—A175474, Abcam; Alexa-647—Ab150115, Abcam, 1:20,000) were stained for 1 h at room temperature, and nuclei were stained using DAPI for 10 min at room temperature. A human gallbladder specimen was used as a positive control for the HIF-1α signal, and a human testicle for the TUBA4A signal (Figure S1, Supplementary Materials).

2.4. Image Analysis

2.4.1. Qualitative Analysis

An initial qualitative analysis of TUBA4A and HIF-1α labeling was performed by the authors RCO and JLS—both with expertise in liver histopathology—first separately and then under consensus. Concerning both HIF-1α and TUBA4A, the distribution of their staining was observed in the cholangiocytes according to our previous study [16]; this included within the portal tracts, at the portal–parenchymal interface, in fibrovascular septa and subcapsular areas, as well as in the ductular reaction spreading to the parenchyma. HIF-1α positivity in cholangiocytes was defined as the strong nuclear positivity exclusively in biliary structures. Although HIF-1α positivity occurred in hepatocytes with varying parenchymal zonal locations and intensities, we did not analyze these data for the present study. In the case of TUBA4A, the location of positivity was classified as cytoplasmic, apical, and ciliary.

2.4.2. Quantitative Analysis

HIF-1α Nuclear Positivity Analysis

The analysis of fluorescence images was conducted using QuPath 0.4.4 software [29], following a standardized protocol to ensure reproducibility. To define a positivity threshold

for HIF-1α staining, a positive control (human gallbladder, large cholangiocytes) was used (Figure S1A, Supplementary Materials). Nuclei were identified and isolated using the DAPI channel, and their intensities in the HIF-1α channel were quantified using the "Positive Cell Detection" tool in QuPath ("Intensity Threshold Parameters: nucleus DAPI mean"). Subsequently, quantifications (mean intensity per nucleus) were exported (QuPath: "measure—show detection results"), and a histogram of the average intensity per nucleus was generated. This histogram revealed two distinct peaks corresponding to positive and negative populations, with the threshold set at the midpoint between these peaks to differentiate the two groups.

Portal tracts, portal–parenchymal or septal–parenchymal interfaces, and areas of ductular reaction extending externally to parenchymal zone 1 were selected as regions of interest (ROIs). These areas were manually delineated using QuPath's annotation tools. The "Positive Cell Detection" tool in QuPath was then applied to count HIF-1α—positive nuclei. Cells were categorized into two groups, negative or positive, based on the predefined threshold.

TUBA4A Analysis—Cytoplasmic Expression

Cytoplasmic expression for TUBA4A was conducted using QuPath 0.4.4 software. To define the positivity threshold, a positive control for TUBA4A staining (human testicle) was used (Figure S1B, Supplementary Materials).

Bile ducts, ductules, and interface structures were selected and, through the "Positive Cell Detection" tool in QuPath ("Intensity Threshold Parameters: cytoplasm TUBA4A mean"), positive cells in the cytoplasm were counted and divided into 2 categories: low expression and high expression for TUBA4A. Cells were isolated using DAPI, and their intensities in the TUBA4A channel were quantified. Subsequently, quantifications (mean intensity per cell) were exported (QuPath: "measure—show detection results"), and the histogram of the average intensity per cell was observed, revealing two peaks (positive and negative). The value between these peaks was set as the positivity threshold and subsequently divided into groups of low and high expression.

TUBA4A Analysis—Cilia Quantification

For cilia analysis, at least 5 images per sample were acquired with a 40× magnification (Axio Imager Z2 Microscope, Zeiss, Germany). Subsequently, the CiliaQ v0.1.4 plugin [30] from the image analysis program ImageJ was used to quantify and calculate the length and bending of cilia in cholangiocytes per image. The plugin presents three steps for image segmentation and processing. In the first step of the workflow, using the CiliaQ v0.1.2 Preparator, the Hysteresis Threshold was applied to segment the fluorescence channel labeling TUBA4A (cilia), and in the second step (CiliaQ v0.0.3 Editor), everything within the threshold that was not cilia (labeling debris) was discarded. Finally, in the CiliaQ v0.1.4 step, cilia were quantified, and the length and bending of each cilium were measured. Given that in BA samples there is a larger number of biliary structures—mainly in the portal–parenchymal interface, ductular reaction, and fibrovascular septa—to calculate the total number of cilia per sample, a ratio of the total number of cilia to the total number of ducts/ductules analyzed, called cilia ratio status (CRs), was computed (Figure S3, Supplementary Materials).

2.5. Correlation of Image Analysis Data with Postoperative Outcomes: Death, LTx, and Native Liver Survival After Portoenterostomy

To evaluate the impact of cholangiocyte cilia characteristics (presence/absence, length, and bending) and cytoplasmic TUBA4A expression on the outcomes following portoenterostomy, we focused on the 1-year native liver survival rate as the primary outcome

measure. Spearman's correlation analysis was conducted to explore associations between the clinical data and image analysis results to provide insights into how these factors may influence the early postoperative prognosis.

2.6. Statistics

Quantitative variables were expressed as individual data points and mean ± SD and categorical data were described as frequencies and percentages. The Shapiro–Wilk and Levene tests were performed to assess the normality of the quantitative variables, and all were found to follow a normal distribution. Therefore, an independent Student's *t*-test was used to compare the groups. Spearman's correlation was applied to correlate quantitative and qualitative variables. The statistical power of significant results was calculated using Cohen's d, with data interpretation according to the Supplementary Materials, Table S1. A two-tailed p-value < 0.05 was accepted as significant. Statistical Package for the Social Sciences (SPSS, version 27.0, IBM Corp., Armonk, NY, USA) was used for data processing and statistical analysis, and GraphPad Prism 8 for graph images and processing.

2.7. Ethics

This study was approved by the Research Ethics Committee at the Hospital de Clínicas de Porto Alegre (HCPA), Brazil (project: arteriopathy in biliary atresia and its relation with the postoperative prognosis after portoenterostomy, number 130030) and performed by the ethical standards outlined in the Declaration of Helsinki. Informed consent for using biological specimens and clinical data of the infants was obtained from parents or legal guardians. Information from the databank used in this study was pseudonymized to protect the privacy of patients and families. The liver samples were stored in the "Biorepository of Biological Specimens in Pediatric Hepatology" of the Center of Experimental Research (CPE) of Hospital de Clínicas de Porto Alegre (HCPA), respecting ethical and methodological aspects. This study was approved by the Board of the Foundation for Science and Technology (FCT) under the European/Portuguese collaboration project Portugal 2020 (title: the role of ischemic cholangiopathy in conditions of hepatic dysfunction, project number 02/SAICT/2017—IsChoHep FCT FEDER).

3. Results

3.1. Clinical and Laboratory Data of the BA Patients

The liver tissue samples of 20 patients were assessed: 14 with BA and 6 with IHC (control group). The demographic and clinical characteristics of patients and controls are presented in Table 2. At the time of surgery, the age ranged from 32 to 110 days (mean 62.7 ± 21.4) in BA patients and from 35 to 78 days (mean 52.7 ± 17.1) in controls, with no significant difference between the groups. During the total follow-up period (2005 to 2018), five patients underwent LTx and five patients died, four of them without having undergone LTx. Age at LTx ranged from 3.8 to 85.7 months (median 46.8 months). Age at death with the native liver ranged from 5.5 to 9.5 months (median 8.3 months), reflecting a native liver survival of less than one year. Nine BA patients survived until the end of the study, five of them with their native liver. Regarding bilirubin serum levels at portoenterostomy, total bilirubin (TB) ranged from 4.6 to 19.1 mg/dL (mean 9.6 ± 4.1), and direct bilirubin (DB) ranged from 3.5 to 14.3 mg/dL (mean 7.1 ± 3.1).

3.2. Detection and Immunolocalization of HIF-1α and TUBA4A by Qualitative Method

HIF-1α nuclear positivity was not detected in any biliary structures in the IHC controls (Figure 1A). In contrast, five out of fourteen (35.7%) patients with BA showed HIF-1α nuclear positivity in cholangiocytes of the portal tracts, portal–parenchyma interface,

ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 1A).

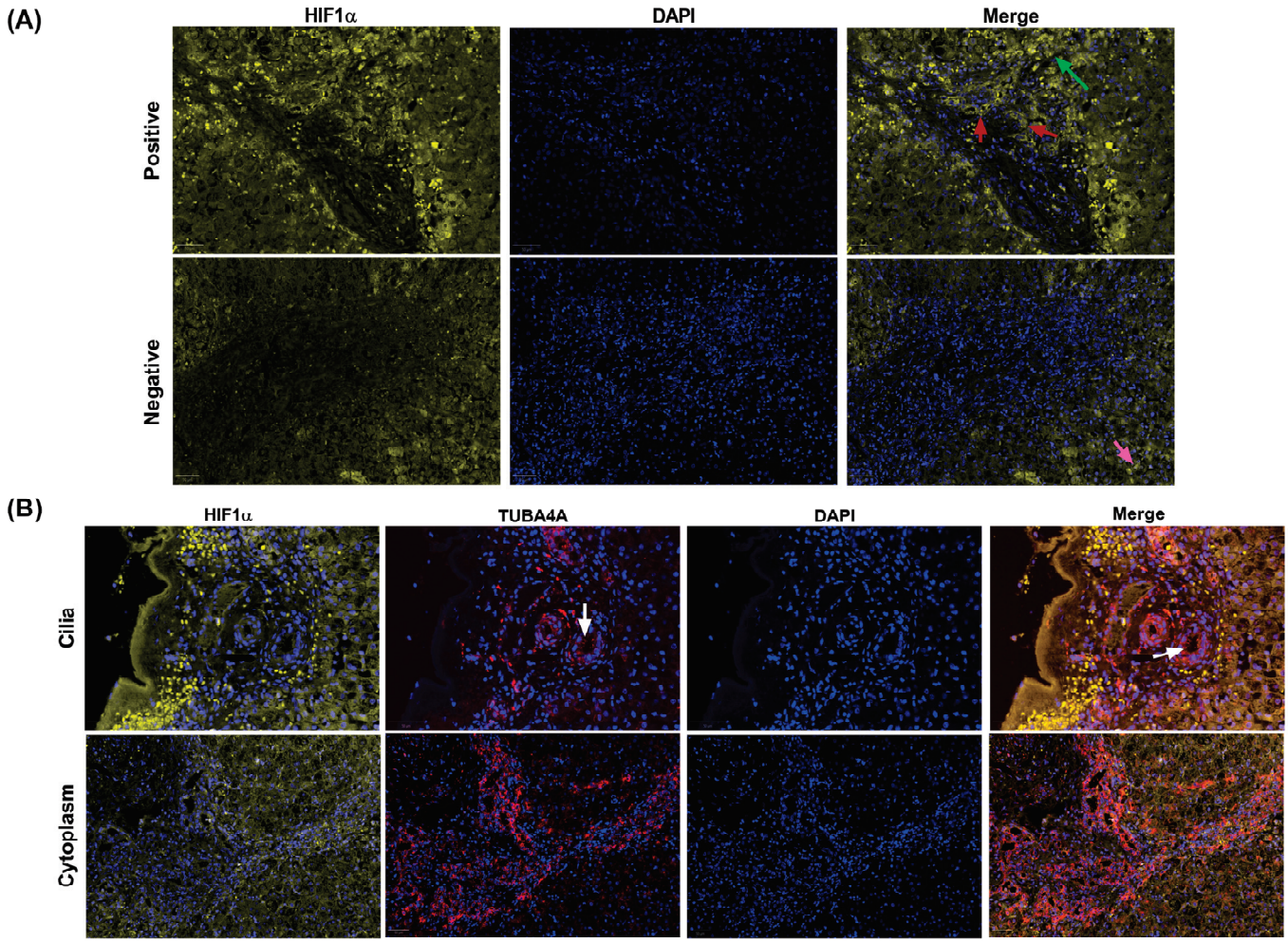


Figure 1. HIF-1α nuclear activation status in cholangiocytes. (**A**) Above (positive): HIF-1α nuclear positivity in the liver of a BA patient. HIF-1α positivity was observed in cholangiocytes of the portal tracts, including interlobular bile ducts (red arrows); the portal–parenchymal interface (green arrow); ductular reaction expanding outwards to parenchymal zone 1; and fibrovascular septa, including intra-septal and septal interface ductular reactions. Below (negative): a patient with idiopathic neonatal cholestasis. Hepatocytes also exhibited HIF-1α nuclear positivity (pink arrow) with variable intensity according to the zonal distribution (pericentral or periportal), in both BA and control samples, independent of the biliary HIF-1α positivity status. Images magnification: 20×. Blue: DAPI staining; Yellow: HIF-1α staining. (**B**) Above (cilia): representative image from a BA sample with TUBA4A staining in cholangiocytes of the portal tracts with ciliary configuration (white arrows). Below (cytoplasm): representative image from BA sample with TUBA4A staining in cytoplasm of cholangiocytes in the portal tract and portal–parenchymal interface. Images magnification: 20×. Blue: DAPI staining; Yellow: HIF-1α staining; Red: TUBA4A staining.

In BA samples, TUBA4A positivity was observed in the cytoplasm and/or apical membrane of cholangiocytes, in the last case, with or without associated ciliary structures (Figure 1B). Cytoplasmic TUBA4A positivity was noted in cholangiocytes located in the portal tracts, portal–parenchymal interface, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 1B).

Qualitative vs. Quantitative Analysis

Qualitative and quantitative methods were used to characterize HIF-1α nuclear positivity. According to the quantitative method, a higher number of cases (six out of fourteen; 42.8% of cases) presented HIF-1α nuclear positivity in cholangiocytes than through the qualitative method (five out of fourteen; 35.7% of cases), showing a small variability between the two evaluation processes.

3.3. Quantitative Analysis of Ciliary Characteristics in Cholangiocytes of Biliary Atresia Patients

When evaluating the CRs across the different assessed areas, no difference was observed between the CRs of BA patients versus controls (Figure 2B). However, regarding primary cilia length, the cilia in the biliary structures of BA patients were notably shorter (Figure 2C).

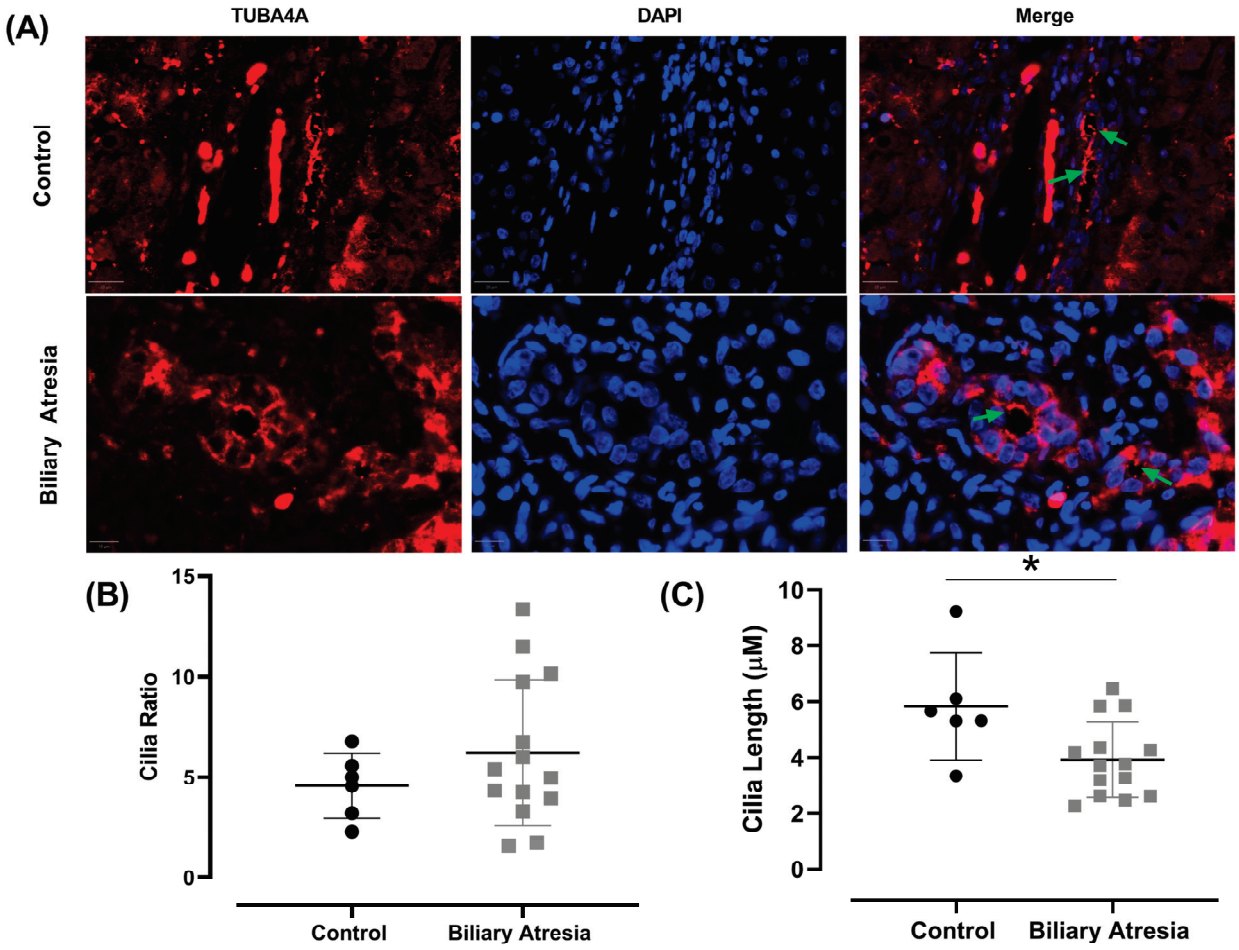


Figure 2. Comparison of cholangiocyte primary cilia features in biliary atresia and controls. (**A**) Representative images showing the differences between controls and BA. Green arrows indicate the cilia of cholangiocytes. Images Magnification: 40×. Blue: DAPI staining; Red: TUBA4A staining. (**B**) Comparison of cilia ratio status between controls and BA. (**C**) Comparison of cilia length between controls and BA (Student's *t*-test, * $p = 0.010$, $d = 1.249$). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

A significant increase in cytoplasmic TUBA4A expression was observed in BA patients compared to the controls (Figure 3A). TUBA4A expression intensity was higher in biliary structures within the portal tracts and along the portal–parenchymal interfaces (Figure 3B,C). The cilia ratio status presented a strong positive correlation with a lower cytoplasmic TUBA4A expression (Table 3).

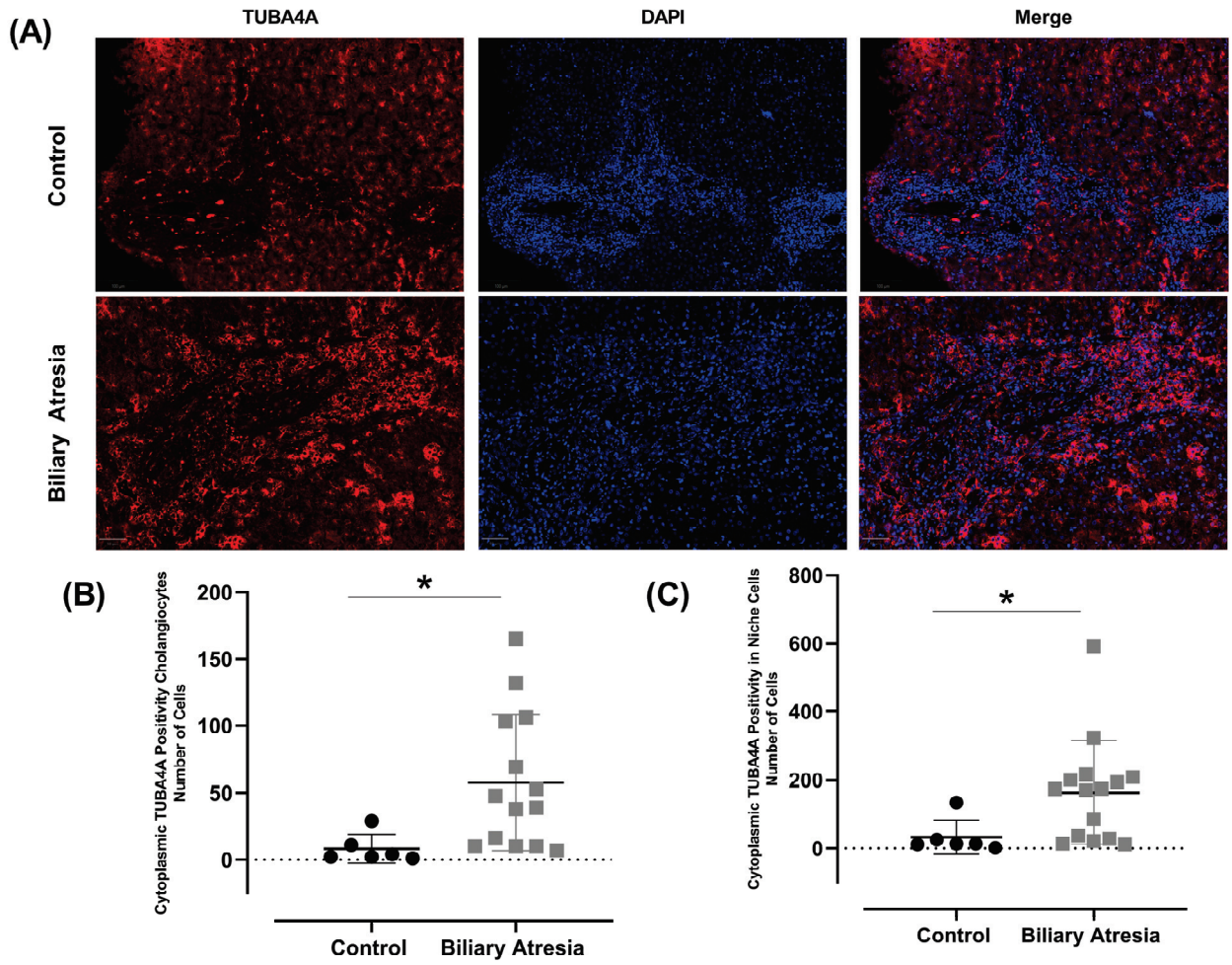


Figure 3. Comparison of the cytoplasmic TUBA4A positivity in cholangiocytes and niche cells. (**A**) Representative images showing the differences between controls and BA. Images Magnification: 40×. Blue: DAPI staining; Red: TUBA4A staining. (**B**) Comparison of cytoplasmic TUBA4A cholangiocyte positivity between controls and BA (*t*-test, * $p = 0.005$, $d = 0.953$). (**C**) Comparison of cytoplasmic TUBA4A positivity in niche cells between controls and BA (*t*-test, * $p = 0.045$, $d = 0.924$). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

Table 3. Spearman's correlation between quantitative and qualitative clinical variables.

Variable Pair	Spearman's Correlation (ρ)	*p*-Value	Significance
	Clinical–laboratory status		
Total Bilirubin vs. *Cilia Length*	−0.665	0.013	*
Direct Bilirubin vs. *Cilia Length*	−0.758	0.003	**
	Morphometric variables		
Lower TUBA4A vs. *CRs*	0.890	<0.001	**
	Outcomes		
LTx vs. *Direct Bilirubin*	0.624	0.023	*
LTx vs. *Cilia Length*	−0.802	0.001	**
Death vs. *HIF-1α Positive Nuclei*	0.692	0.009	**
Death vs. *Higher TUBA4A*	0.626	0.022	*

Abbreviations: CRs—cilia ratio status; HIF-1α: hypoxia-inducible factor-1alpha in cholangiocytes; LTx—liver transplantation; TUBA4A—cytoplasmic alpha-tubulin 4 acetylated; PE—portoenterostomy; NLS—native liver survival. Death and Liver transplantation: represents the outcome until 1 year of life. Characterization of the type of variables under study: Qualitative variables: death; liver transplantation. Quantitative variables: bilirubin serum levels; cilia length; age; levels of TUBA4A and HIF-1α. In the table, * indicates *p*-values between 0.05 and >0.01, while ** indicates *p*-values ≤0.01, representing higher statistical significance.

3.4. Relationship Between HIF-1α Expression and Ciliary Characteristics in BA

Regarding the colocalization of HIF-1α and TUBA4A in BA samples, the presence of HIF-1α nuclear positivity in cholangiocytes often correlated with a cytoplasmic pattern of TUBA4A positivity without identifiable PC. Conversely, HIF-1α negativity was frequently associated with identifiable PC structures in the apical membrane of cholangiocytes. The staining of HIF-1α and TUBA4A was analyzed to understand the behavior of cilia under hypoxic conditions. Cholangiocytes with lower levels of HIF-1α expression exhibited increased TUBA4A staining, indicating a higher rate of PC presence (Figure 4A). Due to the high variability in CRs, the data were divided into two groups based on the median value: normal CRs and decreased CRs (Figure 4B). This division allowed for a clearer understanding of the relationship between HIF-1α expression and cilia presence. Considering the quantitative evaluation of the extent of HIF-1α nuclear expression and the cilia ratio status in the sample of BA, a significant increase in cilia ratio status was observed when the amount of HIF-1α negative cells increased.

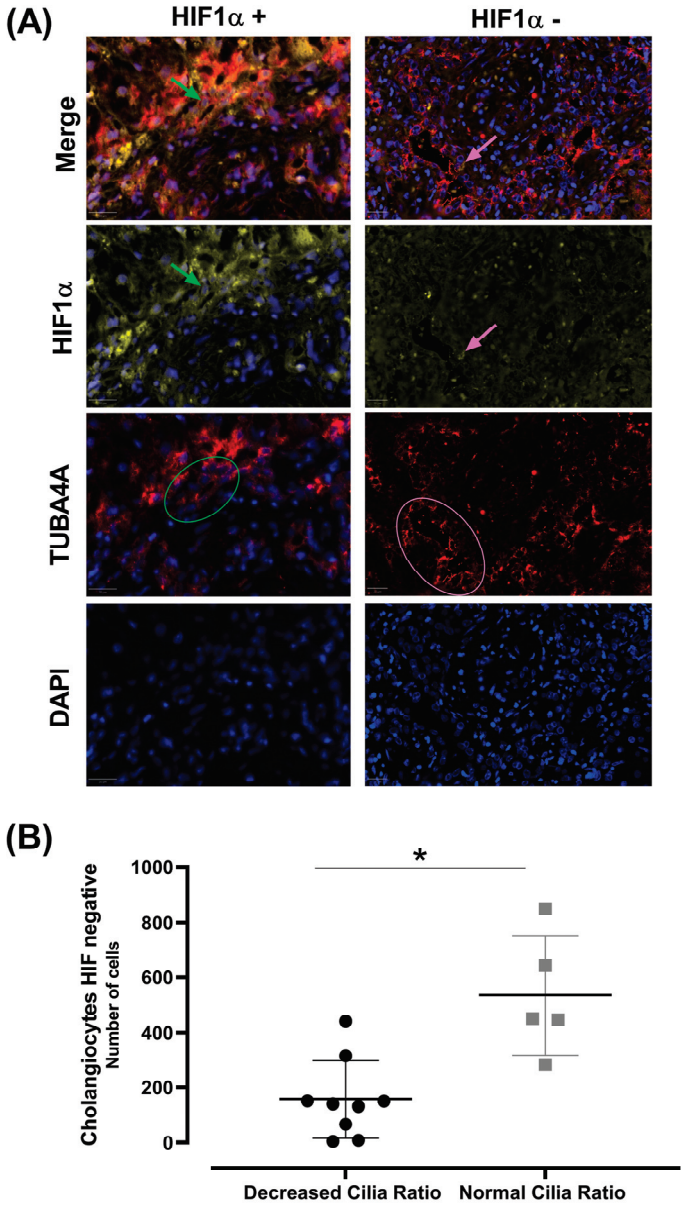


Figure 4. HIF-1α influence over cholangiocyte cilia. (**A**) Representative images demonstrating the influence of HIF-1α positivity on cholangiocyte cilia. The images in the left column display a biliary

duct positive for HIF-1α (indicated by green arrows) with no visible cilia in the duct (highlighted by the green circle). In contrast, the right column shows a biliary atresia sample with HIF-1α-negative cholangiocytes (indicated by pink arrows) where the cilia in the ducts are clearly evident (highlighted by the pink circle). Images magnification: 40×. Blue: DAPI staining; Yellow: HIF-1α staining; Red: TUBA4A staining. (**B**) Considering the quantitative evaluation of the extent of HIF-1α nuclear expression and the cilia ratio status in the sample of BA, a significant increase in cilia ratio status when the amount of HIF-1α negative cells is higher is observed (Student's *t*-test, * $p < 0.001$, $d = 2.216$). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

3.5. Effects of HIF-1α as Well as Ciliary and Cytoplasmic TUBA4A Morphometric Features on the Clinical–Laboratory Status and Native Liver Survival

Table 3 presents the results of the correlations between quantitative and qualitative clinical variables and native liver survival.

3.5.1. Correlations Between Clinical–Laboratory, Image Morphometric, and Outcome Variables

Concerning BA patients, some relevant correlations occurred between the clinical–laboratory data and quantitative morphometric results. Age at portoenterostomy did not present correlations, neither with HIF-1α positivity nor with TUBA4A expression in cholangiocytes. However, cilia length presented negative correlations with total bilirubin ($\rho = -0.665$; $p = 0.013$) and direct reacting bilirubin ($\rho = -0.758$; $p = 0.022$).

Concerning the statistical relationships among the different image morphometric variables, lower TUBA4A cytoplasmic expression presented a strong positive correlation with cilia ratio status ($\rho = 0.890$; $p < 0.001$). Regarding the outcome variables, early LTx (until 1 year of life) was positively correlated with direct-reacting bilirubin serum levels at portoenterostomy ($\rho = 0.624$; $p = 0.023$) and negatively correlated with cilia length ($\rho = -0.802$; $p = 0.001$). On the other hand, early death (until one 1 year of life) showed moderate positive correlations both with HIF-1α nuclear positivity ($\rho = 0.692$; $p = 0.009$) and higher cytoplasmic TUBA4A expression ($\rho = 0.626$; $p = 0.022$) in cholangiocytes.

Confirming our previous immunohistochemical findings [16], HIF-1α nuclear positivity correlated with early death rates ($\rho = 0.692$; $p = 0.009$).

3.5.2. Native Liver Survival

Concerning the correlations between the 1-year NLS after portoenterostomy and the PC features in BA, patients presenting decreased PC length and decreased PC bending either died or needed liver transplantation within 1 year after portoenterostomy (Figure 5). These results suggest that specific alterations in PC morphology are associated with poorer prognosis in BA patients after portoenterostomy.

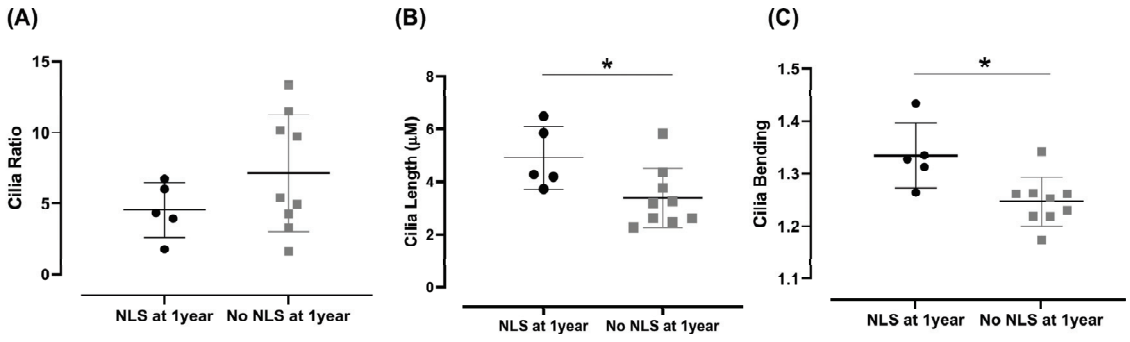


Figure 5. Correlations between native liver survival within 1 year after portoenterostomy and morphometric primary cilia features in the sample of BA patients. (**A**) Comparison of cilia ratio status

between BA patients with and without NLS. (**B**) Comparison of cilia length between BA patients with or without NLS (*t*-test, * $p = 0.018$, $d = 1.317$). (**C**) Comparison of cilia bending between BA patients with or without NLS (*t*-test, * $p = 0.005$, $d = 1.694$). Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

A significant difference concerning the cytoplasmic TUBA4A expression in cholangiocytes occurred between BA patients with and without NLS until 1 year of life (Figure 6A). Increased cytoplasmic TUBA4A expression in cholangiocytes was associated with non-achievement of 1-year NLS (Figure 6A). These findings suggest that higher levels of cytoplasmic TUBA4A in cholangiocytes correlate with poorer outcomes in BA patients.

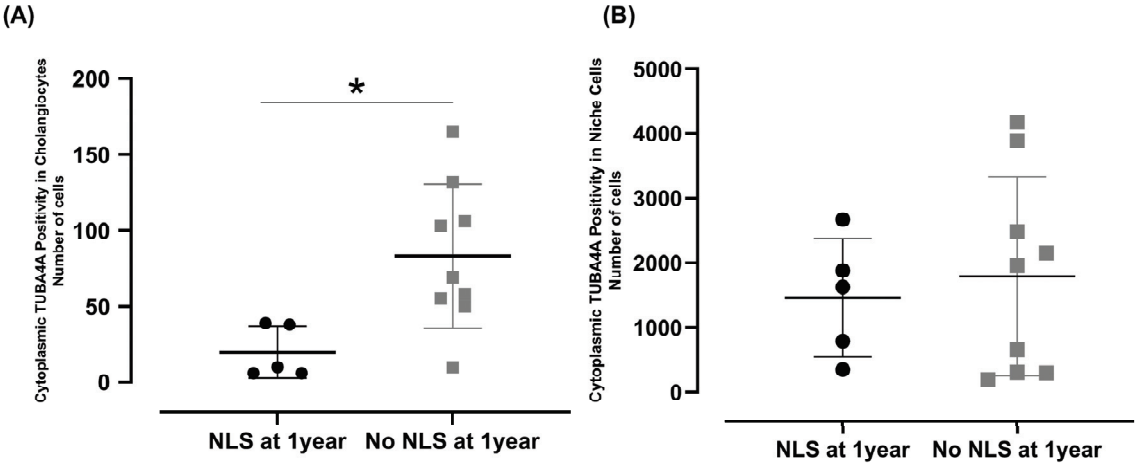


Figure 6. Effects of TUBA4A expression in the cytoplasm of cholangiocytes within the portal tracts and along the portal–parenchymal interface over 1-year rates of native liver survival after portoenterostomy. (**A**) Increased cytoplasmic TUBA4A expression in cholangiocytes was associated with non-achievement of 1-year native liver survival (*t*-test, * $p = 0.020$, $d = 1.005$). (**B**) Difference between TUBA4A cytoplasmic positivity in niche cells regarding native liver survival. Dots represent individual data points. Horizontal lines indicate the mean, and vertical lines represent the SD.

4. Discussion

4.1. Clinical Data of the BA Patients for a Prognostic Evaluation

Regarding the clinical data of BA patients under study, given the small sample available, we were not able to evaluate the 6-month NLS after portoenterostomy. However, during the full follow-up period (2005 to 2018), 7 out of 14 (50%) BA patients died and/or were transplanted in the first year of life, which we consider an early bad prognosis. One of these patients (BA10) with an early bad prognosis underwent LTx at 114 days of life and died 8 days later due to systemic complications of liver cirrhosis and chronic liver failure. Four of the patients with early bad prognosis died with their native liver. Three other patients were transplanted and/or died after two years of life.

4.2. Detection and Immunolocalization of HIF-1α and TUBA4A

In this study, according to the qualitative evaluation, 35.7% of BA patients showed HIF-1α nuclear positivity in the cholangiocytes of portal tracts, portal–parenchymal interfaces, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area, whereas HIF-1α positivity was absent in the biliary epithelium of all the controls (Figure 1A). However, the quantitative analyses of HIF-1α positivity in cholangiocytes showed a higher extent of HIF-1α positivity by using the QuPath 0.4.4 software. Six out of fourteen BA cases (42.8%), and not only 35.7%, presented HIF-1α activation in the intrahepatic biliary epithelium, suggesting that the quantitative method has higher sensitivity rates for HIF-1α detection in immunofluorescence-stained samples.

These findings agree with previous studies published by our team [11,13,14,16,17,31,32] in that, at least in a subset of patients, hypoxia-ischemia seems to have a pathophysiological role in BA, corresponding to an ischemic cholangiopathy [18]. Recently, it was demonstrated that the HIF-1α pathway plays a central molecular role in the development of BA [19]. Chang et al. (2024) confirmed that a precocious hepatic arterial medial layer thickening in BA, apparently associated with the Notch3/Hey1 pathway, leads to peribiliary hypoxia [12]. Another group, using a model of hypoxic injury over fetal extrahepatic bile ducts in sheep [20], endorsed that hypoxic injury over fetal/neonatal extrahepatic bile ducts activates a fetal wound healing program and leads to extrahepatic biliary obstruction. A recent epidemiological genetic publication evaluated 811 BA cases in a genome-wide association study, and observed a strong correlation of BA with genes involved in planar polarity, which integrates ciliary dysgenesis and abnormal vascular and biliary epithelial cell development [33].

Concerning tubulin, TUBA4A positivity was observed in the cytoplasm and/or apical membrane of cholangiocytes, occasionally with a ciliary configuration (Figure 1B), both in BA and controls. In BA, cytoplasmic TUBA4A positivity was noticed in cholangiocytes located in the portal tracts, portal–parenchymal interface, ductular reaction expanding outwards to parenchymal zone 1, fibrovascular septa, and subcapsular area (Figure 1B).

4.3. Quantitative Analysis of Ciliary Characteristics in Cholangiocytes of Biliary Atresia Patients

Concerning the CRs, unlike previous studies [24,26], our morphometric evaluation did not find a difference between BA patients and controls (Figure 2B). However, a remarkable difference was observed concerning cilia length, being notably shorter in BA (Figure 2C). Cilia length showed significant correlations with clinical and laboratory indicators of cholestasis severity, including total and direct bilirubin serum levels. The presence of PC and their correct structure and functioning indicate the ability of cholangiocytes to detect and respond to extracellular stimuli [34] aiming to regulate fundamental signaling pathways [35].

In this context, PC length is an important variable concerning ciliary function [22]. Cilia length is maintained by the addition or removal of tubulin at the tip of PC. Protein synthesis does not occur in PC, and cilia growth involves the import of tubulin monomers from the cytoplasm into the cilium through tightly regulated processes. Changes in cilia length represent a significant alteration in ciliary volume, and altered molecular concentrations within the cilium are associated with processes that affect PC growth [22]. Genetic or environmental factors can lead to reduced cilia length and the development of ciliopathies [36–38].

Given that axoneme length depends on the transit of molecules, including tubulin, between the cytoplasm and cilium, alterations in the microtubule network within the cytoplasm can lead to alterations in the levels of soluble tubulin within PC [27]. Microtubule cytoplasmic stabilization reduces the pool of soluble tubulin and, therefore, may erode the distal tip of the axoneme [38], leading to decreased cilia length or even deciliation.

Additional information obtained in this study was evidenced by the significant increase in cytoplasmic TUBA4A expression in BA patients compared to the controls (Figure 3). Increased TUBA4A expression was particularly evident within the portal tracts and along the portal–parenchymal interfaces. Table 3 shows that higher CRs presented a very strong positive correlation with low or negative cytoplasmic TUBA4A expression ($\rho = 0.890$; $p < 0.001$).

Importantly, elevated cytoplasmic TUBA4A expression was correlated with increased mortality in BA patients ($\rho = 0.626$; $p = 0.022$), Table 3. Concerning deciliation, which can

be identified in the present study through the variable CRs, it is caused by environmental stresses, including hypoxia [37].

4.4. Relationship Between HIF-1α Expression and Ciliary Characteristics in BA

Regarding the colocalization of HIF-1α and TUBA4A in BA samples, in the qualitative analysis, we observed an inverse association between HIF-1α positivity and the presence of PC at the luminal membrane of cholangiocytes, both in biliary cells of portal tracts and at other microanatomic liver areas (Figure 4B). The quantitative analysis of the ciliary characteristics showed that biliary cells without hypoxic characteristics presented preservation of PC, suggesting that hypoxia, indicated by HIF-1α positivity, may negatively impact the formation or maintenance of PC. As previously described, hypoxia constitutes an environmental stress that causes deciliation [37,39].

A correlation between HIF-1alpha pathway activation and decreased early NLS was confirmed in the present study (Table 3). HIF-1α pathway activation occurs within the portal–parenchymal interface, the location of the progenitor cell niche, and can stimulate liver fibrogenesis, with detrimental effects on prognosis [17]. In this study, the CRs correlated with HIF-1α positivity but not with the postoperative prognosis after portoenterostomy.

4.5. Effects of Ciliary and Cytoplasmic TUBA4A Alterations on the Clinical–Laboratory Status and Native Liver Survival

4.5.1. Effects on Clinical–Laboratory Status

Correlations were observed between the need for early LTx, decreased cilia length, and bilirubin serum levels (Table 3). Bilirubin serum levels at the time of portoenterostomy seemed to be affected by decreased cilia length, which can disturb the bile acids' signaling pathways [35,40]. Additionally, increased direct-reacting bilirubin serum levels, a marker of cholestasis severity, as well as decreased cilia length correlated with the need for early LTx.

4.5.2. Effects on Native Liver Survival

This study found correlations between ciliary and cytoplasmic tubulin features and the early postoperative prognosis following portoenterostomy. Decreased cilia length, ciliary bending, and increased cytoplasmic TUBA4A expression in the biliary cells reduced the span of NLS (Figure 5). The detrimental effects of the PC disruptions observed in the present study are understandable since PC regulate several signaling pathways crucial for liver function [35,40].

A unique finding of this study was an increased expression in BA patients of the cytoplasmic TUBA4A in cholangiocytes in comparison to the controls (Figure 6A), suggesting that increased cytoplasmic levels of TUBA4A in cholangiocytes correlate with poorer outcomes in BA patients. Tubulin, a fundamental constituent of the cellular cytoskeleton, is affected by post-translational modifications by diverse molecular factors [41], altering microtubule structures and their associated functions. Toxic compounds, such as alcohol, harm liver cells through tubulin cytoplasmic hyper-stabilization [42–46].

4.6. Limitations of This Study

The limitations of the present study include the small sample size of both BA and the controls. Biliary atresia and intrahepatic neonatal cholestasis are rare hepatobiliary diseases, and the study design of investigations concerning details of pathophysiology and clinical correlations may be affected by the sample size, thus requiring the performance of multicenter clinical studies or the use of experimental models. An additional aggravating factor, specifically concerning BA, is the existence of different clinical variants, which may affect the accuracy of results. Given these concerns, we have chosen to limit our BA sample

to the isolated type and to bypass the sample size constraints by performing exploratory histopathological analyses. Histopathological studies involving digital image analyses enable the evaluation of a large series of microanatomic structures in each patient, making it possible to identify specific histopathological and morphometric patterns related to our subjects of interest. Thus, we used a convenience sample of patients who were cared for and followed in the clinical practice and conducted this exploratory histopathological analysis, being able to obtain statistically significant results with reasonable internal validity. Aiming to confirm the clinical effect of the statistically significant data, we determined the magnitude of the effect size developed by Cohen (1988). Our future next steps will include confirmatory experimental methods. Additionally, studies with larger sample sizes are necessary to extrapolate our findings to the population of BA patients, i.e., to ensure their external validity, not only for the isolated BA variant but also for the remaining clinical types.

5. Conclusions

This study evaluated the presence and clinical–laboratory outcomes attributable to alterations in the primary cilia and cytoplasmic tubulin expression in the intrahepatic biliary epithelium, observing that reduced PC length, decreased PC bending, and increased intensity of cytoplasmic TUBA4A occur in the isolated BA type, and negatively impact the postoperative prognosis after portoenterostomy (Figure 7). These findings suggest the existence of a disruption in the tubulin transport between cytoplasm and PC. The detrimental effect of the HIF-1alpha pathway activation on native liver survival was confirmed but unassociated with PC or cytoplasmic tubulin features.

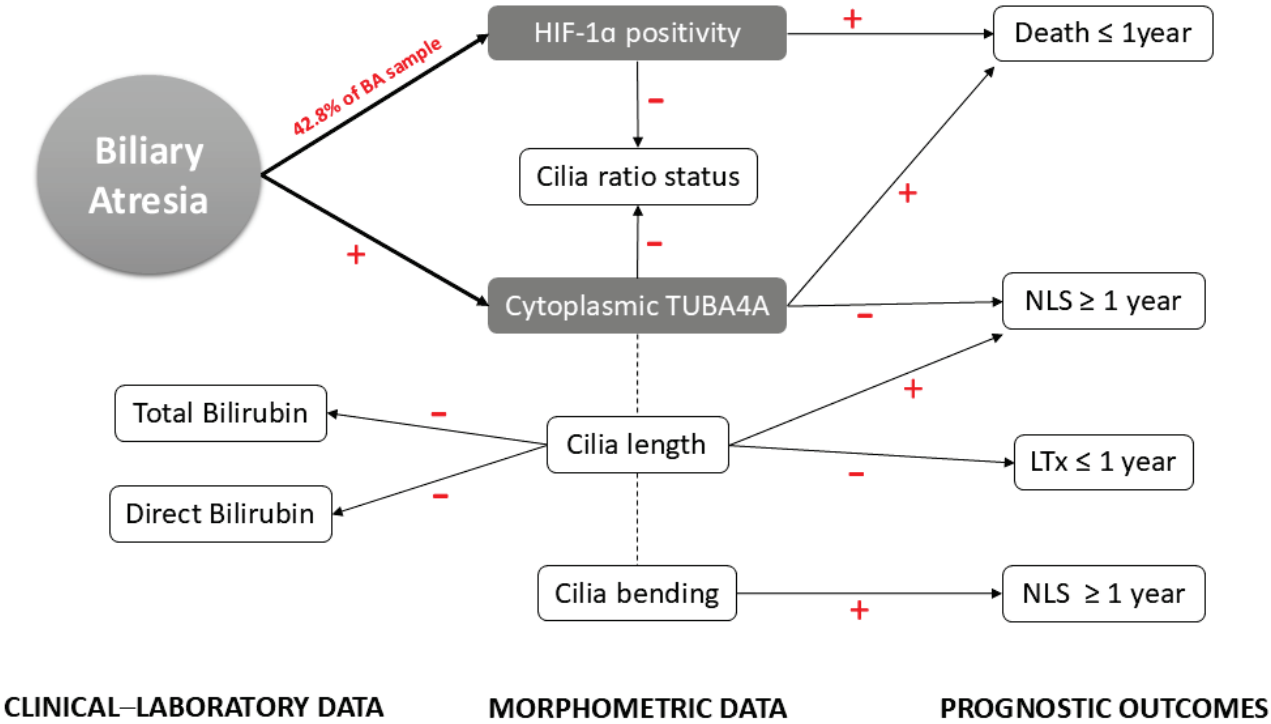


Figure 7. Overview of study findings. The flowchart represents the relationship between BA clinical–laboratory parameters, morphometric data, and prognostic outcomes analyzed in the present study, highlighting the role of PC dysfunction and HIF-1α activation in BA prognostic (+: directly proportional; −: inversely proportional).

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/biomedicines13010087/s1, Figure S1: Positive controls for primary antibodies. (A) Human gallbladder stained for HIF1alpha (yellow). (B) Human testicle stained for TUBA4A (red); Figure S2: Representative images illustrating the microanatomical structures and staining patterns analyzed in this study; Figure S3: Schematic resume of CiliaQ plugin workflow; Table S1: Cohen's effect size. Reference [47] is cited in the supplementary materials.

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Fetal Hemoglobin as a Predictive Biomarker for Retinopathy of Prematurity: A Prospective Multicenter Cohort Study in Portugal

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Abstract: Background/Objectives: Retinopathy of prematurity (ROP) is a leading cause of vision impairment in preterm infants, with its pathogenesis linked to oxygen exposure. Red blood cell (RBC) transfusions, commonly performed in neonatal intensive care units (NICUs), reduce fetal hemoglobin (HbF) fraction, altering oxygen dynamics and potentially contributing to ROP. We aimed to investigate the relationship between RBC transfusions, HbF percentage, and ROP, evaluating HbF as a potential predictive biomarker. **Methods:** A multicenter, prospective study was conducted across eight Portuguese NICUs, involving infants born at <32 weeks gestational age (GA) or <1500 g. ROP staging followed the International Classification of ROP (ICROP2). Clinical data were collected during hospitalization, and HbF fractions were measured from blood samples in the first four weeks of life using standardized methods. Infants were stratified by ROP presence and treatment requirement. Statistical analysis was performed using SPSS 28.0, with $p < 0.05$. **Results:** Eighty-two infants (mean GA: 28.1 ± 2.1 weeks, birth weight: 1055.8 ± 258.3 g) were included. Among them, 29 (35.4%) presented ROP and 4 (4.9%) required treatment. Infants with ROP had more RBC transfusions and lower HbF percentages than those without ROP ($p < 0.05$). Lower HbF was associated with more RBC transfusions ($p < 0.001$). Kaplan–Meier survival curves showed a higher ROP risk in infants with reduced HbF ($p < 0.05$). **Conclusions:** Low HbF percentage in the first four weeks of life may increase ROP risk in preterm infants. HbF could serve as a biomarker for ROP prediction. Interventions preserving HbF may reduce ROP risk. Further studies are needed to validate HbF as a biomarker and refine prevention strategies.

Keywords: retinopathy of prematurity; neonatal diseases; fetal hemoglobin; pathophysiology; innovative therapeutic approaches; neonatal anemia; red blood cell transfusions; preterm infants

1. Introduction

The birth of an extremely preterm infant poses considerable challenges. While advancements in neonatal care have significantly improved survival rates, concerns about long-term morbidities and associated functional impairments remain, with these factors profoundly affecting the well-being and quality of life of preterm infants [1]. Among the many complications of prematurity, retinopathy of prematurity (ROP) stands out as a major cause of visual impairment and childhood blindness worldwide [2].

Globally, the incidence of ROP varies widely, ranging from approximately 20 to 30% among preterm infants in high-income countries to significantly higher levels in low- and middle-income regions, where access to advanced neonatal care is limited [3]. ROP arises from the abnormal development of retinal blood vessels in preterm infants, driven by fluctuating oxygen levels after birth. The pathogenesis of ROP unfolds in two distinct phases: an initial phase marked by high oxygen exposure, which halts normal retinal vascularization, followed by a second phase characterized by retinal ischemia and abnormal neovascularization. This neovascularization is mediated by hypoxia-induced angiogenic factors, such as vascular endothelial growth factor (VEGF) and erythropoietin [4,5]. If untreated, these pathological changes can progress to retinal detachment and vision loss.

ROP is classified according to the International Classification of Retinopathy of Prematurity (ICROP), now in its third edition (ICROP3) [6–8]. This system defines the disease's severity using five stages:

- Stage 1: A thin demarcation line between vascularized and avascular retina.
- Stage 2: A ridge forms at this demarcation line.
- Stage 3: Abnormal neovascularization extends into the vitreous.
- Stage 4: Partial retinal detachment.
- Stage 5: Total retinal detachment, potentially leading to blindness.

Additionally, treatment guidelines are based on evidence from the Early Treatment for Retinopathy of Prematurity (ET-ROP) Study, which established criteria for type 1 and type 2 ROP [9]. These criteria incorporate the zones of the retina, which describe the location of disease relative to the optic disc: zone I is a circle centered on the optic disc with a radius twice the distance to the macula (the area closest to the disc and most critical); zone II extends from the edge of zone I to the nasal ora serrata; and zone III is the remaining peripheral crescent of the retina. The presence of plus disease, characterized by increased vascular dilation and tortuosity, is also a key factor.

Type 1 ROP, requiring prompt treatment, is defined as disease in zone I (any stage with plus disease or stage 3 without plus disease) or zone II (stage 2 or 3 with plus disease). Type 2 ROP, suitable for close monitoring, includes less severe disease in zone I (stage 1 or 2 without plus disease) or zone II (stage 3 without plus disease) [9]. This classification aids in clinical decision-making and ensures timely intervention when necessary.

Despite improved neonatal care and preventive measures, such as optimized oxygen therapy protocols, ROP remains a significant clinical challenge [10]. This highlights the urgent need for innovative strategies to mitigate its impact.

Emerging evidence, including our previous studies, suggests that maintaining higher percentages of fetal hemoglobin (HbF) during the early extra-uterine life of preterm infants may reduce the risk of ROP [11,12]. Fetal red blood cells (RBC) contain a high proportion of HbF, which has a greater oxygen affinity than adult hemoglobin (HbA) [13]. During the third trimester of pregnancy, HbF accounts for 70–80% of total hemoglobin. Between 32 and 36 weeks of gestational age (GA), there is a significant transition to HbA. Subsequently, the HbF fraction gradually decreases to 3–4% by around 24 weeks post-term age [14,15]. Interestingly, the period of HbF reduction between 32 and 36 weeks of GA

coincides with the onset of ROP, suggesting a possible link between HbF percentage and the risk of developing this condition.

Although primarily genetically determined, HbF percentages can also increase in response to hypoxemia, cardiopulmonary insufficiency, or severe anemia in preterm infants. Maternal conditions, such as hypoxia, diabetes, or intrauterine growth restriction, may delay the transition from HbF to HbA [16]. Conversely, adult RBC transfusions, commonly administered to preterm infants during the neonatal period, reduce the HbF fraction, replacing it with HbA. This replacement may contribute to ROP by increasing dissolved oxygen in plasma, exposing the retina to excessive oxygen after a 15 mL/kg RBC transfusion [12]. This elevation induces a rightward shift in the oxygen dissociation curve, leading to an exaggerated, non-physiological supply of oxygen to retinal tissue during a critical phase of susceptibility to hyperoxia, thereby decreasing the angiogenic drive [17–19]. Additionally, the immature antioxidant systems of preterm infants exacerbate oxidative stress following RBC transfusions [20], while inflammatory responses and endothelial activation further contribute to ROP pathogenesis [21]. Notably, preterm infants—especially those critically ill—constitute one of the highest transfusion-requiring cohorts [22].

Given the pivotal role of HbF in oxygen transport and its potential impact on retinal vascularization, this study aims to evaluate HbF as a predictive biomarker for ROP. By exploring HbF's potential to mitigate ROP risk, we hope to enhance the understanding of the disease's mechanisms and improve risk stratification in preterm infants. The broader goal is to advance our comprehension of predictive and preventive measures.

2. Materials and Methods

2.1. Study Design and Population

This multicenter, observational, and analytical study involved a cohort of preterm infants from eight neonatal intensive care units (NICUs) in Portugal, namely Centro Hospitalar Universitário de São João, Centro Hospitalar Universitário de Lisboa Norte, Hospital Prof. Doutor Fernando Fonseca, Centro Materno Infantil do Norte belonging to the Centro Hospitalar Universitário do Porto, Hospital da Senhora da Oliveira, Guimarães, Hospital de Braga, and Maternidade Daniel de Matos and Maternidade Bissaya Barreto belonging to the Centro Hospitalar Universitário de Coimbra. The study is registered as an observational study (ISRCTN16889608).

Recruitment occurred between 19 November 2018, and 19 December 2019, with the study concluding between 30 December 2020, and 21 July 2021. The study followed preterm infants consecutively from birth, including both sexes and all races, if they met at least one of the following inclusion criteria: (1) born before 32 weeks of GA, or (2) birth weight (BW) below 1500 g. Exclusion criteria were as follows: (1) major congenital malformations, (2) congenital or acquired ophthalmological conditions (excluding conjunctivitis, keratitis, and congenital nasolacrimal duct obstruction) unrelated to ROP within the first 12 weeks of life, (3) death prior to the first ROP screening, (4) incomplete clinical data due to patient transfer to another hospital, and (5) lack of informed consent from parents or legal guardians.

The initial cohort for this study consisted of 455 preterm infants enrolled in the GenE-ROP Study, conducted in the eight NICUs mentioned. For the present analysis, a subgroup of 82 infants was selected based on the availability of sufficient blood samples to enable a consistent longitudinal assessment of HbF percentages during the first four weeks of life.

The detailed methodology of the GenE-ROP Study has been described in our earlier publications [23,24].

2.2. Screening for ROP and Gathering Ophthalmological Data

The initial retinal screening was conducted at 31–33 weeks postmenstrual age (PMA) or 4–6 weeks after birth, whichever was later, by qualified ophthalmologists at each NICU. The screening process adhered to the following procedures: (1) adequate mydriasis was achieved using eye drops, following the Portuguese Society of Neonatology's consensus guidelines on ROP [25]; (2) screenings were conducted with either indirect binocular ophthalmoscopy, digital fundus retinography (RetCam), or a combination of both, depending on the specific indication and the protocols of each hospital center; (3) findings were documented for each eye based on the second edition of the ICROP (ICROP2). Follow-up screenings were scheduled according to ROP presence, zone, and severity and were continued until full retinal vascularization or ROP remission post-treatment. Data on the highest stage of ROP, any required treatment, and follow-up examinations were recorded per ICROP2 guidelines [6,7,26]. Treatment criteria followed those from the Early Treatment for Retinopathy of Prematurity (ETROP) Study [9]. Infants diagnosed with type 1 ROP were treated with anti-VEGF or LASER photocoagulation, depending on the disease location and the hospital center.

2.3. Other Data

Data regarding demographics and clinical conditions were gathered from the hospital's medical records during the infants' hospitalization, including GA, BW, sex, and clinical outcomes such as bronchopulmonary dysplasia, peri-intraventricular hemorrhage, cystic periventricular leukomalacia, necrotizing enterocolitis, and hemodynamically significant patent ductus arteriosus.

The analysis of HbF was conducted using the residual blood remaining after routine clinical tests performed on the neonates during their first four weeks of life. All biological samples were coded, anonymized, and stored at -20 °C to ensure patient confidentiality and safeguard their identities.

Hemoglobin analysis was performed using capillary electrophoresis (Capillarys™ Neonat Fast system, Sebia, Lisses, France). Capillary electrophoresis measures the HbF fraction by combining electrophoretic mobility in an alkaline buffer and electro-osmotic flow. A high voltage applied to a silica glass capillary causes hemoglobin to migrate toward a 415 nm wavelength detector. Hemoglobin fractions are quantified and displayed in a pherogram. Automated integration and defined migration zones (N1 to N13) assist in interpreting hemoglobin patterns.

Capillary electrophoresis detects and quantifies HbF, HbA, HbA2, HbS, HbC, HbD-Punjab, HbOArab, HbE, Hb Lepore, and Hb Bart's. It also identifies various Hb variants, including γ- and α-chain abnormalities. Internal quality controls, provided by Sebia (France), contain HbF, HbA, HbS, and HbC. The detection limit for HbA and HbS is approximately 1% of total hemoglobin.

2.4. Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (IBM® SPSS® Statistics, Chicago, IL, USA) version 28.0 for Windows® Software. Categorical variables were expressed as n (%) and continuous variables as mean $\pm$ standard deviation or median (interquartile range). The χ^2 tested differences between the groups for discrete variables, and t-test (parametric test) or Mann–Whitney/Kruskal–Wallis tests (non-parametric tests) for continuous variables, as appropriate. An adjustment was made using binary logistic regression. Survival analysis was conducted using the Kaplan–Meier method and differences between groups were assessed using the log-rank test. Statistical significance was defined as a p-value < 0.05.

In the original cohort of 455 preterm infants, a univariate logistic regression analysis was conducted to evaluate multiple risk factors for ROP, as previously described [23]. Variables identified as significant in the univariate analysis were included in a multivariate logistic regression model, which determined that GA and the number of RBC transfusions were the most statistically significant factors associated with ROP. These findings guided the adjustments performed in the current analysis.

2.5. Ethics Approval

All procedures involving human participants in this study adhered to the ethical standards set forth by the Ethics Committee of Centro Hospitalar Universitário de Lisboa Norte, the Centro Académico de Medicina de Lisboa (CAML), and the Ethics Committees of all other participating hospital centers. These procedures were also conducted in accordance with the 1964 Helsinki Declaration and its subsequent amendments, as well as comparable ethical standards. Written informed consent was obtained from the parents of all infants included in the study.

3. Results

This study included 82 preterm infants, with a mean GA of 28.1 ± 2.1 weeks and a mean BW of 1055.8 ± 258.3 g. Among them, 29 (35.4%) presented ROP, with four (4.9%) requiring treatment: two (2.4%) underwent laser photocoagulation, and two (2.4%) received anti-VEGF therapy. The cohort comprised 41 females (50%). Detailed clinical characteristics, including ROP stage distribution and associated prematurity-related comorbidities, are provided in Table 1.

Table 1. Demographic and clinical characteristics of preterm infants in the study population, including ROP stages.

Number of infants (*n*)	82
GA (weeks), mean ± SD	28.1 ± 2.1
BW (grams), mean ± SD	1055.8 ± 258.3
Female, *n* (%) Male, *n* (%)	41 (50.0) 41 (50.0)
Retinopathy of prematurity, *n* (%) Stage 1 ROP, *n* (%) Stage 2 ROP, *n* (%) Stage 3 ROP, *n* (%) Type 1 ROP, *n* (%)	29 (35.4) 12 (14.6) 9 (11.0) 4 (4.9) 4 (4.9)
Bronchopulmonary dysplasia, *n* (%)	21 (25.6)
Peri-intraventricular hemorrhage, *n* (%)	12 (14.6)
Cystic periventricular leukomalacia, *n* (%)	3 (3.7)
Necrotizing enterocolitis, *n* (%)	14 (17.1)

n, number of individuals; SD, standard deviation.

Preterm infants with ROP received significantly more RBC transfusions during the first 4 weeks of life compared to those without ROP (Table 2).

A significant decrease in HbF percentages during the first four weeks of life was observed with increased RBC transfusions. Infants requiring more transfusions had notably lower HbF percentages than those needing fewer or none ($p < 0.001$) (Table 3).

Table 2. Comparison of RBC transfusions administered during the first 4 weeks of life between infants who developed ROP and those who did not.

Number of RBC Transfusions (First 4 Weeks)	No ROP (*n*, %)	ROP (*n*, %)	*p*	*p**
0	43 (81.1)	8 (27.6)		
1–2	9 (17.0)	13 (44.8)	**<0.001** [1]	**0.009**
3–4	1 (1.9)	8 (27.6)		

p, *p*-value; *p**, binary logistic regression, adjusted for gestational age (GA). [1] Chi-square test. *p*-values less than 0.05 are in bold.

Table 3. Association between the number of RBC transfusions and mean HbF percentages in the first 4 weeks of life.

Number of RBC Transfusions (First 4 Weeks)	Mean HbF (%)—First 4 Weeks *n*; Median (Q1–Q3)	*p*
0	51; 77.3 (75.2–81.8)	
1–2	22; 67.8 (60.4–75.2)	**<0.001** [1]
3–4	9; 44.5 (34.7–53.8)	

[1] Kruskal–Wallis test. *p*-value in bold indicates statistical significance ($p < 0.05$).

Table 4 shows the association between HbF percentages during the first four weeks of life and the presence of ROP. Preterm infants who developed ROP had significantly lower mean HbF percentages during this period compared to those without ROP, even after adjusting for GA. Weekly data analysis revealed that mean HbF percentages were significantly and independently lower in infants with ROP from the second to the fourth week of life.

Table 4. Association between HbF in the first four weeks of life and ROP.

HbF (%)	No ROP *n*; Median (Q1–Q3) or Mean ± SD	ROP *n*; Median (Q1–Q3) or Mean ± SD	*p*	*p**
Mean first 4 weeks	53; 77.3 (74.4–81.6)	29; 62.7 (51.0–72.8)	**<0.001** [1]	**0.003**
1st week of life	50; 79.0 (76.4–81.0)	26; 76.9 (69.2–80.6)	0.100 [1]	0.693
2nd week of life	32; 77.8 (74.6–81.2)	28; 73.3 (54.4–79.5)	**0.005** [1]	**0.020**
3rd week of life	29; 76.1 (69.7–79.8)	26; 52.5 (33.7–73.4)	**<0.001** [1]	**0.031**
4th week of life	24; 70.5 ± 17.3	25; 44.9 ± 19.2	**<0.001** [2]	**0.019**

*p**, binary logistic regression, adjusted for GA; [1] Mann–Whitney test. [2] *t*-test. *p*-values less than 0.05 are in bold.

Additionally, preterm infants who required treatment for ROP (type 1 ROP) had significantly lower mean HbF percentages during the first four weeks of life compared to those with ROP who did not require treatment, even after adjusting for GA (Table 5). When assessed weekly, however, this association was significant only during the second and third weeks of life and was not independent of GA during these periods.

Table 5. Association between HbF in the first four weeks of life and ROP requiring treatment (type 1 ROP).

HbF (%)	No Type 1 ROP *n*; Median (Q1–Q3) or Mean ± SD	Type 1 ROP *n*; Median (Q1–Q3) or Mean ± SD	*p*	*p**
Mean first 4 weeks	25; 64.3 ± 13.3	4; 40.1 ± 7.8	**0.002** [1]	**0.046**
1st week of life	22; 77.4 (71.7–81.8)	4; 56.9 (37.3–77.7)	0.112 [2]	0.119
2nd week of life	24; 75.1 (65.3–79.7)	4; 43.3 (30.8–58.3)	**0.005** [2]	0.057
3rd week of life	22; 59.5 ± 19.5	4; 23.8 ± 5.7	**<0.001** [1]	0.202
4th week of life	21; 46.7 ± 18.5	4; 35.3 ± 22.8	0.287 [1]	0.507

*p**, binary logistic regression, adjusted for GA; [1] *t*-test. [2] Mann–Whitney test. *p*-values less than 0.05 are in bold.

Figure 1 shows Kaplan–Meier survival curves for ROP diagnosis in preterm infants categorized into quartiles for HbF percentage for the first 4 weeks of life and each week within that timeframe at specific PMA and postnatal ages. These survival curves support our previous findings, indicating that groups of preterm infants with lower HbF percentages in the first four weeks of extra-uterine life were more likely to develop ROP than groups with higher HbF percentages. These results remain statistically significant in the HbF groups from the second to fourth week of life.

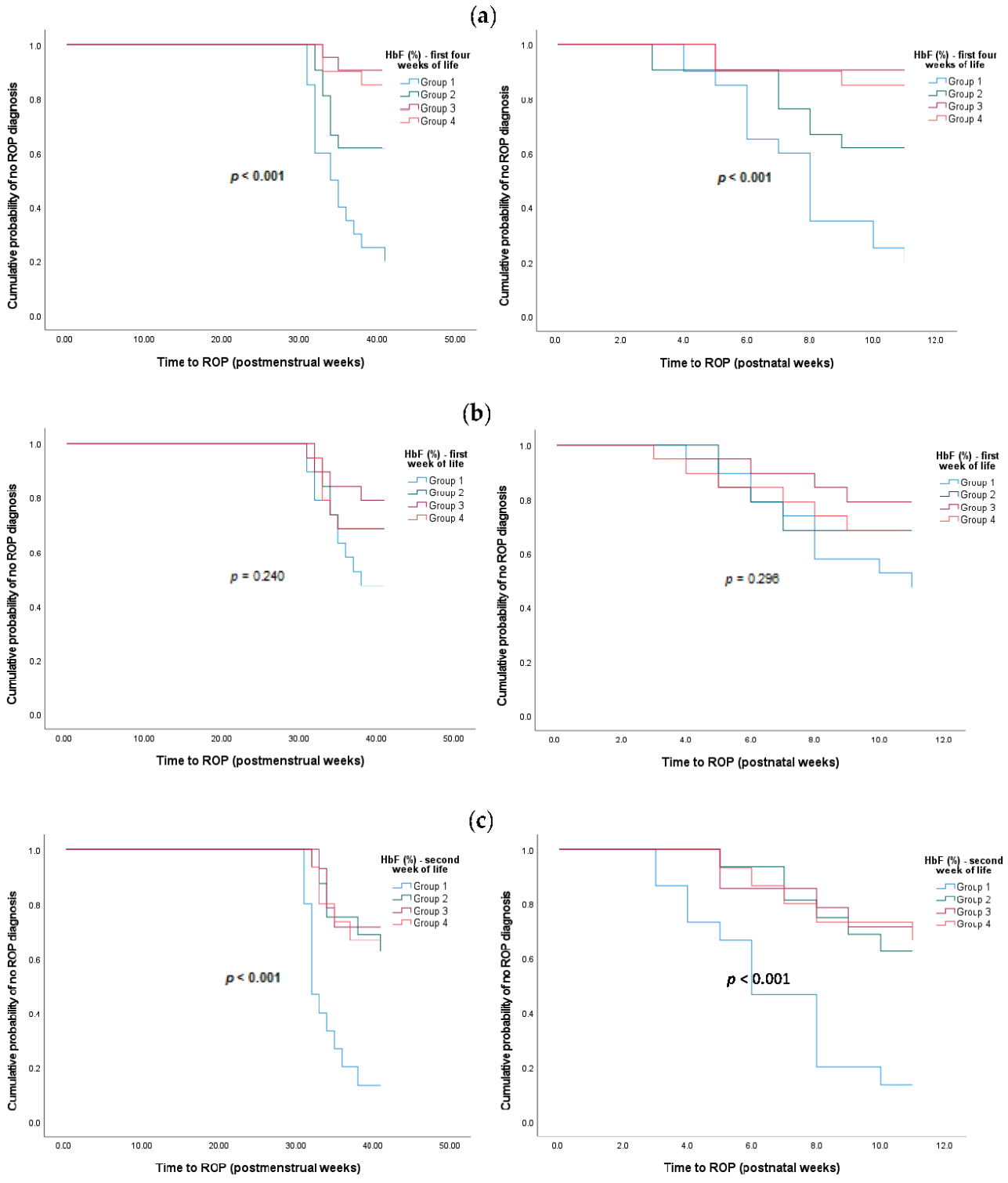


Figure 1. *Cont.*

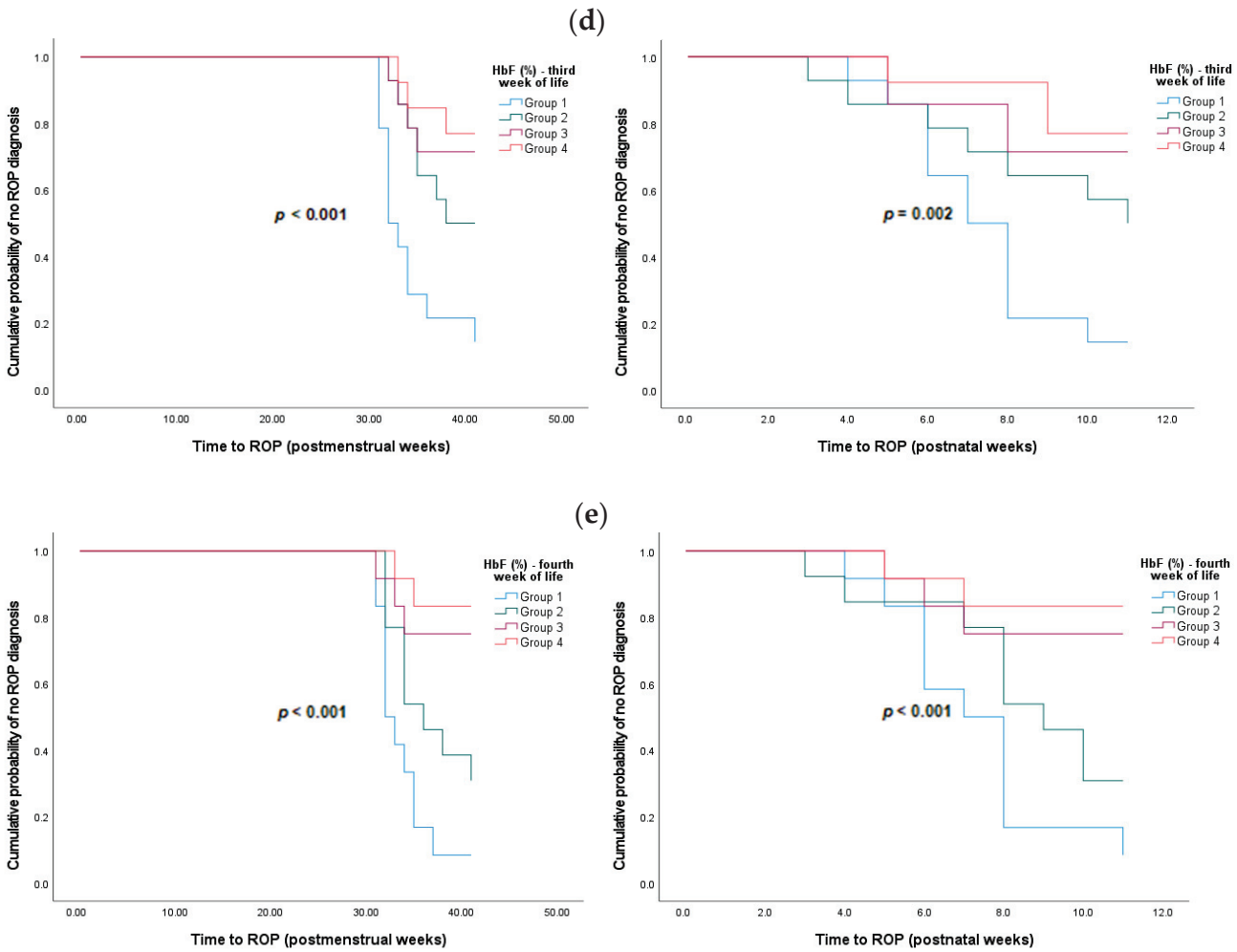


Figure 1. Kaplan–Meier survival curves for ROP diagnosis of preterm infants at specific PMA and postnatal ages, based on HbF percentages divided into quartiles for (**a**) mean of the first four weeks of life (n = 82); (**b**) the first week of life (n = 76); (**c**) the second week of life (n = 60); (**d**) the third week of life (n = 55); (**e**) the fourth week of life (n = 49). Group 1: minimum–first quartile; Group 2: first quartile–second quartile; Group 3: second quartile–third quartile; Group 4: third quartile–maximum. p-values less than 0.05 are in bold.

4. Discussion

RBC transfusions are essential for the survival of prematurely born infants experiencing severe anemia or hemorrhage. However, these transfusions may also contribute to common morbidities observed in NICU settings.

A notable association has been established between RBC transfusions administered during the first four weeks of life and the subsequent development of ROP in our cohort. This association remained significant even after accounting for GA. Previous studies support this link. For example, Lust et al. reported a fourfold increased risk of severe ROP with transfusions during the first ten days of life [19]. Similarly, Teofili et al. found that infants with severe ROP received transfusions earlier, as measured by PMA or postnatal days [22]. Del Vecchio et al. observed that reducing RBC transfusions decreased the incidence of ROP, bronchopulmonary dysplasia, and necrotizing enterocolitis [18].

Emerging evidence suggests that RBC transfusions elevate free iron levels and oxidative stress in preterm infants, contributing to ferroptosis, an iron-dependent form of programmed cell death [27,28]. Our prior research links high erythroblast counts during the first week of extra-uterine life with ROP development, suggesting that anemia and transfusions may play roles in this process [23]. Increased erythroblast production during

anemia results in erythroferrone (ERFE) secretion, which suppresses hepatic hepcidin synthesis, leading to enhanced iron absorption [29]. This iron overload, compounded by free iron from transfusions, raises the risk of retinal ferroptosis, potentially damaging the blood–retina barrier and contributing to ROP pathogenesis [28]. Ferroptosis, therefore, may represent a mechanistic link between anemia, transfusions, and ROP, warranting further investigation.

Approximately 50% of infants born before 32 weeks of GA and 85% of those weighing less than 1000 g receive RBC transfusions during their NICU stay [13].

Neonatal anemia is defined as hemoglobin or hematocrit levels below the average, typically more than two standard deviations below the mean for the infant's age after birth [30]. Except for extremely preterm infants, hemoglobin levels at birth are higher than those later in life, compensating for relative intrauterine hypoxia [13]. After birth, hemoglobin levels decrease as the rate of RBC death exceeds the replacement rate [13]. Physiological anemia is common in nearly all newborns during the first months of life and is more pronounced in very preterm infants, primarily due to their impaired ability to increase erythropoietin production in immature kidneys [13,31]. Other significant causes of neonatal anemia include phlebotomy-related blood losses for laboratory testing and increased RBC degradation [32]. Additionally, in sick preterm infants, hematocrit levels decline further after birth due to various other factors. It has been shown that low hematocrit levels adversely affect short-term outcomes in preterm infants [33].

Given the apparent association between low HbF percentages in early life and the development of ROP in preterm infants, we aimed to evaluate HbF percentages during the first four weeks of life as a potential predictive biomarker for ROP development. Our study found that lower mean HbF percentages during this period were strongly predictive of ROP. In particular, the decline in HbF percentages, especially between the second and fourth week of life, was correlated with an increased susceptibility to ROP. This finding aligns with the cumulative effect of adult RBC transfusions in reducing the HbF fraction. Furthermore, a study by Stutchfield et al. reported that preterm infants with lower HbF fractions during their NICU stay were at a higher risk for ROP development [17]. Similarly, Hellström et al. found that low HbF fractions during the first week of extra-uterine life independently predicted ROP in preterm infants [14]. A study by Teofili et al. also revealed that preterm infants receiving RBC transfusions from adult donors exhibited a 40% decrease in HbF percentages with each transfusion [34]. Notably, these percentages recovered in the absence of further transfusions; however, repeated transfusions resulted in an irreversible decline in HbF levels, underscoring the critical importance of the timing of successive transfusions [34].

In our analysis, lower mean HbF percentages in the first four weeks of life were associated with developing severe ROP requiring treatment. Weekly analysis showed that this correlation remained significant during the second and third weeks of life, but it was not independent of GA. It is worth noting that the treatment-requiring group was small, consisting of only four patients, which may have influenced the results of this sub-analysis.

When adult blood is transfused to preterm infants, it replaces the physiological content of HbF with HbA. HbF differs from HbA because it has two γ subunits, while HbA has two β subunits. The γ subunit in HbF has a lower binding affinity for 2,3-diphosphoglycerate in the deoxygenated state, effectively increasing its oxygen binding affinity [15]. The Bohr effect offsets this potential disadvantage, where hemoglobin releases oxygen in response to increased CO_2 and decreased pH [16]. These characteristics give HbF a higher oxygen affinity, a leftward shift in the oxyhemoglobin dissociation curve, and a steeper oxyhemoglobin dissociation curve. Consequently, HbF delivers oxygen to tissues more effectively by releasing oxygen earlier during hypoxia and with minimal changes in tissue

oxygenation [35]. Additionally, HbF impedes the release of heme groups and demonstrates increased pseudo-peroxidase activity, facilitating the rapid reconversion of reactive ferryl heme. Furthermore, HbF has a greater capacity to produce unbound nitric oxide through oxidative denitrosylation, which regulates vascular tone and blood flow [14,22]. The PacIFiHER study highlighted that low HbF fractions in preterm infants are associated with an increased risk of ROP and poorer systemic oxygenation indices [16].

It is well established that even a small increase in the HbF fraction can significantly benefit the management of sickle cell disease and β-thalassemia. In β-thalassemia, this benefit is linked to HbF's potent antioxidant capacity, which helps reduce the oxidative stress that contributes to hemolytic crises. Additionally, HbF's higher oxygen-binding affinity compared to HbA allows it to capture more oxygen in the lungs and placenta. At the tissue level, its greater nitrite reductase activity promotes vasodilation and facilitates oxygen delivery. Together, these properties underscore HbF's protective role in conditions like sickle cell disease and β-thalassemia, as well as its essential function in supporting preterm infants' transition to an oxygen-rich environment after birth [22].

Therefore, HbF's high oxygen affinity and unique structure may provide significant protective benefits across the two phases of ROP development. In the initial hyperoxic phase, where postnatal oxygen levels inhibit retinal vascularization, HbF may support controlled oxygen delivery, alleviating oxidative stress and reducing the risk of retinal damage. In the subsequent hypoxic phase, an elevated HbF fraction may enhance oxygen transport to retinal tissues, helping to counterbalance the low-oxygen environment and mitigate hypoxia-driven damage. This dual function of HbF underscores its potential protective role against the oxygen fluctuations that contribute to ROP development, suggesting that a lower HbF fraction in early life may serve as both a biomarker and a modifiable risk factor, reinforcing the value of HbF-sparing strategies in mitigating ROP progression.

The current treatments for ROP primarily involve anti-VEGF therapy and laser photocoagulation, which target proliferative ROP. However, it is essential to explore additional interventions focused on preventing ROP. Our research and previous studies highlight the importance of identifying high-risk infants and enhancing their natural protective mechanisms, such as avoiding the rapid decrease in HbF early in life, to minimize or prevent ROP development. Strategies to reduce the use of adult RBC transfusions could be instrumental in achieving this goal. Potential approaches include implementing delayed umbilical cord clamping, adopting blood-sparing sampling techniques to minimize blood loss, and considering the use of placental umbilical cord RBC instead of adult RBC for transfusions in preterm infants [12,14,36]. Another potential alternative is the administration of hydroxyurea to elevate the HbF fraction in high-risk infants [35].

Delayed cord clamping at birth has proven highly effective in preventing neonatal anemia [14]. According to a Cochrane meta-analysis, the resultant autotransfusion of fetoplacental blood reduces the need for RBC transfusions [37], which helps maintain HbF levels [17].

A multicenter study is currently evaluating the impact of microsampling on HbF preservation to reduce neonatal morbidities, including ROP (NCT04239690) [14].

Using umbilical cord blood, rather than adult blood, for RBC transfusions in preterm infants is another promising strategy for preventing complications related to low HbF percentages caused by adult RBC transfusions [38]. Umbilical cord blood, typically discarded after delivery, contains nearly 100% HbF. According to Podraza, approximately 100 mL of blood can be collected from the umbilical cord after the delivery of a full-term newborn. For a preterm infant weighing 500 g, only 7.5 mL of blood is needed for transfusion, based on the standard requirement of 15 mL/kg [12]. While umbilical cord blood transfusion represents an innovative approach to ROP prevention, it poses logistical challenges, in-

cluding the establishment of cord blood banks, distribution systems, and considerations related to cost-effectiveness [12,39]. This approach, although complex, aims to administer RBC exclusively rich in HbF for all transfusions, thereby reducing the HbA content in the neonatal circulation. The ongoing multicenter randomized clinical trial BORN study is investigating the potential benefits of this method (NCT05100212) [40].

Future research should focus on assessing the efficacy and feasibility of implementing these strategies in clinical settings to determine their potential for managing ROP in preterm infants.

Current evidence suggests that stricter transfusion protocols may help mitigate transfusion-related complications in preterm infants [41,42]. A survey conducted in European NICUs revealed variability in transfusion practices, with many centers using more liberal thresholds compared to recent trials advocating for more restrictive practices [43]. Further investigation is required to understand the factors influencing the incorporation of these trial findings in neonatal transfusion protocols.

Our study has several limitations that need to be considered. Firstly, the relatively small sample size, particularly regarding the limited number of infants diagnosed with ROP requiring treatment, increases the risk of Type II error and limits the generalizability of our findings to broader populations. This limitation is inherent to the study design, as we focused on a subgroup of 82 preterm infants with sufficient blood samples for consistent longitudinal HbF assessment during the first four weeks of life. While this approach ensured robust analyses within a consistent cohort, it inherently restricted the scope of our findings. Additionally, our investigation focused solely on the occurrence of ROP and the presence of severe ROP requiring treatment rather than encompassing all stages of ROP, which may also limit the scope of our findings. Another limitation is that our investigation relied on blood samples collected during routine clinical care, without strictly standardized timing for sample collection. Furthermore, although we adjusted for GA, a key risk factor for ROP identified in prior analyses of the full cohort of 455 preterm infants [23], the complexity of ROP pathogenesis and the observational nature of the study limited our ability to fully account for all potential confounders. Despite these limitations, our findings demonstrated a robust association between HbF percentages in the initial four weeks of extra-uterine life and the development of ROP. This supports HbF's potential as a predictive biomarker for ROP.

Future studies with larger, multicenter cohorts are needed to validate our findings, further investigate the role of HbF in ROP pathogenesis, and evaluate preventive strategies aimed at preserving HbF levels, such as reducing adult RBC transfusions or employing alternative transfusion approaches. Such research will be essential to advancing our understanding of ROP and improving outcomes for preterm infants.

To summarize the study's findings and implications, Figure 2 presents a comprehensive flowchart highlighting the key aspects and take-home messages of the research.

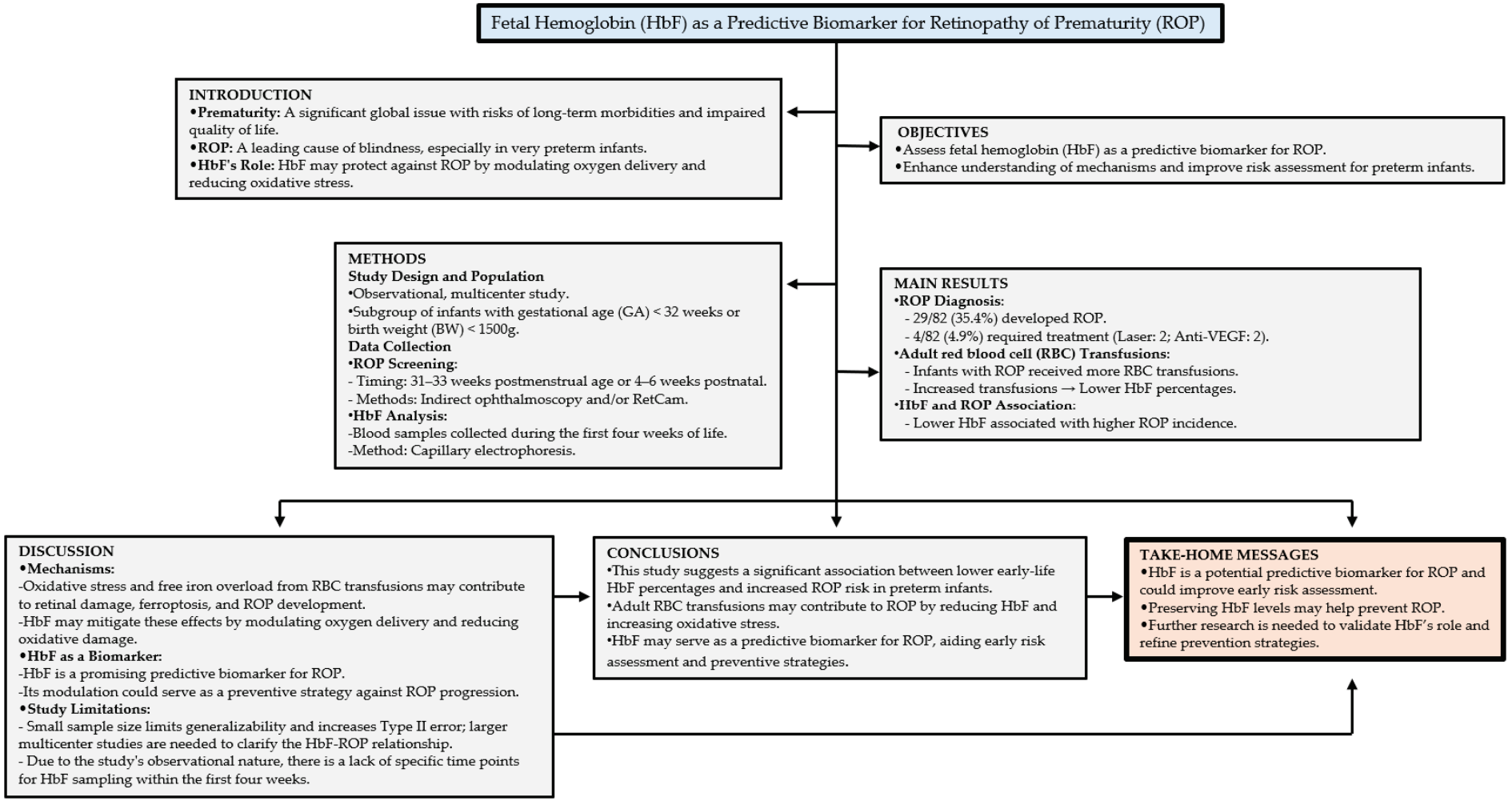


Figure 2. Study overview and key insights. Flowchart summarizing the study's design, results, and practical implications of HbF for ROP.

5. Conclusions

Our study highlights the significant role of hemoglobin fractions, particularly HbF, in the pathogenesis of ROP among preterm infants. The consistent association between lower HbF percentages and increased risk of ROP underscores its potential as a valuable, low-cost predictive biomarker for identifying high-risk infants. Incorporating HbF monitoring into neonatal care could refine risk stratification and enhance early screening protocols, enabling more targeted interventions.

Additionally, the potential benefits of reducing adult RBC transfusions—through strategies such as delayed cord clamping, cord blood transfusions, and restrictive transfusion protocols—warrant further exploration. These interventions may help mitigate the harmful effects of oxidative stress and iron overload, both of which contribute to ROP pathogenesis.

While this study emphasizes HbF's importance, the findings are limited by the relatively small sample size and observational design, necessitating further validation in larger cohorts. Such research will be crucial to confirming HbF's role as a biomarker and evaluating the effectiveness of preventive strategies. Expanding our understanding of HbF's protective role could ultimately improve outcomes for preterm infants at risk of ROP and reduce the global burden of this condition.

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Institutional Review Board Statement: The study (ISRCTN16889608) adhered to the ethical standards of the Helsinki and Tokyo Declarations. The study protocol, data collection instruments, and informed consent forms received approval from the Scientific Council of the Faculty of Medicine of the University of Lisbon and the scientific councils and Ethics Committees of all participating hospital centers, including the Centro Hospitalar Universitário de Lisboa Norte (reference number 340/2018) and other centers (Ethics Committee of Hospital da Senhora da Oliveira Guimarães reference number 01/19-CAc, Ethics Committee of Centro Hospitalar Universitário de São João reference number 20-19, Ethics Committee of Hospital Professor Doutor Fernando Fonseca reference number 70/2018, Ethics Committee of Hospital de Braga reference number 15/2019, Ethics Committee of Centro Hospitalar e Universitário de Coimbra reference number CHUC-014-019, Ethics Committee of Centro Hospitalar Universitário do Porto reference number 031-DEFI/032-CE). All data were handled confidentially, with participants' anonymity preserved. Written informed consent was obtained from the parents or legal guardians of all infants.

Informed Consent Statement: Written consent was obtained from the parents of all infants participating in the study.

Data Availability Statement: The datasets analyzed in this study are available from the corresponding author upon reasonable request.

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Review

Insights into the Global and Mexican Context of Placental-Derived Pregnancy Complications

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Abstract: Placental-derived pregnancy complications encompass a range of disorders that hinder optimal fetal development, significantly impacting maternal and neonatal health outcomes. Key conditions include placental insufficiency, preeclampsia, fetal growth restriction (FGR) or intrauterine growth restriction (IUGR), fetal overgrowth, and gestational diabetes mellitus (GDM), which together contribute to a heightened risk of preterm birth, perinatal mortality, and long-term developmental challenges in affected infants. These complications are particularly notable because they generate approximately 80% of pregnancy disorders and pose significant public health concerns across diverse global contexts. Their management continues to face challenges, including a lack of consensus on diagnostic criteria and varied implementation of care standards. While imaging techniques like magnetic resonance imaging (MRI) and Doppler ultrasound have emerged as critical tools in clinical assessment, disparities in access to such technologies exacerbate existing inequalities in maternal and fetal health outcomes. Maternal and pregnancy care is a broad range of services aimed at promoting the well-being of women throughout the perinatal period. However, access to these services is often limited by economic, geographical, and sociocultural barriers, particularly for marginalized groups and women in low- and middle-income countries (LMICs). The implementation of targeted interventions designed to address specific obstacles faced by disadvantaged populations is a crucial component of bridging the gap in health equity in maternal care. Public health authorities and policymakers strive to develop evidence-based strategies that address the interplay between healthcare access, socioeconomic factors, and effective interventions in order to mitigate the adverse effects of placental-derived pregnancy complications. Continued research and data collection are essential to inform future policies and practices to improve outcomes for mothers and infants.

Keywords: pregnancy complications; incidence; prevalence; trends; healthcare strategies; public policies

1. Introduction

The placenta is a vital temporary organ that develops during the first three months of pregnancy and expands in tandem with the growth of the uterus, serving as a life-sustaining mechanism for fetal development [1]. It is responsible for numerous crucial functions, among which are (1) the provision of essential nutrients to the fetus, facilitating the transfer

of nutrients from the mother's bloodstream to the fetal circulation [2], (2) the oxygen transmission from the maternal blood to the fetal blood, while simultaneously removing waste products, such as carbon dioxide, urea, and creatinine from the fetus [3], (3) the secretion of various hormones, including human chorionic gonadotropin (hCG), human placental lactogen (hPL), estrogens, and progesterone, which are crucial for regulating maternal metabolism and preparing the body for childbirth [4], and (4) the provision of fetal protection from certain infectious pathogen transmissions, as well as the transfer of maternal antibodies during the early stages of life [5].

Several maternal factors, including hormonal and nutritional statuses, environmental exposures, and genotypes and epigenotypes, interfere with placental functions, which can have a significant impact on fetal outcomes [6,7] (see Figure 1). Pregnancy complications arising from abnormal placental function can lead to significant maternal and fetal health issues in the short and long terms [8]. In this timing sense, abnormalities in placental function can lead to various pregnancy complications in relation to placental insufficiency, maternal metabolism, and fetal growth, which have significant long-term health implications for both the mother and the newborn [6,9].

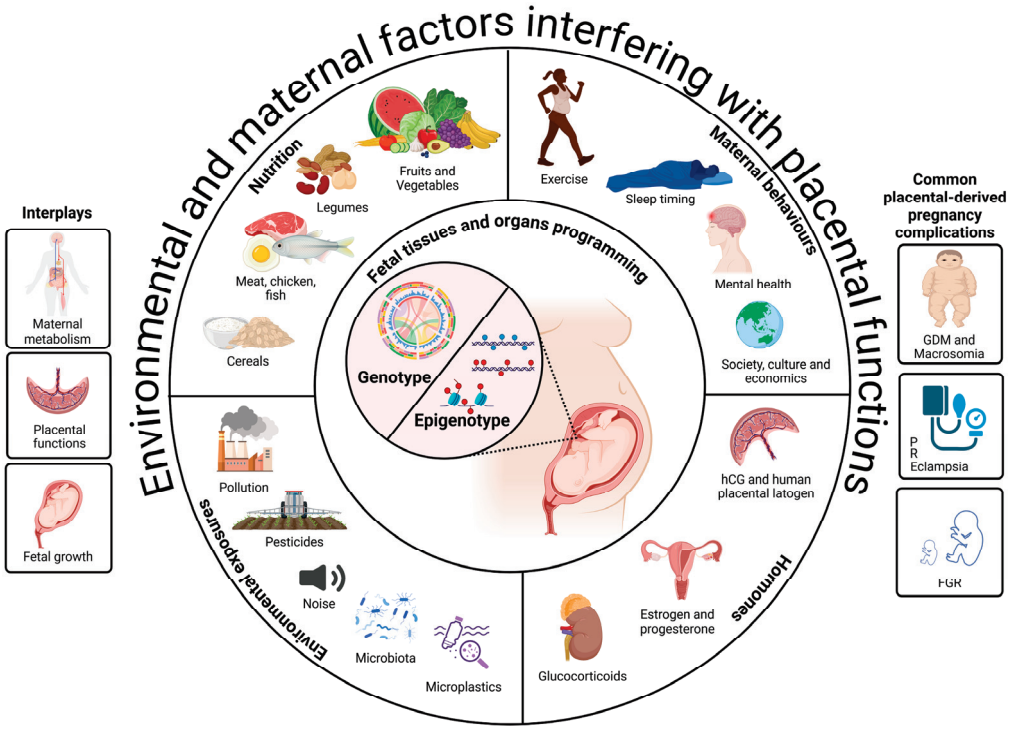


Figure 1. The intricate relationship between maternal metabolism, placental function, and fetal growth health. This scheme depicts the interplay between maternal metabolism, placental functions, and fetal growth as critical aspects for shaping the development of the fetus and influencing long-term health outcomes for both mother and neonate. Environmental factors, maternal nutrition and behaviors, and circulating maternal hormones interfere with components of the placental function that, consequently, impact fetal and neonatal health. During pregnancy, exposure to these different components can alter the genotype and epigenetic composition of the placenta, affecting its hormone production and creating a feedback loop that affects both nutrient availability and fetal health outcomes. The primary severe complications of pregnancy resulting from placenta dysfunction are DGM, preeclampsia, and FGR. These diseases have significant public health concerns across the globe and in diverse contexts. Abbreviations: hCG, human chorionic gonadotrophin; FGR, fetal growth restriction; GDM, gestational diabetes mellitus. Created in https://BioRender.com (access date: 3 January 2025).

Long-term studies on placental disorders have increasingly demonstrated that the outcomes of pregnancy complications have an impact on the health of both mothers and their newborns [10]. For instance, women who experience preeclampsia or GDM are at a heightened risk for chronic health conditions, including cardiovascular disease, hypertension, cerebrovascular events, and metabolic disorders, later in life [11]. In a similar manner, neonates who have been exposed to preeclampsia exhibit a significant predisposition to cardiovascular diseases and metabolic issues as young adults [12]. These phenomena underscore the concept of developmental programming, which suggests that certain prenatal conditions can lead to chronic health issues later in life, regardless of genetic predisposition. In conjunction with this concept, the Barker hypothesis proposes that prenatal stressors, such as maternal malnutrition or psychological stress, may result in an elevated risk of metabolic syndrome and related diseases in offspring, thereby establishing a cycle of health challenges that may impact maternal health as well [13]. Additionally, exposure to obesity and a high-fat diet during pregnancy has been linked to adverse outcomes, such as increased appetite in offspring and a predisposition to obesity and metabolic diseases [10]. The importance of maternal nutrition cannot be overstated; inadequate intake of micronutrients can adversely impact fetal growth and development, particularly in low-income groups where access to nutrient-dense foods may be restricted [14,15].

Research on placental dysfunction has predominantly focused on high-income countries (HICs), resulting in a disparity in comprehension among LMICs. Placental disorders are linked to higher rates of maternal and perinatal mortality in LMICs, with over 95% of these deaths occurring in these regions. Factors such as malnutrition, inaccessibility to healthcare, and infectious diseases compound the hazards associated with placental dysfunction [16].

Health equity refers to the principle that all individuals should have fair access to quality healthcare, resulting in equal health outcomes across different population groups [17]. The achievement of health equity remains a significant global challenge, particularly in the field of maternal and pregnancy-related care, where disparities are evident. Evidence suggests that healthcare inequities disproportionately impact women, especially those who are pregnant or birthing, as well as their infants and families [18]. Despite the advancements in healthcare policies, substantial disparities persist, particularly among underrepresented groups, commonly referred to as underreported (U3) populations. These disparities lead to elevated rates of maternal mortality and morbidity, as well as adverse health outcomes for infants, largely attributed to social determinants of health, such as poverty, discrimination, and inadequate healthcare resources [19]. Placental disorders further complicate the landscape of maternal health, encompassing diverse conditions that have the potential to adversely affect pregnancy outcomes [6,7].

In order to reduce pregnancy complications related to placental dysfunction, with the most common and high prevalence in LMICs, it is crucial to consider local and regional health determinants. The aim of this review is to focus on the impacts of placental dysfunction-related pregnancy complications in a global and Mexican context, discussing public policies, emerging insights, technological advances, gaps in research, and future directions.

2. Pregnancy Complications with a High Prevalence in Human Populations Related to Placental Dysfunction

Placental abnormalities are conditions in which the placenta does not develop or function properly during pregnancy. These abnormalities can arise at any stage of placental development and may significantly impact both maternal and fetal health. They can disrupt essential functions of the placenta, leading to various complications during pregnancy and delivery. Placental anomalies, such as placental previa, placental accreta

spectrum, placental abruption, vasa previa, chorioangioma, and choriocarcinoma, are distinguished by distinctive characteristics, with a prevalence ranging from 0.3% to 2% among populations [20]. Although the identification, monitoring, and treatment of placental abnormalities during pregnancy pose significant challenges, the present review will solely focus on pregnancy complications with a high prevalence.

Pregnancy complications, which are prevalent among pregnant women, are frequently associated with placental anomalies, resulting in distinct risks and management challenges. Complications such as FGR, fetal overgrowth, small and large for gestational age (SGA and LGA, respectively), preeclampsia, GDM, and preterm birth, among others, are the main pregnancy complications that have significant long-term health implications for both the mother and the newborn [6,9]. Understanding the interplay between maternal metabolic status, placental function, and fetal growth is crucial for improving pregnancy and neonatal outcomes.

2.1. Maternal Metabolic Status-Associated Disorders

Maternal metabolism throughout pregnancy undergoes significant adaptations, including increased insulin resistance, altered fat metabolism, and modifications in glucose homeostasis (normal physiological responses), ensuring an adequate supply of glucose to the fetus. In this regard, a healthy metabolic state is essential for optimal placental function and fetal development [21].

For instance, maternal obesity can lead to an increased risk of GDM, characterized by glucose intolerance diagnosed between 24 and 28 weeks of gestation [22]. It poses risks not only to the mother, including an increased likelihood of developing type 2 diabetes postpartum [23], but also to the fetus (overgrowth), leading to structural and functional changes in the placenta, including delayed maturation, inadequate nutrient and oxygen transfer to the fetus, and involving a combination of oxidative stress and inflammatory responses. These alterations may result in severe complications such as macrosomia and metabolic disorders later in life [8]. The implications of these disorders are not only in the placenta but extend beyond the immediate postpartum period, and they can lead to a cycle of obesity and metabolic disease across generations, with fetal exposure to an unhealthy intrauterine environment playing a critical role in developmental programming [23].

2.2. Placental Insufficiency-Associated Disorders

Abnormal placental function can lead to conditions such as FGR (also known as IUGR), preterm birth, and preeclampsia, which are severe complications associated with adverse pregnancy outcomes [24]. FGR, which is used to define fetuses with an estimated fetal weight that is less than the 10th percentile for gestational age, can arise from various factors affecting the placenta, umbilical cord, and maternal (hypertension and diabetes) or fetal (genetic disorders or congenital anomalies) conditions [9,25]. Maternal hypertension and inadequate placental blood flow are factors also related to preterm birth [26]. Preeclampsia is another complication associated with placental insufficiency that shares some pathophysiological traits with FGR, as both conditions can stem from inadequate remodeling of maternal blood vessels supplying the placenta [24].

Research indicates that maternal health prior to conception, including nutritional status and metabolic health, influences placental development and function [1]. Some of the causes of placental dysfunction or placenta insufficiency are in connection with vascular remodeling issues, maternal chronic diabetes, hypertension, and autoimmune disorders, as well as lifestyles factors such as smoking and drug use. In connection, the few observable symptoms reported by healthcare providers include decreased fetal movement and smaller-than-average fundal height measurements [27].

2.3. Fetal Growth-Associated Disorders

Fetal growth is influenced by the interplay between maternal metabolic status and placental function (Figure 1). Adequate nutrient supply from the mother through a well-functioning placenta is necessary for proper fetal development [25]. On the contrary, fetal growth disorders may arise from a variety of etiological factors, such as placental insufficiency, maternal health conditions such as hypertension and diabetes, inadequate nutrition, substance abuse, infections, and genetic abnormalities [9,25]. The timing of delivery is critical, particularly in cases of FGR, as it must balance the risks of prematurity and stillbirth [26].

Fetal growth disorders, including SGA and LGA, arise when a fetus fails to meet expected growth standards during pregnancy, resulting in complications such as increased risks of delivery challenges and neurodevelopmental and metabolic issues. The term SGA refers to newborns whose birth weight is less than the 10th percentile for their gestational age, compared to the term LGA that refers to those whose birth weight exceeds the 90th percentile [28]. Both SGA and LGA infants may face challenges during delivery, such as necessitating cesarean sections or experiencing birth injuries. Additionally, they may encounter complications such as feeding difficulties, temperature instability, and elevated risks of subsequent health issues [28,29].

Studies have shown that interventions aimed at improving maternal nutrition can lead to better pregnancy outcomes, particularly in populations with high rates of undernutrition. Furthermore, maternal education on nutrition is vital for ensuring that women understand the importance of dietary choices during pregnancy [30].

3. Global Versus Mexican Impact of Placenta-Derived Pregnancy Complications

The relevance of pregnancy complications is underscored by rising maternal mortality rates in various regions, particularly in the United States, where the maternal mortality rate has more than doubled from 1987 to 2018, reaching 17.4 deaths per 100,000 live births. Globally, the maternal mortality ratio of pregnancy complications has declined by 38% between 2000 and 2017 [30]. However, most maternal deaths still occur in LMICs, where access to quality healthcare remains limited [31]. Racial and geographic disparities are significant; black women are three times more likely to die from pregnancy-related causes compared to white women, highlighting systemic inequities in healthcare access and outcomes [32]. Understanding the mechanisms behind placental function is crucial for improving pregnancy outcomes and reducing the risks associated with placental-related disorders.

3.1. Impact of Placental-Derived Pregnancy Complications in the Globe

The incidence of pregnancy complications is influenced by various factors, including socioeconomic status, access to healthcare services, and environmental conditions [31]. In the U.S., over 60,000 birthing individuals experience severe maternal morbidity annually, with a notable rise in such cases in recent years [30]. The current situation regarding pregnancy complications is alarming in that the implications of placental insufficiency extend beyond individual pregnancies, affecting public health systems globally [8].

Around the globe, ten million women develop preeclampsia annually, resulting in significant morbidity and mortality. The global burden includes an estimated 76,000 maternal deaths and 500,000 infant fatalities each year attributed to hypertensive disorders of pregnancy, including preeclampsia. In Latin America, preeclampsia is recognized as the leading cause of maternal mortality [33].

The management of complications arising from abnormal placental function contributes significantly to healthcare expenditure. Increased rates of neonatal intensive care unit (NICU) admissions for FGR and LGA infants elevate costs [6]. Children who experience FGR are at a higher risk for stillbirth, neonatal complications, cardiovascular diseases, and metabolic disorders. LGA infants face risks during delivery, including birth trauma and increased likelihood of cesarean delivery. They are also at risk for metabolic syndrome costs later in life [25]. The prevalence of these conditions can lead to broader societal challenges, including workforce productivity losses and increased healthcare demands as affected individuals age [34].

Abnormalities in placental function can lead to conditions such as FGR or fetal overgrowth, each associated with increased morbidity and mortality. FGR is characterized by the failure of the fetus to reach its genetically determined growth potential, primarily due to placental insufficiency [25]. This condition affects 5–10% of pregnancies and is linked to various maternal factors, including poor nutrition, smoking, and pre-existing health conditions like hypertension. In developed countries, the prevalence tends to be lower due to better prenatal care compared to developing regions, where it can be significantly higher [35]. Conversely, fetal overgrowth can occur due to maternal obesity, diabetes, or excessive gestational weight gain [36]. The incidence of LGA has been rising in recent years, correlating with increasing rates of maternal obesity [25]. Current estimates suggest that LGA affects about 10–15% of pregnancies, with ongoing research indicating a potential rise in prevalence linked to lifestyle changes and dietary habits [36].

A systematic review of 25 studies involving 2895 pregnancies complicated by severe early-onset FGR found that the antenatal mortality rate was approximately 12%. Specifically, 355 fetuses died antenatally out of 2895 pregnancies. The neonatal mortality rate was reported at about 8%, with 192 live-born neonates dying in the neonatal period. Overall, 81% of pregnancies complicated by early-onset FGR survived to birth. However, long-term follow-up indicated that about 12% of survivors experienced neurodevelopmental impairment or cerebral palsy [37].

Pregnancies complicated by fetal overgrowth (often linked to conditions like gestational diabetes) have been associated with increased perinatal morbidity and mortality. Specific mortality rates for fetal overgrowth were not detailed in the sources, but it is known that such conditions elevate risks for complications during delivery and long-term health issues for the offspring. Children born from pregnancies with fetal overgrowth are at higher risk for developing obesity, diabetes, and cardiovascular diseases later in life [25].

3.2. Impact of Placental-Derived Pregnancy Complications in Mexico

The incidence of fetal overgrowth, specifically referred to as fetal macrosomia (FM), in Mexico has been assessed in several studies. Historically, the prevalence of FM in Mexico has varied. A previous estimate from 2004 to 2005 indicated a prevalence of 3.8%, which aligns more closely with recent findings than earlier studies that reported higher rates, such as an 18.6% incidence observed in a hospital study in Saltillo. In 2020, the national incidence rate of macrosomia is approximately 2.75%, with a notable difference between genders; it is higher in males at 3.17% compared to 2.31% in females. The rates can vary by state, with some regions reporting higher incidences of LGA births. The states with the highest incidences were Sonora (6.2%), Baja California Sur (5.44%), and Sinaloa (5.36%). In contrast, Mexico City reported the lowest incidence at 1.28% [38]. Several factors contribute to fetal overgrowth, including maternal obesity and gestational diabetes, which are increasingly prevalent in Mexico [22]. Socioeconomic factors also play a role, as certain populations, such as the Maya community, exhibit different growth patterns influenced by their sociodemographic conditions [39].

The incidence of preeclampsia in Mexico is a significant public health concern, with estimates suggesting a prevalence ranging from 10% to 14% according to the World Health Organization (WHO) [40]. Preeclampsia is responsible for approximately 4000 maternal deaths annually in Mexico, highlighting its severity as a health issue. Among reported cases, about 94% are classified as mild preeclampsia, while severe cases account for approximately 3.75%, and eclampsia for about 1.75% [41]. Other studies show a prevalence that can reach up to 16.7%, indicating significant variability depending on the population studied and the methodologies employed [42]. The prevalence rates are influenced by several factors, including access to healthcare, socioeconomic conditions, and the presence of risk factors such as obesity. For instance, obesity is identified as a significant risk factor for developing preeclampsia, with women having a body mass index (BMI) ≥ 30 kg/m^2 being at higher risk [33].

The prevalence of GDM in Mexico is generally estimated to be between 10% and 12% based on various studies; this figure may not fully capture the national prevalence due to insufficient data [43]. A recent study reported a prevalence of 44.5% using the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria and 37.5% using the National Institute for Health and Care Excellence (NICE) criteria in a cohort from Northeast Mexico [44]. A survey among healthcare practitioners indicated a clinician-reported prevalence of 23.7%, reflecting regional differences in GDM incidence across Mexico [45]. This finding indicates that the choice of diagnostic criteria can significantly affect reported prevalence rates.

In another instance, a national retrospective study indicated that approximately 28.8% of adolescents in certain regions of Chiapas experienced FGR, highlighting a substantial prevalence among vulnerable populations [46]. In Yucatan, a study found the prevalence of FGR to be 9.06%, with higher rates among the Maya population at 12.4% [26]. For instance, Baja California Sur reported an LGA prevalence of 16.8% [47].

The incidence of FGR varies significantly across different regions and populations in Mexico. Recent studies indicate that approximately 28.8% of infants experience FGR, which is a specific type of FGR characterized by low birth weight (LBW). A study in Chiapas found that 12% of the sample had LBW, with FGR being prevalent among adolescents from indigenous communities. This suggests a strong correlation between socioeconomic factors and fetal growth outcomes [46]. In Guanajuato, the leading causes of infant mortality are conditions originating during the perinatal stage, with a sizable portion attributed to congenital malformations and chromosomal abnormalities. The region has reported high antenatal care coverage, yet disparities remain for marginalized populations [48]. Regional research indicates that FGR is more common among Maya women compared to non-Maya women, highlighting the influence of ethnic and socioeconomic conditions on fetal growth [39].

The perinatal mortality rate has been historically underreported in Mexico, particularly concerning stillbirths [49]. The linkage of live birth and death records from 2008 to 2019 revealed that neonatal mortality rates were significantly higher for preterm births compared to term births, as well as for SGA infants compared to those appropriate for gestational age (AGA). However, LGA infants did not show an increased mortality risk compared to AGA infants [50]. In 2020, Mexico reported 22,637 fetal deaths, with a considerable proportion attributed to conditions related to FGR. The study emphasized that conditions originating during the perinatal stage are major contributors to infant mortality [48]. The State of Mexico has been identified as having particularly high rates of morbidity and mortality related to preeclampsia, emphasizing the need for targeted public health interventions in this region [41].

4. Impact of Public Policies on Placental Dysfunction Disorders

Public policies related to maternal and fetal health significantly influence neonatal growth and development. These policies encompass a range of healthcare practices, monitoring protocols, and regulations regarding environmental exposures [30]. The following sections outline key areas where public policies are affecting fetoplacental and neonatal growth.

4.1. Maternal Health Monitoring

Several guidelines, such as those from the Royal College of Obstetricians and Gynaecologists (RCOG), emphasize the importance of accurately assessing fetal growth through ultrasound measurements and Doppler assessments. This is crucial for identifying SGA fetuses and managing conditions like FGR effectively [51].

In the UK, the National Health Service (NHS) has implemented the 'Saving Babies' Lives' Care Bundle initiative aiming to reduce stillbirth rates by 50% by 2025. It highlights evidence-based practices to improve outcomes, particularly for high-risk pregnancies [52]. This policy reflects an increasing focus on targeted interventions that can enhance fetoplacental health.

4.2. Environmental Exposures and Endocrine Disruptors

Air pollution has emerged as a critical risk factor, with multiple studies indicating that exposure to particulate matter (PM2.5, PM10, etc.) can elevate blood glucose levels in pregnant women. Findings from several studies demonstrate a significant association between air pollution and abnormal glucose tolerance during pregnancy, highlighting the need for attention to air quality as a component of prenatal care [53]. For example, a large cohort study found that increased levels of ambient air pollutants correlated with adverse glycemic outcomes [54].

Ambient temperature is another significant environmental factor affecting glycemic control during pregnancy. Increased environmental temperatures have been associated with maternal beta-cell dysfunction and elevated blood glucose levels, suggesting that extreme weather conditions may exacerbate the risk of GDM. In fact, rising temperatures have been consistently linked to higher rates of GDM, with studies indicating that temperatures exceeding 25 °C could lead to detrimental glycemic effects [55].

Exposure to endocrine-disrupting chemicals (EDCs) such as bisphenol A (BPA), persistent organic pollutants (POPs), heavy metals, and phthalates during pregnancy has been linked to adverse outcomes in fetoplacental development, including altered placental function and fetal growth metrics [6]. Elevated levels of arsenic in maternal blood or meconium have been linked to an increased risk of GDM. Other metals such as nickel, antimony, cobalt, and vanadium also show positive associations with GDM risk [54]. Public policies that regulate or limit the use of these chemicals in consumer products can significantly impact maternal environments and subsequently fetoplacental health.

4.3. Healthcare Accessibility and Quality

Policies that ensure equitable access to prenatal care are vital. Disparities in healthcare access can lead to higher rates of stillbirth and complications among marginalized communities. For instance, stillbirth rates are disproportionately higher among Black and Asian women in the UK compared to their white counterparts [34]. In LMICs, there is a pressing need for innovative monitoring technologies to accurately assess fetoplacental health, both in clinical settings and remotely. Current tools have limitations that hinder timely interventions during high-risk pregnancies [31].

On other hand, maternal nutrition plays a critical role in placental development. Policies promoting adequate maternal nutrition, including iron supplementation during pregnancy, are essential, as deficiencies can lead to placental dysplasia and affect fetal growth [52].

The significance of maternal vaccination is also pivotal in safeguarding both mothers and infants from infectious diseases during pregnancy and early life. Vaccines such as the inactivated influenza vaccine (IIV) and the Tetanus, Diphtheria, and Pertussis (Tdap) vaccine have been recognized for their safety and efficacy in pregnant women [56]. Vaccination has the potential to mitigate the risks associated with viral pathogens, as unvaccinated pregnant women exhibit higher rates of severe illness, hospitalization, and complications in comparison to their vaccinated counterparts. This holds particular significance for outbreaks such as influenza and SARS-CoV-2, which have been associated with heightened maternal mortality and adverse fetal outcomes, including placental lesions and fetal mortality in severe cases [57].

4.4. Future Policy Directions

There is a need for continued research into the mechanisms of placental function and the relationship with fetal growth. This includes exploring novel therapeutic strategies aiming to restore normal growth patterns in fetuses affected by placental dysfunction. Effective public health policies should integrate findings from ongoing research to implement guidelines supporting maternal health and fetal monitoring practices. Such policies may involve developing standardized protocols for monitoring fetal growth and managing pregnancies at risk for FGR or overgrowth. Enhancing training for healthcare providers on the latest monitoring techniques and interventions would improve outcomes in pregnancies complicated by abnormal fetal growth.

Access to healthcare is recognized as a universal social right; however, the reality in LMICs often contrasts sharply with that in HICs due to limited health resources that necessitate careful rationing. This situation raises critical issues regarding the equity and efficiency of healthcare systems in these regions [58]. For instance, while HICs generally have more substantial healthcare budgets, leading to better health outcomes, LMICs struggle with inadequate funding and infrastructure, which hampers their ability to address placental disorders effectively. The contrasting experiences of HICs and LMICs underscore the necessity for tailored policy interventions that consider each country's unique context and healthcare challenges. For effective health financing reforms, it is crucial to address the specific performance issues within each country's health financing arrangements [59]. By learning from successful models in HICs and adapting them to the realities of LMICs, policymakers can aim to enhance access, efficiency, and ultimately, the outcomes for mothers and infants affected by placental disorders.

5. Emerging Insights into Placental Function

Emerging insights into placental function reveal significant advancements in understanding the mechanisms and genetic factors influencing placental development, which are crucial for fetal health. Recent studies highlight various aspects of placental function, genetic influences, and technological innovations in research.

5.1. Genetic Influences on Placental Growth

A groundbreaking study conducted by researchers from Copenhagen University Hospital and other institutions identified 40 genetic variations associated with placental growth. This genome-wide association study involved a large cohort of approximately 65,000 children and highlighted that variations in the fetal genome are pivotal for placental

weight. Notably, faster placental growth was linked to an increased risk of preeclampsia and earlier delivery, suggesting that an imbalance between this two factors are crucial. These findings underscores the placenta's essential role as a mediator of maternal–fetal interactions and its impact on pregnancy outcomes [36].

Nutrigenomics is a burgeoning scientific field that investigates the complex interactions between nutrition and gene expression, particularly focusing on how dietary components can influence genetic pathways and overall health outcomes. This discipline is especially significant in the context of fetal health and placental disorders, as maternal nutrition directly affects both fetal development and pregnancy outcomes [60]. The causal mechanisms underlying these associations remain partially unresolved, particularly concerning the relative contributions of maternal and fetal genetic effects. Research should focus on identifying the nutritional components that enhance placental health and prevent associated disorders, paving the way for targeted interventions. By addressing these key areas, future research in nutrigenomics will contribute significantly to enhancing fetal health and preventing placental disorders, ultimately improving maternal and child health on a global scale [61].

5.2. Hypoxia and Placental Function

Another significant insight pertains to the role of hypoxia-inducible factors (HIFs) in placental function. Research indicates that chronic activation of HIF-2α can impair placental development, contributing to conditions such as FGR and preeclampsia. Specifically, increased levels of HIF-2α were observed in pathological placentas, while silencing this factor enhanced trophoblast differentiation and placental growth factor availability. This suggests potential therapeutic avenues for addressing placental dysfunction [62].

5.3. Oxygenation and Fetal Brain Development

The importance of oxygenation levels in the placenta has also been emphasized in recent studies. Research shows that adequate oxygenation during the third trimester is crucial for cortical brain development in the fetus. Insufficient oxygen can hinder cognitive outcomes later in life, linking placental health directly to neurological development. This connection was established through advanced imaging techniques that provided a clearer understanding of how placental conditions affect fetal brain growth [63].

5.4. Role of Biomarkers in Diagnosis

The clinical application of biomarkers to diagnose placental dysfunction remains an evolving field. However, the role of biomarkers in the diagnosis and management of placental dysfunction, particularly in conditions such as GDM, preeclampsia, and FGR, has gained considerable attention in recent research. Biomarkers such as pregnancy-associated plasma protein A (PAPP-A), placental protein 13 (PP13), cell-free DNA (cfDNA), sex-hormone binding globulin (SHBG), follistatin, and myostatin show promise in predicting GDM, with varying sensitivities [64,65]. As counterparts, soluble fms-like tyrosine kinase (sFlt-1) and placental growth factor (PlGF) are integral to the angiogenic profile of preeclampsia and are effective indicators of placental dysfunction [66]. These biomarkers can predict the onset of clinical signs and symptoms, which will facilitate risk assessment and timely intervention for affected mothers and their fetuses. Despite this, current studies underscore the necessity of validating these findings through larger, prospective studies with standardized methodologies.

Currently, several crucial molecules involved in preeclampsia and FGR have emerged as prominent biomarkers in various studies. These include 1-deoxyspinglolipids (1-deoxySLs: 1-deoxysphinganine and 1-deoxysphingosine), polyamines (spermine and spermidine), and enzymatic markers (ornithine decarboxylase, spermidine/spermine

N1-acetyltransferase, and spermine oxidase) [67]. The incorporation of these biomarkers into clinical settings holds promise for augmenting diagnostic precision regarding placental dysfunction. However, prospective validation studies are necessary to establish their clinical utility and further clarify their roles in the pathogenesis of conditions such as preeclampsia and FGR. Research into these biomarkers is advancing our understanding of the complex metabolic alterations associated with placental health and disease, paving the way for potential therapeutic strategies in the future.

5.5. Technological Advances

Innovative technologies are transforming the study of placental health. The introduction of AI-driven tools like HAPPY enables comprehensive analysis of placental histopathology, allowing for rapid assessment of cellular interactions and structural integrity. This technology enhances our ability to predict complications related to placental health, thereby improving maternal and infant care. Such advancements highlight the growing recognition of the placenta's role in pregnancy and the need for detailed investigation into its biology [68].

6. Good Practices for Healthy Placental Function

Public policies related to maternal and fetal health significantly influence neonatal growth and development. These policies encompass a range of healthcare practices, monitoring protocols, and regulations regarding environmental exposures [30]. The following sections outline key areas where public policies affect fetoplacental and neonatal growth.

6.1. Clinical Management

Early detection of growth issues: Implementing routine ultrasound assessment to monitor placental development, fetal growth velocity, and the use of customized growth charts can enhance the detection of SGA infants. Early identification allows for timely interventions that improve outcomes [34]. Regular umbilical artery Doppler assessments are recommended for pregnancies at risk of FGR to monitor blood flow and make informed decisions regarding delivery timing [69].

Interventions for high-risk pregnancies: For pregnancies complicated by obesity or other risk factors, interventions such as low-dose aspirin have been shown to reduce the incidence of FGR by improving placental perfusion. Additionally, managing maternal health through lifestyle modifications, such as smoking cessation, is critical in preventing SGA outcomes [29].

6.2. Addressing Environmental Influences

Endocrine disruptors: Exposure to environmental EDCs such as BPA can negatively impact placental function and fetal growth [70]. Limiting exposure to these substances during pregnancy is essential for maintaining healthy fetoplacental dynamics.

Epigenetic considerations: Understanding the role of epigenetics in fetoplacental development can provide insights into how maternal stressors affect fetal outcomes [71]. Research suggests that epigenetic modifications may allow the fetus to adapt to adverse conditions at the expense of optimal placental function [72].

7. Gaps and Future Research Directions

Despite advancements in understanding placental dysfunctions, significant research gaps remain regarding the effects of various environmental factors and genetic influences on placental development. Public policies to fund research in these areas can help to bridge

these gaps, leading to better-informed guidelines and practices supporting healthy fetal development [31].

Continued research into novel therapeutic strategies targeting placental function may offer new avenues for managing abnormal fetal growth patterns. They include exploring gene targeting and nanoparticle drug delivery systems to restore normal placentation and nutrient transfer. Investigating biomarkers for early detection of preeclampsia and other complications related to abnormal placentation could improve clinical outcomes. Creating predictive models based on machine learning can improve early detection and intervention efforts nationwide.

8. Conclusions

Placental-derived pregnancy complications remain a significant concern around the globe as well as in Mexico. The increasing prevalence of macrosomia highlights the need for targeted public health interventions to address underlying causes, such as maternal health and socioeconomic disparities. Along this line, the prevalence of FGR in Mexico is a multifaceted issue influenced by regional disparities and socioeconomic factors. Targeted interventions to improve maternal health education and access to prenatal care are essential for reducing the incidence of placental-derived pregnancy complications and its associated health risks in early infancy. Addressing these challenges requires a comprehensive approach that considers the unique needs of diverse populations across the country.

A holistic approach that addresses maternal health before conception and throughout pregnancy is needed to improve pregnancies and infant outcomes. This includes improving maternal nutrition and managing metabolic conditions, as well as ensuring optimal placental function. Community-based interventions promoting preconception care and maternal education have shown promise in improving health outcomes for mothers and their infants. By focusing on these interrelated factors, healthcare providers can better support women during their reproductive years and ultimately improve pregnancy and neonatal outcomes.

Current public policies play a crucial role in shaping the environment for placental function through monitoring practices, regulation of harmful substances, accessibility to care, and support for research initiatives. As the significance of these factors increases, it is imperative to sustain efforts to refine policies that facilitate optimal outcomes for maternal and neonatal health. Enhancing healthcare accessibility and quality regarding fetal growth and development requires improving monitoring techniques, understanding placental functions better, and developing effective interventions. Finally, addressing these areas is essential for reducing the risks associated with placental-derived pregnancy complications, leading to better maternal and neonatal health outcomes.

Placental health is further constrained by methodological limitations, as the predominant focus on HICs often overlooks the experiences of vulnerable populations in the Global South. This geographical bias raises concerns about the generalizability of findings and the potential for epistemic injustices, which ignore the unique health challenges faced by marginalized communities. Moreover, observational biases stemming from self-reported data and the misclassification of Indigenous populations may further obscure the true landscape of health disparities. In Mexico, despite the implementation of reforms aimed at enhancing maternal health, challenges persist, particularly with regards to the availability of high-quality healthcare for Indigenous populations and those residing in rural areas. The centralization of healthcare services and modifications in insurance models have sparked concerns regarding the continuity of care and equitable access to maternal health services. In summary, the persistent disparities in healthcare access and outcomes highlight the necessity for more inclusive and representative research methodologies to effectively address the complications of placental-derived pregnancy complications in diverse settings.

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Abbreviations

The following abbreviations are used in this manuscript:

FGR	Fetal growth restriction
IUGR	Intrauterine growth restriction
GDM	Gestational diabetes mellitus
MRI	Magnetic resonance imaging
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen
HICs	High-income countries
LMICs	Low- and middle-income countries
LGA	Large for gestational age
NICU	Neonatal intensive care unit
FM	Fetal macrosomia
SGA	Small for gestational age
AGA	Appropriate for gestational age
EDCs	Endocrine-disrupting chemicals
BPA	Bisphenol A
HIFs	Hypoxia-inducible factors

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Impact of Pulmonary Hypertension and Patent Ductus Arteriosus in Preterm Infants with Presumed Pulmonary Hypoplasia †

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Abstract

Objectives: Pulmonary hypertension and hemodynamically significant PDA (hsPDA) involve seemingly opposite physiological features—decreased pulmonary blood flow and pulmonary overcirculation, respectively—but the literature demonstrates variable respiratory consequences in association with each of these morbidities. The aim of this study is to evaluate whether the two factors representing pulmonary circulation provide different contributions to respiratory outcomes in preterm infants with and without pulmonary hypoplasia. **Methods**: The medical records of preterm very low birth weight (VLBW) infants admitted to our unit during the study period from January 2013 to December 2020 were retrospectively reviewed. Preterm VLBW infants were divided into groups according to the presence of presumed pulmonary hypoplasia (PPH). Multivariable logistic regression analysis was performed to assess the association of PPH and pulmonary hypertension or delayed PDA closure with in-hospital outcomes. **Results**: Postnatal age at final treatment for PDA was significantly later [median 33 vs. 19 days, $p = 0.025$] in the PPH group. Multivariable analysis indicated that early pulmonary hypertension was significantly associated with neonatal death [aOR (95%CI) 11.575 (2.988–44.833) for no-PPH vs. 9.981 (1.334–74.647) for PPH]. Delayed PDA closure was associated with increased odds of adverse respiratory and composite outcomes [aOR (95%CI) 4.929 (1.613–15.055) and 3.320 (1.048–10.515), respectively] but decreased odds of neonatal death in the no-PPH group. However, Cox proportional hazards models did not demonstrate statistically significant associations for PPH, early pulmonary hypertension, or delayed PDA closure with mortality, likely due to time-varying effects and the absence of death events in the subgroup of infants with both PPH and delayed PDA closure. **Conclusions**: PPH is associated with a higher prevalence of air leak syndrome and pulmonary hypertension. Delayed PDA closure exerts different effects on respiratory outcomes in preterm VLBW infants with and without PPH. Although early pulmonary hypertension appears to be a key circulatory factor contributing to neonatal death, its effect may vary over time. These findings underscore the importance of accounting for time-dependent effects when interpreting pulmonary circulatory risk factors in clinical practice.

Keywords: patent ductus arteriosus; preterm premature rupture of membrane; pulmonary circulation; pulmonary hypertension; very low birth weight

1. Introduction

Mid-trimester preterm premature rupture of membranes (PPROM) and oligohydramnios are risk factors for pulmonary underdevelopment, which is induced by altered angiogenesis and disrupted alveolarization [1,2]. Such alterations of lung volume and abnormal pulmonary vasculature in premature birth may further complicate the infant's pulmonary circulatory status, leading to increased pulmonary vascular resistance [3]. In affected infants, the clinical manifestations of pulmonary hypertension, such as labile or consistent hypoxemia, can be alleviated using pulmonary vasodilators such as inhaled nitric oxide [4]. Echocardiographic findings of pulmonary hypertension include tricuspid regurgitation and/or interventricular septum flattening, bidirectional or right-to-left shunt at the atrial level, or via patent ductus arteriosus (PDA) [4].

Conversely, a left-to-right shunt through the PDA, commonly observed in preterm infants, increases pulmonary blood flow. When the shunt is large, it can lead to pulmonary overcirculation and congestion, as well as decreased systemic perfusion. Clinical manifestations include feeding intolerance, hypotension, and oliguria [5]. Such cases are referred to as "hemodynamically significant PDA (hsPDA)" and carry a risk of congestive heart failure.

Furthermore, sustained pulmonary overcirculation imposes shear stress on the pulmonary endothelium, leading to vascular injury and remodeling. These structural changes, including intimal proliferation and medial hypertrophy, result in increased pulmonary vascular resistance. Chronic exposure to hsPDA may thus contribute to a gradual rise in pulmonary vascular resistance, eventually leading to the development of pulmonary hypertension. As pulmonary hypertension progresses, the right heart is placed under increasing strain. In severe or prolonged cases, this may result in shunt reversal and ultimately culminate in Eisenmenger physiology [6].

Pulmonary hypertension and hsPDA involve seemingly opposite physiological features—decreased pulmonary blood flow and pulmonary overcirculation, respectively—but the literature demonstrates variable respiratory consequences in association with each of these morbidities [7–10]. Here, we sought to evaluate whether these two factors representing pulmonary circulation status contribute differently to respiratory outcomes in preterm infants depending on fetal pulmonary underdevelopment.

2. Materials and Methods

2.1. Patient Inclusion and Data Collection

The electronic medical records of preterm very low birth weight (VLBW) infants admitted to our unit (a level III NICU) during the study period from January 2013 to December 2020 were retrospectively reviewed. The included infants were divided according to the presence of presumed pulmonary hypoplasia (PPH), which was defined as (a) PPROM > 7 days and/or (b) oligohydramnios. This definition was based on the threshold time interval associated with adverse outcomes, as described in the previous literature [11,12].

During the study period, 503 VLBW infants were admitted to the NICU. Of these, outborn infants (n = 61) and those with major congenital anomalies (n = 29), polyhydramnios, a lack of information concerning amniotic fluid (n = 27), and preterm infants born at less than 23 weeks of gestation (n = 7) were excluded. Figure 1 summarizes the inclusion and exclusion process, group allocation according to PPH status. A total of 379 infants (295 in the no-PPH group and 84 in the PPH group) were included. The flow also outlines descriptive outcome frequencies for each group and the statistical approaches used in the analysis.

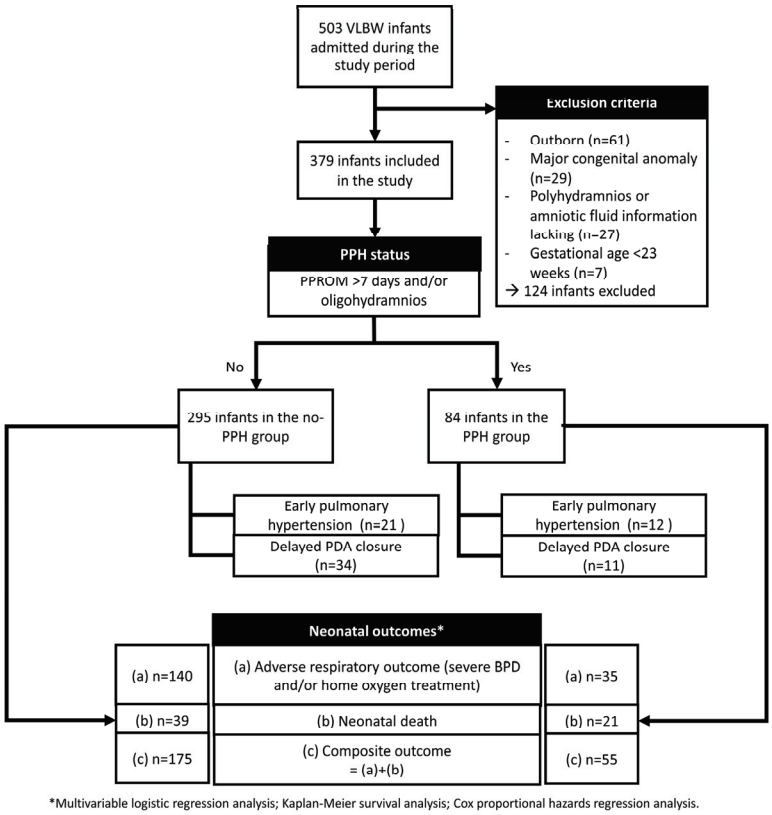


Figure 1. Flowchart of patient selection; group allocation based on PPH status. Descriptive outcome data (deaths and adverse outcomes) are summarized in each group for reference. Full outcome analyses are presented in the Results Section.

The following information related to baseline neonatal and maternal characteristics was obtained: gestational age (GA, weeks), birth weight (BW, g), whether the infant was small for gestational age (SGA) (BW < 10th percentile based on Fenton's growth chart), 1 and 5 min Apgar scores, sex, single/multiple births, delivery mode, maternal age (years), maternal morbidities (diabetes and hypertension), histologic chorioamnionitis, method of conception (natural or via assisted reproduction therapy), and antenatal corticosteroid administration. The onset and length of PPROM (h) and the presence of oligohydramnios were also obtained.

Data on respiratory morbidities and hsPDA treatment were collected. Respiratory distress syndrome was diagnosed when surfactant was administered via an endotracheal tube in case of failure to achieve a target SpO2 of $\geq$95% despite the provision of FiO2 $\geq$ 40%, accompanied by typical ground-glass appearance and/or air-bronchograms on chest radiographs. The number of surfactant doses administered was recorded. Air leak syndrome was defined only when interventions, such as chest tube insertion or angio-needle aspiration, were performed to relieve hypoxia and/or hypotension due to pneumothorax/pneumomediastinum. Massive pulmonary hemorrhage was diagnosed when blood from the airway was identified with concomitant or sequential cardiopulmonary collapse.

Infants with pulmonary hypertension were defined as those who presented with labile hypoxemia with or without differential hypoxia (>5–10% difference between preductal and postductal SpO2) that could not be improved by conventional treatment with appropriate ventilatory management and surfactant administration. The requirement for pulmonary vasodilator therapy (e.g., inhaled nitric oxide, milrinone infusion, sildenafil and/or bosentan administration, and iloprost nebulizer) was a requisite for the clinical diagnosis of pulmonary hypertension. Echocardiography was performed at or immediately after the

diagnosis of clinical pulmonary hypertension (i.e., as soon as possible) by the pediatric cardiologist on duty. Echocardiographic diagnosis of pulmonary hypertension was based on the findings of bidirectional or right-to-left shunt at the atrial level or through PDA, tricuspid regurgitation, interventricular septum flattening, and right atrial enlargement/right ventricular hypertrophy with or without right heart dysfunction [13,14]. Early pulmonary hypertension was defined as the occurrence of pulmonary hypertension < 7 days after birth. The development of pulmonary hypertension beyond this period was defined as late pulmonary hypertension.

PDA was diagnosed via echocardiography. Hemodynamic significance was defined based on clinical symptoms and signs such as murmur, bounding pulse, blood pressure fluctuation or hypotension, increased pulse pressure, decreased urine output not attributable to underlying anatomical or functional genitourinary tract disease or etiologies other than shunt steal, exacerbating respiratory condition not attributable to pulmonary disease, and pulmonary edema or cardiomegaly on chest radiographs. Echocardiographic severity was determined based on PDA size, shunt flow pattern (pulsatile, growing, or continuous), peak systolic velocity of the shunt flow, end-diastolic velocity of the left pulmonary artery (>20 cm/s), left atrium-to-aorta ratio (>1.4), and hyperdynamic heart motion. When hsPDA was diagnosed, either medical treatment using a cyclooxygenase inhibitor (COX-I) or surgical treatment was selected. Ibuprofen (intravenous or oral) was the only drug available for the treatment of PDA in our unit during the study period. Surgical PDA ligation was performed in infants who experienced medical treatment failure and those in whom ibuprofen use was contraindicated. The surgery was performed at the bedside of the neonatal intensive care unit (NICU) by a dedicated pediatric cardiac surgical team. Data on postnatal age and corrected age at medical or surgical treatment for hsPDA were collected. The time point of PDA closure was defined based on echocardiographic confirmation of closure after medical or surgical treatment. PDA identified at ≥21 days of life was defined as "delayed PDA closure."

Data related to the following in-hospital outcome variables were also collected: duration of invasive mechanical ventilation (days), severe bronchopulmonary dysplasia (BPD) [15], length of NICU stay (days), corrected age at discharge, need for home oxygen treatment, and mortality before 28 days after birth and before discharge.

2.2. Statistical Analysis

The included infants were categorized into the PPH and no-PPH groups. Baseline characteristics, respiratory morbidities, and in-hospital outcomes were compared between the two groups. Continuous variables were analyzed using the Wilcoxon rank-sum test, and categorical variables were analyzed using the chi-square test or Fisher's exact test, as appropriate. Kaplan–Meier survival analysis was performed to compare survival between infants with and without PPH.

Multivariable logistic regression analysis was performed to assess the association of PPH and delayed PDA closure (≥21 days after birth) with the following neonatal outcomes: (a) adverse respiratory outcome (severe BPD and/or home oxygen treatment), (b) neonatal death, and (c) composite outcome (adverse respiratory outcome and/or neonatal death).

Cox proportional hazards regression analyses were performed to evaluate the associations between variables of interest and in-hospital mortality, using the length of hospital stay (days) as the time scale.

Statistical analyses were performed using SPSS ver. 28 (IBM Corp., Armonk, NY, USA), and p-values less than 0.05 were considered significant.

The study was approved by the institutional review board of our hospital (approval number: KC22WASI0911). The need for informed consent was waived due to the retrospective nature of the study.

3. Results

Patient Characteristics

The baseline characteristics of the no-PPH and PPH groups are described in Table 1. BW was significantly lower in the PPH group than in the no-PPH group (median 931 g vs. 1070 g, $p = 0.009$), whereas GA did not significantly differ. The prevalence of SGA was higher in the PPH group than in the no-PPH group; however, the difference was not statistically significant. The prevalence of multiple births was greater in the no-PPH group than in the PPH group (40% vs. 27.4%, $p = 0.035$). PPROM duration was significantly longer (median 188 h vs. 18 h, $p < 0.001$) and onset was earlier (23.9 vs. 26.8 weeks' gestation, $p < 0.001$) in the PPH group than in the no-PPH group. Sixty-eight of the 84 infants (81.0%) in the PPH group were affected by oligohydramnios.

Table 1. Baseline characteristics of the included patients according to PPH status.

	No PPH (n = 295)	PPH (n = 84)	*p*
Gestational age (weeks)	28.1 [26.1–30.1]	27.7 [25.4–30.1]	0.220
Birthweight (g)	1070 [820–1290]	931 [730–1227]	0.009
Male	144 (48.8%)	39 (46.4%)	0.700
SGA	48 (16.3%)	21 (25.0%)	0.067
Multiple births	118 (40.0%)	23 (27.4%)	0.035
Cesarean delivery	259 (87.8%)	76 (90.5%)	0.499
1 min Apgar	3 [2–5]	3 [1–4]	0.060
5 min Apgar	6 [5–7]	6 [4–7]	0.146
Maternal age (years)	34 [31–36]	33 [30–36]	0.156
Primiparity	179 (60.7%)	47 (56.0%)	0.436
Maternal diabetes	29 (9.8%)	5 (6.0%)	0.272
Maternal hypertension	46 (15.6%)	14 (16.7%)	0.812
Histologic chorioamnionitis	97 (33.3%)	37 (44.0%)	0.071
Conceived via ART	67 (22.7%)	16 (19.0%)	0.474
PPROM	94 (31.9%)	49 (58.3%)	<0.001
PPROM (hours) [1]	18 [6–61]	188 [45–521]	<0.001
PPROM onset (weeks) [1]	26.8 [25.3–29.0]	23.9 [22.6–27.6]	<0.001
PPROM before 25 weeks of gestation [1]	20 (21.3%)	30 (61.2%)	<0.001
Oligohydramnios	0 (0.0%)	68 (81.0%)	<0.001
ACS administration	203 (69.0%)	67 (79.8%)	0.055

[1] Analyzed in those with PPROM (94 in no PPH, 49 in PPH).

Neonatal morbidities associated with respiratory status and pulmonary circulation are shown in Table 2. The PPH group had a significantly higher prevalence of air leak syndrome (19.0% vs. 8.1%, $p = 0.004$) and pulmonary hypertension (28.6% vs. 15.6%, $p = 0.007$). When analyzed specifically by the day of onset for pulmonary hypertension, early pulmonary hypertension occurred twice as often in the PPH group than in the no-PPH group in the whole study population (14.3% vs. 7.1%, $p = 0.040$). Meanwhile, the proportion of early

and late pulmonary hypertension among those affected by pulmonary hypertension was similar regardless of the presence of PPH. Although the prevalence of hsPDA was similar between the PPH and no-PPH groups, the postnatal age at final treatment for PDA was significantly later in the PPH group than in the no-PPH group [median 33 vs. 19 days, $p = 0.025$]. The prevalence of delayed PDA closure was similar between the two groups when analyzed across the entire study population. When analyzed in those who were affected by hsPDA, the prevalence of delayed PDA closure was higher in the PPH group (11/16 = 68.8%) than in the no-PPH group (34/78 = 43.6%).

Table 2. Respiratory comorbidities and factors associated with pulmonary circulation status in the included infants.

	No PPH (n = 295)	**PPH (n = 84)**	***p***
RDS	265 (89.8%)	75 (89.3%)	0.885
Surfactant ≥ 2 doses	82 (27.9%)	20 (23.8%)	0.457
Air leak syndrome	24 (8.1%)	16 (19.0%)	0.004
Massive pulmonary hemorrhage	48 (16.3%)	18 (21.4%)	0.271
Pulmonary hypertension, total	46 (15.6%)	24 (28.6%)	0.007
Pulmonary hypertension depending on onset			
Early pulmonary hypertension *	21 (7.1%)	12 (14.3%)	0.040
Late pulmonary hypertension	25 (8.5%)	12 (14.3%)	0.113
HSPDA **	78 (26.9%)	16 (20.3%)	0.230
COX-I use	25 (32.1%)	2 (12.5%)	0.140 †
COX-I failure	8 (32.0%)	0 (0.0%)	>0.99 †
Surgical ligation	61 (78.2%)	14 (87.5%)	0.512 †
PNA at initial Tx.	18 [9–26]	33 [20–47]	0.09
CA at initial Tx.	29.7 [28.1–31.3]	30.9 [28.4–32.0]	0.199
PNA at final Tx.	19 [10–29]	33 [20–47]	0.025
CA at final Tx.	29.9 [28.1–31.4]	30.9 [28.4–32.0]	0.324
PDA closure ≥ 21 d	34 (11.5%)	11 (13.1%)	0.695

* Analyzed in 46 in no PPH, 24 in PPH. ** Analyzed in 290 in no PPH, 79 in PPH. † Fisher's exact test.

Table 3 shows the neonatal outcomes during the later days of NICU stay. While the duration of invasive mechanical ventilation, severe BPD prevalence, and need for home oxygen treatment did not significantly differ, mortality before NICU discharge was significantly higher in the PPH group than in the no-PPH group (25.0% vs. 13.2%, $p = 0.009$). Kaplan–Meier survival analysis (Figure 2) also revealed a significantly lower survival in the PPH group compared to the no-PPH group (log-rank $\chi^2 = 7.046$, $p = 0.008$). The median survival time was 160.6 days (95% CI, 139.8–181.5) in the PPH group, whereas it was 223.0 days (95% CI, 77.3–374.4) in the no-PPH group.

Table 3. In-hospital outcomes of the included infants.

	No PPH (n = 295)	**PPH (n = 84)**	***p***
MV duration	11 [3–37]	10 [2–44]	0.890
Severe BPD *	95 (37.1%)	23 (35.9%)	0.862
Length of stay	56 [39–82]	53 [24–95]	0.382
CA at discharge	37.3 [35.7–39.9]	37.1 [34.9–40.0]	0.598
Home oxygen treatment **	89 (36.6%)	21 (36.8%)	0.976
Death before 28 days after birth	39 (13.3%)	13 (15.5%)	0.612
Death before discharge †	39 (13.2%)	21 (25.0%)	0.009

* Analyzed in 256 with no PPH and 64 with PPH. ** Analyzed in 293 in no PPH, 57 in PPH. (excluding infants transferred before 28 days of age). † Analyzed in 295 in no PPH, 84 in PPH. (excluding infants transferred to other hospitals).

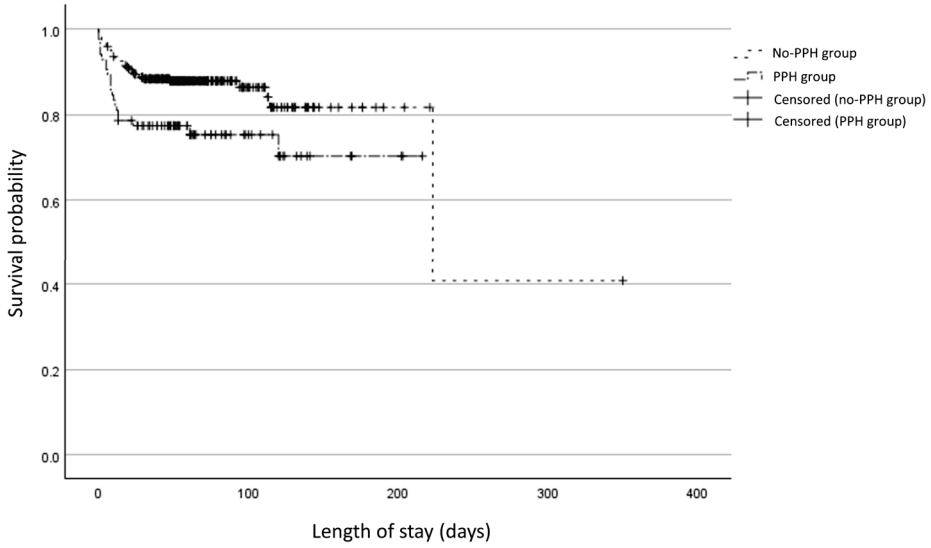


Figure 2. Kaplan–Meier survival analysis, PPH group vs. no-PPH group.

Multivariate logistic regression analysis was performed to evaluate the impact of early pulmonary hypertension and delayed PDA closure on neonatal outcomes (Table 4). BW (per 100 g), multiple births, and antenatal corticosteroid administration were added to adjust for confounding effects. Early pulmonary hypertension was associated with increased odds of neonatal death [odds ratio (OR): 11.165, 95% confidence interval (CI): 3.657–34.088] and composite outcomes (OR: 5.800, 95%CI: 1.263–26.633) when analyzed in the total cohort; however, when analyzed separately in the no-PPH and PPH groups, early pulmonary hypertension was significantly associated with neonatal death only (OR: 11.575, 95%CI: 2.988–44.833 and OR: 9.981, 95%CI: 1.334–74.647, respectively). Delayed PDA closure increased the odds of adverse respiratory and composite outcomes and decreased the odds of neonatal death in the total cohort. A separate analysis of the no-PPH group revealed similar results for adverse respiratory outcomes (OR: 4.929, 95%CI: 1.613–15.055), neonatal death (OR: 0.036, 95%CI: 0.003–0.383), and composite outcomes (OR: 3.320, 95%CI: 1.048–10.515). Delayed PDA closure was not associated with any outcome in the PPH group.

Table 4. Multivariable logistic regression analysis for the effect of early pulmonary hypertension and delayed PDA closure (≥21 days of life) on neonatal outcomes.

	Early Pulmonary Hypertension		**Delayed PDA Closure**	
Included infants, total	*p*	OR (95%CI)	*p*	aOR * (95%CI)
Adverse respiratory outcome	0.293	2.129 (0.520–8.713)	0.002	4.849 (1.783–13.189)
Neonatal death	<0.001	11.165 (3.657–34.088)	<0.001	0.020 (0.002–0.176)
Composite outcome	0.024	5.800 (1.263–26.633)	0.044	2.906 (1.030–8.197)
No-PPH infants	*p*	OR (95%CI)	*p*	aOR * (95%CI)
Adverse respiratory outcome	0.310	2.379 (0.446–12.683)	0.005	4.929 (1.613–15.055)
Neonatal death	<0.001	11.575 (2.988–44.833)	0.006	0.036 (0.003–0.383)
Composite outcome	0.068	6.920 (0.867–55.267)	0.041	3.320 (1.048–10.515)
PPH infants	*p*	OR (95%CI)	*p*	aOR * (95%CI)
Adverse respiratory outcome	0.388	4.158 (0.164–105.578)	0.544	2.166 (0.179–26.279)
Neonatal death	0.025	9.981 (1.334–74.647)	>0.990	- **
Composite outcome	0.091	8.390 (0.710–99.135)	0.962	1.059 (0.099–11.360)

* Factors included in the model: birth weight (per 100 g), multiple births, antenatal corticosteroid administration, early pulmonary hypertension, and PDA closure ≥ 21 days after birth. ** In the PPH group, the mortality for delayed PDA closure was 0%.

In the Cox proportional hazards regression analyses, six models were constructed (Table 5). Models 1–4 included baseline covariates (i.e., birthweight, multiple births, and air leak syndrome) that were also adjusted for in the multivariable logistic regression analyses, with additional inclusion of PPH, early pulmonary hypertension, or delayed PDA closure. Models 5 and 6 examined the interaction effects between PPH and early pulmonary hypertension, and between PPH and delayed PDA closure, respectively. Across all models, higher birthweight and absence of air leak syndrome were consistently and significantly associated with reduced mortality ($p < 0.001$). In contrast, none of the variables of interest—PPH, early pulmonary hypertension, or delayed PDA closure—showed statistically significant associations with mortality, either as independent predictors or in interaction terms.

Table 5. Cox proportional hazards regression analyses were used to evaluate the association between clinical variables and in-hospital mortality.

Model #	Variables	Hazard Risk (95%CI)	*p*-Value
Model 1	Birth weight (100 g)	0.703 (0.629–0.785)	<0.001
	Multiple births	0.669 (0.395–1.134)	0.128
	Air leak syndrome	0.263 (0.146–0.472)	<0.001
	PPH	0.912 (0.518–1.607)	0.768
Model 2	Birth weight (100 g)	0.704 (0.630–0.787)	<0.001
	Multiple births	0.671 (0.396–1.138)	0.130
	Air leak syndrome	0.261 (0.145–0.470)	<0.001
	PPH	0.915 (0.519–1.615)	0.778
	Delayed PDA closure	1.153 (0.910–1.462)	0.215
Model 3	Birth weight (100 g)	0.702 (0.627–0.785)	<0.001
	Multiple births	0.662 (0.391–1.121)	0.126
	Air leak syndrome	0.263 (0.146–0.474)	<0.001
	PPH	0.912 (0.518–1.608)	0.768
	Early pulmonary hypertension	0.983 (0.712–1.356)	0.915
Model 4	Birth weight (100 g)	0.705 (0.629–0.791)	<0.001
	Multiple births	0.673 (0.397–1.141)	0.132
	Air leak syndrome	0.259 (0.143–0.471)	<0.001
	PPH	0.916 (0.515–1.629)	0.775
	Delayed PDA closure	1.154 (0.910–1.465)	0.215
	Early pulmonary hypertension	0.986 (0.715–1.361)	0.936
Model 5	Birth weight (100 g)	0.707 (0.632–0.791)	<0.001
	Multiple births	0.663 (0.395–1.113)	0.128
	Air leak syndrome	0.263 (0.146–0.474)	<0.001
	PPH	0.911 (0.518–1.603)	0.765
	Early pulmonary hypertension	0.971 (0.704–1.339)	0.868
	PPH × Early pulmonary hypertension	0.973 (0.469–2.021)	0.943
Model 6	Birth weight (100 g)	0.713 (0.637–0.797)	<0.001
	Multiple births	0.661 (0.390–1.121)	0.123
	Air leak syndrome	0.266 (0.148–0.478)	<0.001
	PPH	0.920 (0.519–1.630)	0.781
	Delayed PDA closure	1.174 (0.924–1.492)	0.180
	PPH × Delayed PDA closure	0.805 (0.406–1.596)	0.531

4. Discussion

The present results suggest that neonatal death before discharge is more prevalent in infants with PPH than in those without. Early pulmonary hypertension was also associated with an increased risk of neonatal death, regardless of PPH status. Delayed PDA closure contributed to an increased risk of adverse respiratory outcomes and composite outcomes overall, but not specifically in infants affected by PPH. However, based on the Cox proportional hazards models, PPH, early pulmonary hypertension, and delayed PDA closure were not significant factors in terms of their time-to-event associations.

Amniotic fluid plays a pivotal role in the development of a normal lung. Mechanical compression of the fetal thorax due to loss of amniotic fluid hampers normal fetal breathing movements and generation of distended pressure by amniotic fluid [1,16], leading to interference with epithelial and endothelial development and reduction in lung cell size and the number of airway branches [17]. Animal studies have shown decreased collagen levels and elastic tissue development in hypoplastic lungs in cases of oligohydramnios [1,18]. The loss of amniotic fluid due to PPROM and oligohydramnios is linked to the pulmonary underdevelopment [19–21]. Despite a few reports suggesting diagnostic criteria [2,22,23], there are limited guidelines that concretely aid in diagnosing pulmonary hypoplasia in clinical settings. Therefore, prolonged PPROM latency and/or oligohydramnios were chosen to define PPH in our study.

Infants with PPH in our study exhibited a higher prevalence of several adverse outcomes, including neonatal mortality and respiratory morbidities like air leak syndrome, pulmonary hypertension, and BPD. The significantly higher prevalence of air leak syndrome observed in the PPH group may reflect greater pulmonary structural fragility, potentially linked to impaired alveolar development or relative surfactant deficiency due to reduced expression of the lamellar bodies of type II pneumocytes in the context of hypoplastic lung disease [24].

Both air leak syndrome and pulmonary hypertension in preterm infants are independently associated with an increased risk of neonatal death. In a recent study comparing neonatal outcomes of preterm VLBW infants of the Brazilian Network on Neonatal Research (Rede Brasileira de Pesquisas Neonatais, RBPN) vs. the Neonatal Research Network of Japan (NRNJ), the infants from RBPN exhibited 9.06-fold higher odds of mortality, with air leak syndrome being one of the leading associated factors [25]. In another recent report based on the National Kids' Inpatient Database, the association of pneumothorax with increased odds for mortality was identified in all gestational age groups > 24 weeks (greatest in preterm infants 29–32 weeks) [26].

Impaired development of the pulmonary vasculature in association with pulmonary hypoplasia includes decreased size of the total pulmonary vascular bed, decreased number of vessels per unit of lung tissue, and increased pulmonary arterial smooth muscle, which are characteristic features leading to increased pulmonary vascular resistance [2]. This is the basis for investigating the effect of pulmonary vasodilators on suspected pulmonary hypoplasia in infants exposed to PPROM and/or oligohydramnios [3,27]. Although early pulmonary hypertension and neonatal death were both more prevalent in the PPH group than in the no-PPH group, early pulmonary hypertension itself was associated with increased odds of neonatal death, regardless of whether the infant had been affected by PPH. Arjaans et al. [7] reported that the persistent pulmonary hypertension phenotype, which corresponds to early pulmonary hypertension features in our study, was associated with a death rate of 42% before 36 weeks postmenstrual age (PMA).

Meanwhile, while early pulmonary hypertension showed consistent associations with neonatal death across both groups, the impact of delayed PDA closure was confined to the no-PPH group, and composite outcomes did not always follow the same directional trend.

Our analysis indicated that PDA treatment occurred at a significantly later postnatal age in infants with PPH than in those without, indicating that delayed treatment of PDA in this setting may be due to increased pulmonary resistance and possibly early pulmonary hypertension in a subset of infants during the early days after birth. Since the clinical signs and symptoms of hsPDA are caused by left-to-right shunt flow, elevated pulmonary vascular resistance fundamentally hinders progression of hemodynamical compromise caused by "ductal steal." Furthermore, in the case of pulmonary hypertension, which involves either complete or partial flow of the PDA shunt in the opposite direction (i.e., right-to-left shunt or bidirectional shunt, respectively), it may take more time to recover the left-to-right direction of the shunt and progress into a true hsPDA status. In other words, "delayed PDA closure" may be less delayed considering the relatively shorter duration of left-to-right shunt flow after restoration of the shunt direction. Recent studies have demonstrated that the duration of PDA shunt exposure contributes to adverse neonatal outcomes [10,28–30]. As such, it is plausible that PDA closure at $\geq$21 days of birth, which is shorter than the expected duration of actual hsPDA exposure, is not associated with adverse neonatal outcomes in infants affected by PPH.

On the other hand, delayed PDA closure in the no-PPH group was associated with increased odds of adverse respiratory outcomes and composite outcomes, and with decreased odds of neonatal death. This result does not represent a cause-and-effect relationship; it is logical to think that those who survived throughout the NICU stay were likely to experience delayed PDA closure. As the conservative approach to hsPDA became popular worldwide [31–33], a longer duration of PDA exposure was associated with the deterioration of neonatal respiratory conditions. For instance, Mirza et al. [29] and Deng et al. [28] both reported that prolonged exposure to hsPDA was associated with an increased risk of the composite outcome of BPD/death. The physiological explanation for their work is discussed in another recent publication [30], which demonstrated that in infants who underwent a later PDA closure, transcatheter hemodynamic variables indicated significantly higher pulmonary vascular resistance, highlighting the unfavorable impact of prolonged hsPDA exposure on the development of early pulmonary vascular disease. Our study results are consistent with previous studies in that the adverse effects of pulmonary vascular shear stress caused by prolonged ductal shunt flow may lead to an increased risk of bronchopulmonary dysplasia and/or pulmonary hypertension [29,34,35]. Taken together, these findings emphasize the importance of interpreting the impact of delayed PDA closure in the context of pulmonary vascular status. A physiologically tailored approach to PDA management with careful consideration of individualized pulmonary hemodynamics rather than uniform time-based criteria should be pursued.

It is noteworthy that in contrast to the logistic regression model and Kaplan–Meier survival analysis, Cox proportional hazards regression analyses did not demonstrate a statistically significant association between PPH, early pulmonary hypertension, or delayed PDA closure and mortality. This discrepancy can be partly explained by violations of the proportional hazards assumption, particularly for PPH and early pulmonary hypertension, whose mortality effects declined over time as indicated by significant time interaction terms ($p < 0.001$). Such time-varying effects may have led to the underestimation of their impact in the Cox models. From a clinical standpoint, this suggests that infants with PPH or early pulmonary hypertension face a heightened risk of early deterioration, underscoring the importance of early recognition and timely initiation of supportive therapies such as pulmonary vasodilators. Establishing clear screening thresholds for echocardiographic evaluation in infants with PPH may facilitate rapid diagnosis and early treatment, potentially mitigating the time-sensitive mortality risk.

By comparison, the absence of death events in the subgroup of infants with both PPH and delayed PDA closure likely precluded reliable estimation of hazard ratios in this group. Therefore, the findings from logistic regression and Kaplan–Meier analyses described above may offer more reliable guidance in this context.

Notably, both lower birthweight and the presence of air leak syndrome consistently showed significant time-dependent associations with mortality across all Cox models. While birthweight is a non-modifiable risk factor, this finding underscores the importance of early risk stratification and tailoring respiratory management accordingly. Optimizing ventilation strategies to minimize lung injury while ensuring adequate gas exchange and pulmonary and systemic circulatory balance is essential, particularly in extremely low birthweight infants. In addition, as air leak syndrome remains a major contributor to neonatal mortality as described above, clinicians should prioritize gentle ventilation approaches to reduce the likelihood of barotrauma. In addition, heightened vigilance is warranted to detect and respond promptly to sudden deterioration, especially in infants at high risk for pulmonary compromise.

Our study is meaningful in that we focused on two disease entities representing different pulmonary circulatory statuses (poor pulmonary flow vs. increased pulmonary flow) and investigated the consequential impact on specific respiratory outcomes in preterm infants with suspected pulmonary underdevelopment. It would be prudent to consider the baseline structural and volumetric characteristics of the lung and pulmonary vasculature when discussing "true hsPDA" exposure.

We acknowledge the limitations of this study, which include a retrospective design and a small sample size from a single institution. As in other retrospective studies, cause-and-effect relationships cannot be concluded from our study results, and only associative relationships can be inferred. In addition, the final treatment decision was made by the attending neonatologist, and the possibility of individual preferences may have introduced bias. However, the need for hsPDA treatment was fundamentally assessed in accordance with previously published criteria [36]; deviation from our unit strategy is likely to have been minimal. Finally, we used PPROM latency and the presence of oligohydramnios to define "presumed" and not "proven" pulmonary hypoplasia. Further, the degree of pulmonary underdevelopment in the infants who experienced prolonged PPROM latency and/or oligohydramnios may be variable, and classifying all these infants in one "PPH group" may obscure the potential impact of the severity of pulmonary underdevelopment. Future prospective studies using an appropriate diagnostic methodology to accurately define and stratify pulmonary hypoplasia in terms of severity should aim to complement the results of our study.

5. Conclusions

In conclusion, the impact of different pulmonary circulation statuses on neonatal mortality and respiratory morbidities in preterm infants varied depending on the presence of PPH. Delayed PDA closure may represent different durations of hemodynamically significant left-to-right shunt flow and thus differentially affect subsequent adverse respiratory outcomes in those with and without PPH. In comparison, early pulmonary hypertension appears to be the principal circulatory factor associated with neonatal mortality, regardless of PPH status. However, as such associations may vary over time, cautious interpretation and clinical application of these findings are warranted.

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Abbreviations

The following abbreviations are used in this manuscript:

ART	Assisted reproduction therapy
CA	Corrected age
COX-I	Cyclo-oxygenase inhibitor
hsPDA	Hemodynamically significant patent ductus arteriosus
MV	Mechanical ventilation
PNA	Postanal age
PPH	Presumed pulmonary hypoplasia
PPROM	Preterm premature rupture of membranes
SGA	Small-for-gestational age
Tx	Treatment

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Article

Neuropsychomotor Development of Children Exposed to SARS-CoV-2 in Utero During COVID-19 Pandemic

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Abstract

Introduction: Little is known about the effects of intrauterine exposure to SARS-CoV-2, especially on growth and neurodevelopment in children. **Objective**: We wished to verify the effect of intrauterine exposure to SARS-CoV-2 on neurological development in children. **Methods**: Infants born to mothers presenting with SARS-CoV-2 infection during pregnancy were enrolled in a prospective descriptive–analytical study involving outpatient appointments performed 6 and 12 months after birth. Their neurological development was assessed using the Bayley-III Scale, using a score of >85 as the cutoff threshold for identifying developmental delay. Differences between groups were assessed through an ANOVA, using Bonferroni correction for multiple comparisons. Regression models were

employed to examine the associations between the Bayley-III scores and maternal features. **Results**: Two hundred and sixty-nine infants were evaluated, most of whom were born full-term and with birth weights appropriate for gestational age at delivery. Developmental delays were observed in 26% of the infants in at least one of the Bayley-III domains. The language domain was particularly affected, with impairments observed in children exposed to SARS-CoV-2 closer to the time of delivery. These findings were statistically significant ($p < 0.05$). **Conclusions**: Infants born to mothers presenting with SARS-CoV-2 infection during pregnancy presented developmental delays at 6 and 12 months, particularly in the language domain. These findings reinforce the relevance of long-term clinical follow-ups of newborns exposed to SARS-CoV-2 infection during pregnancy.

Keywords: neonatal disease; child development; COVID-19; pandemic; SARS-CoV-2; neuropsychological tests; infants

1. Introduction

Neuropsychomotor developmental delays in children exposed to SARS-CoV-2 in utero during the COVID-19 pandemic have been suggested, particularly in the language and motor domains. Previous research has been conducted to understand the relationship between maternal complications and SARS-CoV-2 infection during pregnancy [1]. However, the perinatal repercussions caused by SARS-CoV-2 have not yet been elucidated. There are gaps in the understanding of this process, which still requires more comprehensive elucidation, especially regarding the impact of maternal SARS-CoV-2 infections on fetal and neonatal neurological and psychiatric development [2,3].

It has been shown that maternal infections during pregnancy, followed by congenital fetal infections, can affect fetal development through direct infections in fetal cells. Exposure of the fetus to maternal inflammation is associated with placental damage, which may compromise fetal development. Several congenital infections, such as Cytomegalovirus, Zika, and Rubella viral infections, as well as Syphilis and Toxoplasmosis, have been associated with neurodevelopmental impairments [4]. Although children exposed to infectious agents during pregnancy may not present symptoms at birth, they may display neurodevelopmental commitment over time, with the sequelae varying according to the pathogen and the severity of the infection. Previous research on neurodevelopment in infants born with congenital infections suggests that even without brain damage or birth defects, children may experience impaired neurodevelopment. This process could be associated with the inflammatory cytokine response and placental immune activation, which can interfere with brain growth and plasticity [5,6].

As maternal SARS-CoV-2 infections during pregnancy lead to an acute inflammatory status, they can impact fetal development, resulting in impaired neurological features. The current state of the knowledge provides evidence that SARS-CoV-2 exposure during pregnancy is associated with fetal neurodevelopmental impairments, suggesting that specific guidelines are needed to monitor and mitigate its long-term effects on children's health [7]. Some studies have suggested that SARS-CoV-2 infections during pregnancy do not appear to be associated with early childhood developmental concerns [8]. Recent reports from our group argue against this point by demonstrating pediatric structural encephalic changes associated with SARS-CoV-2 infection during pregnancy [9]. As some developmental commitments are undiagnosed or inapparent until later in infancy, clinical follow-up should continue over time after birth. In this sense, the constant monitoring of neurodevelopment is essential during the first two years, a period when developmental milestones and neu-

rocognitive acquisitions occur most significantly. Longitudinal studies have demonstrated that newborns exposed to SARS-CoV-2 prenatally had lower neurodevelopmental scores, particularly in motor development and interactive behavior [10]. Furthermore, Suffrey et al. [11] found that children born during the pandemic had lower-than-average scores in developmental tests. As suggested previously [2,10], further research is warranted to elucidate the full extent of these effects.

The present work was designed as a prospective cohort investigation aiming at providing evidence of the impact of in utero SARS-CoV-2 infection on infant neurological development. This study comprises two major goals, as follows: (i) examine comprehensively the neurological development of infants exposed in utero to SARS-CoV-2 during a 1-year follow-up and (ii) compare the development of the infants according to clinical aspects of SARS-CoV-2 exposure during pregnancy and the maternal education level. The findings of this study will contribute significantly to our understanding of the effects of prenatal exposure to SARS-CoV-2 on infant neurodevelopment as well as to clinical pediatric practice and public health policies.

2. Material and Methods

2.1. Population

This is a prospective cohort study performed at the University Hospital of Brasília, in Brasília, Brazil. Children were eligible for enrollment from the first 15 days of life to 12 months of age if born from June 2020 to November 2020 and if their mothers had had SARS-CoV-2 infection proven by nasopharyngeal RT-PCR during pregnancy. The primary outcome was evaluated with the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) at 6 and 12 months of life. The participants were recruited with non-probabilistic convenience sampling, using media and social networks from 1 May 2020 to 31 May 2021. Infants enrolled in the study were followed prospectively from 1 July 2020 to 31 December 2021. This study was performed at two public hospitals—the University Hospital of Brasília and Asa Norte Regional Hospital, both considered public reference centers for COVID-19 in the Federal District of Brazil.

The exclusion criteria applied in this investigation comprised pregnant women vaccinated for COVID-19 before or during pregnancy. In addition, smokers and excessive alcohol/illicit drug users were excluded. The exclusion criteria also encompassed neonatal features, including requirement of Intensive Care Unit admission, congenital malformations, and chromosomal abnormalities, as well as clinical and/or serological diagnoses of congenital infections (Cytomegalovirus, Zika, Rubella, Hepatitis B and C viral infection, Chagas disease, Syphilis, or Toxoplasmosis). To reduce the risk of confounding bias for the clinical features and outcomes, pregnant women with a confirmed diagnosis of Cytomegalovirus, Zika, Rubella, Herpes, Human Immunodeficiency Virus infection, Chagas disease, Syphilis, or Toxoplasmosis were excluded from the study. Upon enrollment, no children were excluded from the study population.

All participants underwent mandatory neonatal screenings as per Brazilian Health Ministry guidelines, including biological screening (TANDEM methodology), pulse oximetry test with subsequent echocardiogram if abnormalities were detected, auditory screening (evoked otoacoustic emissions), and ophthalmic fundus examination.

The evaluation took place with pregnant women infected by SARS-CoV-2 in Brazil between June 2020 and April 2022. The guardians of each participant signed an informed consent form and were informed of the test results. Children with developmental delays were advised and referred to a rehabilitation center.

Figure 1 presents a compendium flowchart of study design, population, and methods.

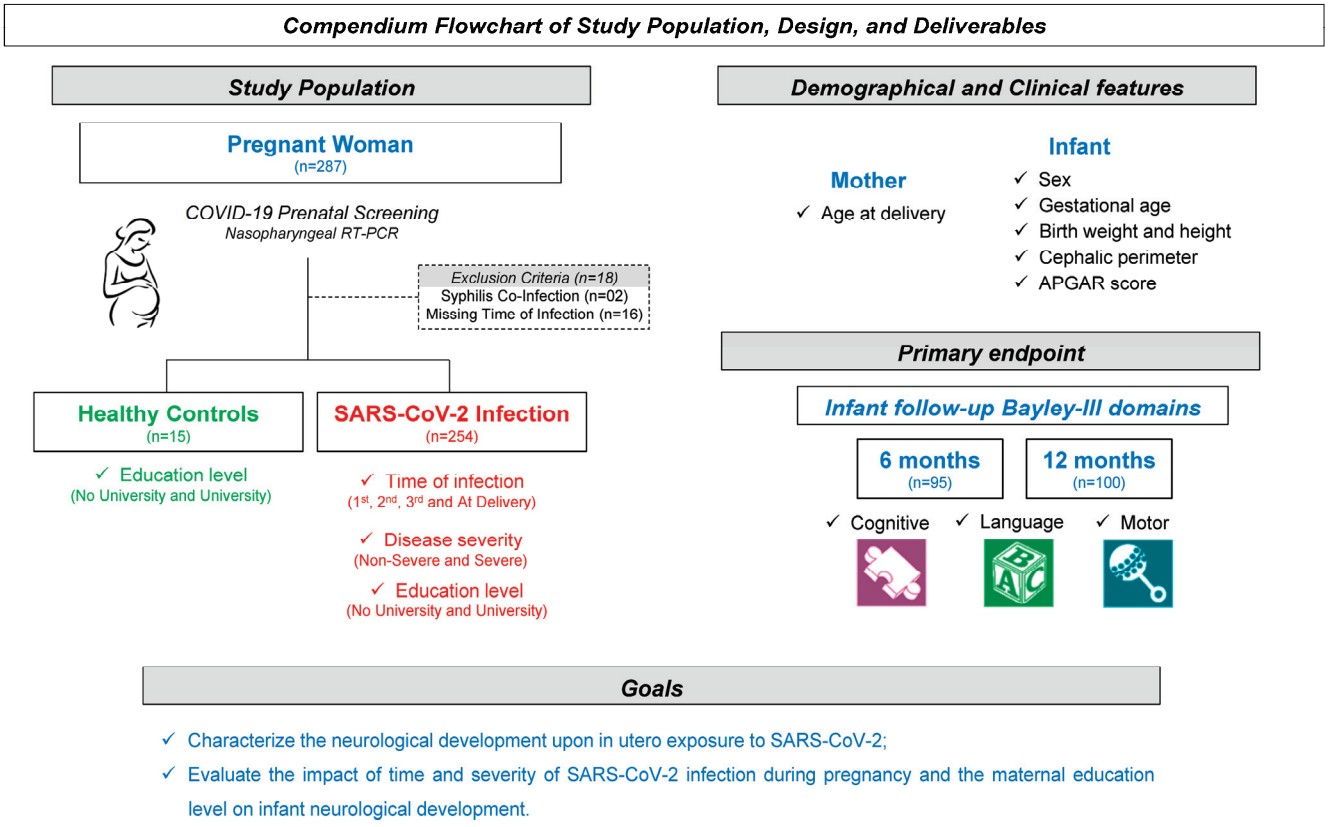


Figure 1. Compendium flowchart of study design, population, and methods.

2.2. Data Collection Procedures, Instruments, and Analyzed Variables

A detailed study description has been previously published [3]. In this manuscript, we provide a summary of the overall methodology employed in the study. Information of the clinical status of the pregnant women was collected and analyzed during initial consultations, whereas data on infants and children were gathered during follow-up visits. Maternal infection severity was graded as non-severe, severe, and critical according to the criteria of the World Health Organization (WHO), and pregnant women were divided into 2 groups, non-severe and severe (including critical) groups, for analysis purposes. The trimester of infection was defined as follows: 1st trimester (up to 13 weeks and 6 days), 2nd trimester (from 14 weeks to 26 weeks and 6 days), and 3rd trimester (more than 27 weeks). Acute infection at delivery was considered a separate clinical subgroup. The caregiver educational level was determined according to the presence or absence of a university degree.

The Bayley-III Scale is the most used assessment tool for infant neurodevelopment [12]. The original version presents appropriate parameters of validity and reliability, in addition to good sensitivity and specificity indices to identify children with developmental delay [13–15]. These scales were translated into Portuguese and validated in the Brazilian context in 2006, and the Bayley-III Scale has become the gold standard for monitoring children's developmental progress since then [13,14]. The Bayley-III Scales were applied by four independent examiners, previously trained by professionals from the Occupational Therapy Department at the University of Brasília, with at least 10 years of experience with Bayley Scales application to ensure the accuracy and consistency of data collection. In this study, the Bayley-III Scales were used to assess infant neurodevelopment, focusing on the motor, cognitive, and language subscales, while the complementary scales (socio-emotional and adaptive behavior) were not utilized. The original version of Bayley-III demonstrates adequate validity and reliability parameters, with strong sensitivity and specificity indices

for identifying children with developmental delays [10,11]. Furthermore, as an additional external control, inter-rater reliability was applied by performing measurements in 10 children who were not part of the present study, resulting in an excellent correlation index (ICC = 90).

To evaluate the performance of the investigated children, a score of 0 or 1 was attributed to each item on the scale. The weighted average of the scores obtained by the Bayley-III at 6 and 12 months was used. The Bayley scores were expressed as continuous variables and converted into categorical data, considering the cutoff threshold of <85 to indicate an altered score, considered a delay in neurological development [16,17].

2.3. Statistical Analysis

Normality tests (Shapiro–Wilk and Kolmogorov–Smirnov) were carried out to determine the dataset distribution profile. The clinical and demographic parameters were presented as means (± standard deviations—SDs) and as frequency values. Multiple comparisons amongst groups were performed using the analysis of variance (ANOVA) test with Bonferroni correction. Comparative analyses of categorical data were carried out by the Chi-square test (χ^2). Multinomial and logistic regression models were employed, adjusting for infant sex and gestational age at birth to verify associations of the cognitive, language, and motor domains of the Bayley-III Scale with maternal features (time of SARS-CoV-2 infection, disease severity, and educational level). The statistical analyses were performed using Statistical Analysis System software (SAS; version 9.4), Graphpad Prism Software (version 10.0), and Minitab software (Minitab, LLC; version 17). In all cases, p-values < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of Study Population

Table 1 summarizes the clinical and demographic features of the study population. Most babies were born full-term (90.3%), and the mean gestational age at birth was 38.40 (±1.66) weeks. Around half of the participants were females (51.3%) with a mean birth weight of 3137.80 (±532.84) grams. Most babies (80.1%) had an appropriate weight for their gestational age at birth. The mean birth height was 51.46 (±6.69) cm. The cephalic perimeter was 35.50 (±3.62) cm.

Table 1. Study population.

Clinical and Demographic Parameters	Healthy Controls * (*n* = 15)	SARS-CoV-2 Infection (*n* = 254)
Full term, *n* (%)	12/15 (80.0)	220/247 (89.1)
Gestational age (weeks), mean ± SD	38.6 (±2.3)	38.2 (±1.7)
Female, *n* (%)	10/15 (56.7)	129/254 (50.8)
Birth weight (grams)	3241.4 (±523.0)	3131.7 (±533.4)
Appropriate weight for gestational age, *n* (%)	14/15 (93.3)	199/247 (80.6)
Birth height (cm), mean ± SD	48.5 (±3.12)	48.2 (±2.57)
Cephalic perimeter (cm), mean ± SD	35.0 (±1.01)	34.4 (±1.98)
Mother age at delivery (years), mean ± SD	31.7 (±3.65)	34.5 (±3.19)
APGAR 1st-minute score		

Table 1. *Cont.*

Clinical and Demographic Parameters	Healthy Controls * (*n* = 15)	SARS-CoV-2 Infection (*n* = 254)
Score (mean ± SD)	7.1 (±2.0)	8.0 (±1.2)
≤7	5/14 (35.7)	50/241 (20.7)
>7	9/14 (64.3)	191/241 (79.3)
APGAR 5th-minute score		
Score (mean ± SD)	8.8 (±0.70)	8.9 (±0.69)
≤7	1/14 (7.1)	10/241 (4.1)
>7	13/14 (92.9)	231/241 (95.9)
Bayley-III Scale at 6 months of age		
Normal, *n* (%)	5/5 (100.0)	59/95 (62.1)
Delay, *n* (%)	0/5 (0.0)	36/95 (37.9)
Cognitive, *n* (%)	0/5 (0.0)	20/36 (55.6)
Language, *n* (%)	0/5 (0.0)	23/36 (63.9)
Motor, *n* (%)	0/5 (0.0)	12/36 (33.3)
Bayley-III Scale at 12 months of age		
Normal, *n* (%)	3/3 (100.0)	67/100 (67.0)
Delay, *n* (%)	0/3 (0.0)	33/100 (33.0)
Cognitive, *n* (%)	0/3 (0.0)	4/33 (12.2)
Language, *n* (%)	0/3 (0.0)	26/33 (78.8)
Motor, *n* (%)	0/3 (0.0)	13/33 (39.4)
Time of Maternal SARS-CoV-2 Infection		
1st trimester, *n* (%)	n/a	50/254 (19.7)
2nd trimester, *n* (%)	n/a	78/254 (30.7)
3rd trimester, *n* (%)	n/a	105/254 (41.3)
At delivery, *n* (%)	n/a	21/254 (8.3)
Severity of Maternal SARS-CoV-2 Infection		
Non-severe, *n* (%)	n/a	212/250 (84.8)
Severe, *n* (%)	n/a	42/250 (16.8)
Caregiver Education Level		
No university degree, *n* (%)	11/15 (73.3)	173/243 (71.2)
University degree, *n* (%)	4/15 (26.7)	70/243 (28.8)

* n/a—not applicable.

The mother mean age at delivery was 30.52 (±6.34) years. The time of SARS-CoV-2 infection during pregnancy comprised 19.1% in the first trimester, 34.5% in the second trimester, 38.9% in the third trimester, and 7.5% with acute infection at delivery. Most mothers presented non-severe illness (84.6%), with 15.4% presenting severe disease. The caregiver educational level indicated that a high proportion of mothers (72.6%) did not have a university degree (Table 1).

3.2. Bayley-III Developmental Delays Regardless of Unaltered APGAR Scores

The categorical analysis of the Bayley-III scores to assess the infant neurological development status at 6 and 12 months of age is presented in Figure 2. The results indicate that at 6 months of age, 7% of the infants born to mothers infected with SARS-CoV-2 during

pregnancy experienced cognitive delays, 23% had language delays, and 11% had motor delays. As for 12 months of age, cognitive delays were observed in 2% of the participants, language delays in 26%, and motor delays in 7%. The percentage of children with a delay according to each group can be observed in Figure 2.

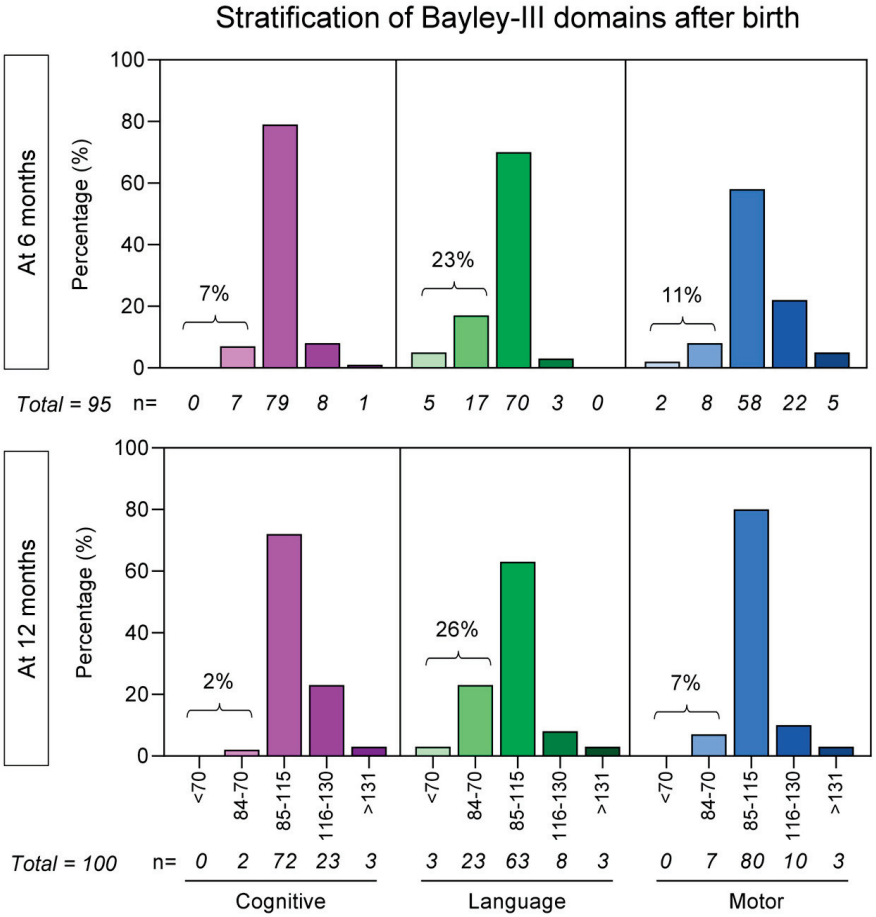


Figure 2. Stratification of Bayley-III cognitive (purple bars), language (green bars), and motor (blue bars) domains at 6 and 12 months after birth for infants born to mothers with SARS-CoV-2 infection during pregnancy. Data are presented as percentages of children with Bayley-III categorized as <70 (<−2 SD below average), 84–70 (−1 to −2 SD below average), 85–115 (−1 to 1 SD around average), 116–130 (1–2 SD above average), and >131 (>2 SD above average), illustrated by distinct shades of colors. SD = standard deviation.

No significant differences were observed in the frequency of the APGAR 1st- and 5th-minute scores (p = 0.190 and 0.4695, respectively) between infants born to mothers infected with SARS-CoV-2 during pregnancy (≤7–20.7% and 4.1%, respectively) and healthy controls (≤7–35.7% and 7.1%, respectively). Therefore, there is no evidence suggesting an association between APGAR scores and cognitive, language, and motor development (Table 1).

3.3. Bayley-III Domains According to the Time of Maternal SARS-CoV-2 Infection

Table 2 presents the comparative analysis between cognitive, language, and motor domains of infants at 6 and 12 months, according to the trimester during pregnancy in which maternal SARS-CoV-2 infection occurred. The data analysis demonstrated that at 6 months, a lower cognitive domain was observed for infants born to mothers who acquired SARS-CoV-2 infection in the third trimester or with acute infection at delivery compared with those infected in the first pregnancy trimester.

Table 2. Bayley-III cognitive, language, and motor domains at 6 and 12 months after birth, according to the time of maternal SARS-CoV-2 infection.

Bayley-III Domains *	HC	Time of Maternal SARS-CoV-2 Infection				*p-Values*
		1st Trimester	2nd Trimester	3rd Trimester	At Delivery	*ANOVA or* χ^2
6 months after birth						
Cognitive, mean (±SD)	110.0 (±11)	111.7 (±23)	102.0 (±12)	**98.3 (±12) 1st**	**90.0 (±10) 1st**	***0.0037***
Normal, *n* (%)	5/5 (100.0)	21/23 (91.3)	23/29 (79.3)	28/38 (73.7)	3/5 (60.0)	
Altered, *n* (%)	0/5 (0.0)	**2/23 (8.7) #**	**6/29 (20.7) #, 1st**	**10/38 (26.3) #, 1st**	**2/5 (40.0) #, 1st, 2nd, 3rd**	***0.0001***
Language, mean (±SD)	92.0 (±2)	94.4 (±17)	95.5 (±12)	93.3 (±14)	88.8 (±22)	*0.8994*
Normal, *n* (%)	5/5 (100.0)	21/23 (91.3)	23/29 (79.3)	29/38 (73.7)	3/5 (60.0)	
Altered, *n* (%)	0/5 (0.0)	**2/23 (8.7) #**	**6/29 (20.7) #**	**9/38 (26.3) #**	**2/5 (40.0) #, 1st, 2nd, 3rd**	***0.0001***
Motor, mean (±SD)	112.4 (±12)	106.6 (±21)	107.3 (±16)	104.3 (±16)	92.2 (±17)	*0.2961*
Normal, *n* (%)	5/5 (100.0)	19/23 (82.6)	26/29 (89.7)	35/38 (92.1)	3/5 (60.0)	
Altered, *n* (%)	0/5 (0.0)	**4/23 (17.4) #**	**3/29 (10.3) #**	**3/38 (7.9) #**	**2/5 (40.0) #, 1st, 2nd, 3rd**	***0.0001***
12 months after birth						
Cognitive, mean (±SD)	115.0 (±9)	113.8 (±15)	108.2 (±12)	110.6 (±12)	103.8 (±19)	*0.3503*
Normal, *n* (%)	3/3 (100.0)	15/15 (100.0)	33/34 (97.1)	44/45 (97.8)	4/6 (66.7)	
Altered, *n* (%)	0/3 (0.0)	0/15 (0.0)	1/34 (2.9)	1/45 (2.3)	**2/6 (33.3) #, 1st, 2nd, 3rd**	***0.0001***
Language, mean (±SD)	98.0 (±5)	107.0 (±12)	92.5 (±15)	98.2 (±17)	**83.5 (±19) 1st**	***0.0161***
Normal, *n* (%)	3/3 (100.0)	15/15 (100.0)	22/34 (64.7)	34/45 (75.6)	3/6 (50.0)	
Altered, *n* (%)	0/3 (0.0)	0/15 (0.0)	**12/34 (35.3) #**	**11/45 (24.4) #**	**3/6 (50.0) #, 1st, 2nd, 3rd**	***0.0001***
Motor, mean (±SD)	95.0 (±5)	97.3 (±13)	101.5 (±12)	103.5 (±16)	102.4 (±18)	*0.5351*
Normal, *n* (%)	3/3 (100.0)	12/15 (80.0)	31/34 (91.2)	39/45 (86.7)	5/6 (83.0)	
Altered, *n* (%)	0/3 (0.0)	**3/15 (20.0) #**	**3/34 (8.8) #**	**6/45 (13.3) #**	**1/6 (17.0) #, 2nd, 3rd**	***0.0001***

* Data are expressed as mean values (standard deviations—SDs) of Bayley-III cognitive (purple), language (green), and motor (blue) domains at 6 and 12 months after birth for infants born to mothers with SARS-CoV-2 infection during pregnancy. The *p*-values were calculated by multiple comparisons carried out by ANOVA with Bonferroni correction. The comparative analysis of the categorical data was carried out by the Chi-square test (χ^2). Significant differences are highlighted in bold and identified by # when compared with HC and by 1st, 2nd, and 3rd when compared with the 1st trimester, 2nd trimester and 3rd trimester, respectively.

At 12 months, lower language development was observed in infants born to mothers with acute infection at delivery compared with those born to mothers infected in the first pregnancy trimester.

The analysis of the categorical Bayley-III scores demonstrated that except for the cognitive domain at 12 months, most Bayley-III scores presented altered patterns in infant born to mothers infected with SARS-CoV-2 during pregnancy compared with healthy controls.

Of note, infants born to mothers with acute infection at delivery presented a significant higher frequency of altered Bayley-III scores in most domains compared with those born to mothers infected earlier during pregnancy, especially in the first trimester (Table 2).

3.4. Bayley-III Domains According to the Severity of Maternal SARS-CoV-2 Infection

The Bayley-III cognitive, language, and motor domains at 6 and 12 months were also evaluated according to the severity of maternal SARS-CoV-2 infection during pregnancy (Table 3). The data analysis carried out by taking the dataset as continuous variables did not demonstrate significant differences in the Bayley-III domains at 6 and 12 months.

Table 3. Bayley-III cognitive, language, and motor domains at 6 and 12 months, according to the severity of maternal SARS-CoV-2 infection.

Bayley-III Domains *	HC	Severity of Maternal SARS-CoV-2 Infection		*p-Values*
		Non-Severe	Severe	*ANOVA or* χ^2
6 months after birth				
Cognitive, mean (±SD)	110.0 (±11)	102.6 (±17)	97.9 (±15)	*0.3477*
Normal, *n* (%)	5/5 (100.0)	65/80 (81.3)	10/14 (71.4)	
Altered, *n* (%)	0/5 (0.0)	**15/80 (18.7) #**	**4/14 (28.6) #**	***0.0001***
Language, mean (±SD)	91.6 (±1)	94.7 (±15)	89.8 (±14)	*0.4640*
Normal, *n* (%)	5/5 (100.0)	62/80 (77.5)	9/14 (64.3)	
Altered, *n* (%)	0/5 (0.0)	**18/80 (22.5) #**	**5/14 (35.7) #,N-S**	***0.0001***
Motor, mean (±SD)	112.4 (±12)	106.5 (±17)	96.8 (±17)	*0.0972*
Normal, *n* (%)	5/5 (100.0)	71/80 (88.8)	11/14 (78.6)	
Altered, *n* (%)	0/5 (0.0)	**9/80 (11.2) #**	**3/14 (21.4) #,N-S**	***0.0001***
12 months after birth				
Cognitive, mean (±SD)	115.0 (±9)	109.6 (±13)	110.0 (±13)	*0.7815*
Normal, *n* (%)	3/3 (100.0)	82/85 (96.5)	11/12 (91.7)	
Altered, *n* (%)	0/3 (0.0)	**3/85 (3.5) #**	**1/12 (8.3) #**	***0.0095***
Language, mean (±SD)	98.0 (±5)	96.5 (±17)	100.3 (±16)	*0.7444*
Normal, *n* (%)	3/3 (100.0)	61/85 (71.8)	11/12 (91.7)	
Altered, *n* (%)	0/3 (0.0)	**24/85 (28.2) #**	**1/12 (8.3) #,N-S**	***0.0001***
Motor, mean (±SD)	95.0 (±5)	101.4 (±14)	107.8 (±15)	*0.2238*
Normal, *n* (%)	3/3 (100.0)	72/85 (84.7)	12/12 (100.0)	
Altered, *n* (%)	0/3 (0.0)	**13/85 (15.3) #**	**0/12 (0.0) #,N-S**	***0.0001***

* Data are expressed as mean values (standard deviations—SDs) of Bayley-III cognitive (purple), language (green), and motor (blue) domains at 6 and 12 months after birth for infants born to mothers with SARS-CoV-2 infection during pregnancy. The *p*-values were calculated by multiple comparisons carried out by ANOVA with Bonferroni correction. The comparative analysis of the categorical data was carried out by the Chi-square test (χ^2). Significant differences are highlighted in bold and identified by # when compared with HC and by N-S when compared with the non-severe subgroup.

However, the analysis of the categorical Bayley-III scores at 6 and 12 months demonstrated that regardless of the disease severity, infants born to mothers with SARS-CoV-2 infection presented altered Bayley-III scores compared with those born to healthy control mothers.

Additionally, infants born to mothers with severe SARS-CoV-2 infection presented a higher frequency of altered language and motor domains at 6 months compared with the group with non-severe maternal disease. At 12 months, the frequency of infants with

altered language and motor domains were lower in the subgroup born to mothers with severe SARS-CoV-2 infection (Table 3).

3.5. Bayley-III Domains According to the Caregiver Educational Level

Table 4 shows the comparison between the cognitive, language, and motor domains at 6 and 12 months according to the caregiver educational levels. The data analysis carried out by taking the dataset as continuous variables did not show significant differences in the Bayley-III domains at 6 and 12 months.

Table 4. Bayley-III cognitive, language, and motor domains at 6 and 12 months, according to the caregiver educational level.

Bayley-III Domains *	HC	Caregiver Educational Level		*p-Values*
		No University Degree	University Degree	*ANOVA or* χ^2
6 months after birth				
Cognitive, mean (±SD)	110.0 (±11)	102.0 (±18)	103.0 (±15)	*0.5784*
Normal, *n* (%)	5/5 (100.0)	55/66 (83.3)	19/25 (76.0)	
Altered, *n* (%)	0/5 (0.0)	**11/66 (16.7) #**	**6/25 (24.0) #**	***0.0001***
Language, mean (±SD)	91.6 (±1)	93.3 (±13)	95.2 (±19)	*0.8032*
Normal, *n* (%)	5/5 (100.0)	50/66 (75.8)	19/25 (76.0)	
Altered, *n* (%)	0/5 (0.0)	**16/66 (24.2) #**	**6/25 (24.0) #**	***0.0001***
Motor, mean (±SD)	112.4 (±12)	103.6 (±17)	109.6 (±20)	*0.2356*
Normal, *n* (%)	5/5 (100.0)	57/66 (86.4)	22/25 (88.0)	
Altered, *n* (%)	0/5 (0.0)	**9/66 (13.6) #**	**3/25 (12.0) #**	***0.0010***
12 months after birth				
Cognitive, mean (±SD)	115.0 (±9)	109.9 (±13)	110.7 (±13)	*0.7926*
Normal, *n* (%)	3/3 (100.0)	63/66 (95.5)	27/28 (96.4)	
Altered, *n* (%)	0/3 (0.0)	3/66 (4.5)	1/28 (3.6)	*0.1466*
Language, mean (±SD)	98.0 (±5)	97.5 (±17)	96.6 (±15)	*0.9680*
Normal, *n* (%)	3/3 (100.0)	49/66 (74.2)	22/28 (78.6)	
Altered, *n* (%)	0/3 (0.0)	**17/66 (25.8) #**	**6/28 (21.4) #**	***0.0001***
Motor, mean (±SD)	95.0(±5)	102.2(±15)	103.3(±14)	*0.6285*
Normal, *n* (%)	3/3 (100.0)	57/66 (86.4)	24/28 (85.7)	
Altered, *n* (%)	0/3 (0.0)	**9/66 (13.6) #**	**4/28 (14.3) #**	***0.0005***

* Data are expressed as mean values (standard deviations—SDs) of Bayley-III cognitive (purple), language (green), and motor (blue) domains at 6 and 12 months after birth for infants born to mothers with SARS-CoV-2 infection during pregnancy. The *p*-values were calculated by multiple comparisons carried out by ANOVA with Bonferroni correction. The comparative analysis of the categorical data was carried out by the Chi-square test (χ^2). Significant differences are highlighted in bold and identified by # when compared with HC.

The analysis of the categorical data showed that higher frequencies of altered Bayley-III domains were found in infants born to mothers with SARS-CoV-2 infection during pregnancy compared with healthy controls. However, no differences in the categorical data were found regarding the caregiver educational level (Table 4).

3.6. Regression Models of Association Between Bayley-III Domains and Maternal Features

Multinomial and logistic regression models were employed to examine the associations between the Bayley-III scores of each domain and maternal features including time of infection, disease severity, and educational level (Table 5). The data analysis indicated a significant negative relationship between altered cognitive (coef = −14.5) and language (coef = −14.01) domains at 12 months and acute SARS-CoV-2 infection at delivery (Table 5).

Table 5. Associations between Bayley-III domains at 6 and 12 months and maternal features.

Bayley-III Domains *	Maternal Features **					
	Acute SARS-CoV-2 Infection at Delivery	***p-Value***	**Severe Disease**	***p-Value***	**University Degree**	***p-Value***
6 months after birth						
Cognitive, Coef (SE)	−12.33 (7.58)	*0.107*	−4.71 (4.82)	*0.332*	1.03 (3.95)	*0.795*
Language, Coef (SE)	−6.86 (6.63)	*0.304*	−4.89 (4.19)	*0.247*	1.92 (3.45)	*0.579*
Motor, Coef (SE)	−15.80 (7.83)	*0.047*	−9.68 (4.97)	*0.055*	5.94 (4.12)	*0.153*
12 months after birth						
Cognitive, Coef (SE)	**−14.65 (5.30)**	***0.007***	0.41 (4.08)	*0.920*	0.79 (2.98)	*0.792*
Language, Coef (SE)	**−14.01 (6.76)**	***0.041***	3.83 (5.07)	*0.452*	−0.88 (3.67)	*0.812*
Motor, Coef (SE)	0.12 (5.89)	*0.983*	6.40 (4.29)	*0.140*	1.07 (3.21)	*0.740*

* Multinomial and logistic regression models were employed to examine the associations between Bayley-III scores (cognitive—purple; language—green; motor—blue) and maternal features. The regression models were adjusted for infant sex and gestational age at delivery. ** Reference groups: infections in the 1st, 2nd, and 3rd trimesters; non-severe; and no-university degree. *p*-Values of <0.05 denoted statistical significance. The analyses were performed with Minitab version 17 (Minitab, LLC, State College, PA, USA). Significant coefficients are highlighted in bold.

4. Discussion

This study observed that women exposed to SARS-CoV-2 during pregnancy had children with delayed neuropsychomotor development, specifically in the language domain. For this, the individuals studied (all within the context of a pandemic and social isolation) were divided according to the trimester of infection onset, the severity of the infection in the pregnant woman, and the educational level of the caregivers. The analysis of the Bayley-III domains based on categorical classification demonstrated that at 6 months of life, delays in cognitive, language, and motor development were observed in infants born to SARS-CoV-2-infected mothers, especially in those from subgroups of mothers with acute infection at delivery, with severe COVID-19, and without a university degree. At 12 months, a language delay was observed particularly in children whose pregnant mothers were acutely infected with SARS-CoV-2 at delivery. Multinomial and logistic regression models indicated a significant negative relationship between altered cognitive and language domains at 12 months and acute SARS-CoV-2 infection at delivery.

Given these findings, once the SARS-CoV-2 infection has been diagnosed in a pregnant woman, an orderly and serial follow-up of these children is necessary, with a strong focus on developmental assessment for diagnosis and for promoting early intervention in order to reduce the impact of this delay on the child's further development, since language acts as a driver of many other skills to be acquired in childhood and adult life [17].

In two recent studies, developmental tests were administered indirectly by asking parents to complete surveys. Despite observing differences in developmental scores between infants exposed to SARS-CoV-2 and those not exposed, these studies did not find any statistically significant differences between the groups according to the trimester of infection onset during pregnancy or the severity of the maternal disease [6,18]. However, children born during the pandemic exhibited a considerable reduction in verbal, motor, and general cognitive performance compared with those born prior to the pandemic, regardless of their exposure to the virus [18].

The mitigation of the effect of viral infection during pregnancy on speech was observed with the increase in age in clinical studies, which could be explained by routine pediatric interventions in response to any developmental delay. The early identification of language delays allows for targeted stimulation, parental guidance, and when necessary, speech therapy or other developmental interventions. These strategies help alleviate the initial impact of viral exposure on neurodevelopment, promoting language acquisition over time. Additionally, neural plasticity in early childhood may play a role in compensating for initial delays, as children have an inherent capacity to reorganize and strengthen alternative neural pathways in response to early-life challenges.

Furthermore, a recent survey has uncovered a concerning increase in pre-existing social disparities during the pandemic. Specifically, the survey, which interviewed 500 parents from disadvantaged backgrounds in terms of education, revealed that their 0-to-2-year-old children were less likely to participate in enriching activities. This social deprivation in engaging in activities may contribute to developmental delays, as was observed in the current study, particularly among children whose mothers did not hold a university degree [19,20]. These findings highlight the need for greater support and resources to be made available to vulnerable families during times of crisis [21,22].

Shuffrey et al. revealed a difference in gross and fine motor development and personal/social scores in pandemic babies compared with those born before the pandemic. These authors did not observe associations between in utero exposure to maternal SARS-CoV-2 infection and neurodevelopmental delay at 6 months of age. These results differ from our data, which show an impact of maternal SARS-CoV-2 infection during pregnancy and delayed neurological development at 6 and 12 months of age. Together, these findings support the need for the long-term monitoring of children born during the SARS-CoV-2 pandemic to assess putative late outcomes [11].

A previous study by our group was pioneering in utilizing both the gold-standard neurodevelopmental assessment scale and ultrasound elastography for the purpose of investigating possible impacts of the pandemic on children [9]. In this previous study, Junior and colleagues presented elastography data from children exposed and not exposed to SARS-CoV-2 during the pandemic, which showed statistically significant differences, particularly a reduction in the elasticity coefficient in the deep white matter in the exposed group. Notably, children with altered elasticity coefficients, possibly with prior inflammation, also exhibited developmental delays in the language subdomain of the Bayley-III Scale at 12 months. A plausible neurological model to explain these events is that viral exposure may lead to changes in myelin content or induce mild myelin edema. Inflammatory processes could increase water content in the brain parenchyma, altering its stiffness and reducing elasticity coefficients. Since myelin is essential to efficient neural transmission, these structural changes might disrupt connectivity within language-related brain networks, ultimately contributing to the observed language delays.

The present study presents key methodological differences compared with previous investigations. Unlike other studies that incorporated parental impressions in developmental evaluations, the current investigation has employed the Bayley-III Scale applied by experienced examiners who had no emotional bond or acquaintance with the child prior to the test. This approach minimizes potential biases, such as the overestimation of developmental outcomes due to parental perception. However, it is important to mention that this study has some limitations. The primary limitations include the relatively small sample size. In this sense, it is also important to point out that correlational studies do not establish cause–effect principles. Given that data collection began during the COVID-19 pandemic—during a period of social isolation and prior to vaccine availability—the population's fear of attending a clinic located within a hospital complex (and the potential

exposure to asymptomatic carriers, particularly via public transportation) could have impacted the recruitment of a larger control group. The inclusion of a pre-pandemic control group was inadequate, since the presence or absence of social isolation could introduce confounding variables, potentially affecting developmental outcomes. Additionally, the study population sample consisted of outpatients, meaning that maternal infections were predominantly mild. Furthermore, this study was conducted in a single center, which may limit the generalizability of our findings. Future studies with larger sample sizes and multicenter designs are necessary to validate and expand upon our results. Another limitation of our study is the number of maternal socio-demographic features assessed during the questionnaire application. The analysis of other maternal features such as income level, access to healthcare, social support, family composition, number of family members, the birth order of the children, and previous motherhood experience should be included in future investigations, since they may have a direct impact on child development. Possible interventions for these children and the impact of readjusting neurodevelopment scores, especially language, should also be monitored.

The research aims outlined in this study encompassed a comprehensive examination of the developmental trajectory in children exposed to SARS-CoV-2 in utero using the Bayley-III over a one-year period, as well as a comparative analysis based on factors such as the timing of fetal exposure, maternal infection severity, and maternal educational level. It is imperative to pursue further investigations into developmental delays and to understand the right moment for intervention as well as whether the intervention could change the course of the delay. Additionally, the evaluation of children's development within a cohort of vaccinated pregnant women becomes paramount, as vaccinated individuals demonstrate distinctive responses to SARS-CoV-2 infection and associated conditions. This evaluation will enable an assessment of whether such children are also susceptible to developmental delays as in the case of unvaccinated mothers.

In conclusion, our findings indicate that children born to mothers exposed to SARS-CoV-2 during the COVID-19 pandemic exhibited delays in neuropsychomotor development, particularly in the language domain. This association was most evident in children whose mothers were infected at the time of delivery and in those with severe infections. Additionally, we observed that children whose caregivers did not have a university degree, which is considered a high educational level for the Brazilian population, were more likely to experience language developmental delays, suggesting that socioeconomic disparities may have played a significant role in shaping developmental outcomes during the pandemic. This finding underscores the need to consider environmental and social factors when evaluating the potential effects of in utero viral exposure. Given these results, we emphasize the importance of continuous follow-up and early intervention for children exposed to SARS-CoV-2 during gestation. Targeted screening and developmental support strategies may help mitigate potential long-term consequences, particularly in language acquisition, which is fundamental to cognitive development, socialization, and future academic achievement. Our findings also open new avenues for research into the effects of maternal infection on infant neurodevelopment. The longitudinal follow-up of these children could provide valuable insights into the consequences of in utero exposure to SARS-CoV-2 and its impact on the central nervous system. The observed association between SARS-CoV-2 exposure and altered neurodevelopment highlights the need for healthcare professionals to be vigilant in monitoring children born to mothers infected during pregnancy. Early screening and timely referral to appropriate interventions could mitigate developmental deficits and promote more favorable long-term outcomes.

Finally, our results suggest that public health policies aimed at reducing disparities in access to healthcare and parental education may be crucial to minimizing the adverse effects

of the pandemic on early childhood development. Building on our findings, we propose the following topics for future research: (i) the recruitment of patients from existing databases for subsequent Bayley-III assessments at 24 months of age to evaluate the trajectory of language developmental delays; (ii) advanced neuroimaging studies using brain MRI in children with developmental delays beyond 24 months, post-myelination, to identify potential structural and functional alterations associated with in utero viral exposure; and (iii) comparative studies on neurodevelopmental outcomes in children exposed to SARS-CoV-2 in utero, differentiating between those born to vaccinated and unvaccinated mothers, given the potential influence of maternal immune responses on fetal neurodevelopment.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: No new data were created or made available due to privacy and ethical restrictions on pregnant woman and pediatric data. Therefore, the data cannot be shared to ensure the confidentiality and protection of the participants' privacy.

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Systematic Review

Antenatal Sildenafil for Congenital Diaphragmatic Hernia: A Systematic Review and Bayesian Meta-Analysis of Preclinical Studies

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Abstract

Background: In congenital diaphragmatic hernia (CDH), pulmonary hypoplasia and pulmonary hypertension are major causes of morbidity and mortality. Antenatal treatment with sildenafil has shown some promising protective effects in experimental CDH, but no systematic review has yet evaluated the preclinical evidence on this topic. **Methods**: PubMed and EMBASE databases were searched for studies using antenatal sildenafil in animal models of CDH. Bayesian model-averaged (BMA) meta-analysis was used to calculate Bayes factors (BFs). The BF_{10} is the ratio of the probability of the data under the alternative hypothesis (presence of effect) over the probability of the data under the null hypothesis (absence of effect). Risk of bias was assessed by the SYRCLE tool. **Results**: We included 18 studies (14 nitrofen and 4 surgical CDH). The BMA analysis showed inconclusive evidence (BF_{10} between 0.33 and 3) for the presence of an effect of sildenafil in fetal survival (7 studies, BF_{10} = 1.25) or in lung hypoplasia as assessed by the lung-to-body weight ratio (16 studies, BF_{10} = 2.04). In contrast, the BMA analysis showed conclusive evidence (BF_{10} > 3) in favor of a positive effect of sildenafil on small pulmonary arteries medial wall thickness (12 studies, BF_{10} = 1499), radial alveolar count (6 studies, BF_{10} = 167.57), interalveolar septa thickness (4 studies, BF_{10} = 56.86), distal airway complexity (3 studies, BF_{10} = 7.95), mean saccular airspace diameter (2 studies, BF_{10} = 7.61), total lung capacity (2 studies, BF_{10} = 6.91), lung compliance (2 studies, BF_{10} = 5.19), and VEGF expression (5 studies, BF_{10} = 10.62). **Conclusions**: In preclinical models of CDH, antenatal sildenafil rescues pulmonary vascular remodeling and airway/airspace morphometric alterations.

Keywords: congenital diaphragmatic hernia; sildenafil; nitrofen; animal models; Bayesian statistics

1. Introduction

Congenital Diaphragmatic Hernia (CDH) is a rare congenital malformation characterized by incomplete diaphragm development that leads to herniation of abdominal organs

into the thorax, resulting in disruption of physiologic cardiopulmonary development [1,2]. The incidence of CDH ranges from 1.7 to 5.7 per 10,000 live births. The mortality rate for affected individuals varies across studies, ranging from 20% to 60%, with significant disparities based on the presence of associated anomalies and the type of registry reporting the data [1,2].

The degree of pulmonary hypoplasia, postnatal pulmonary hypertension (PH), and cardiac dysfunction are pivotal in determining clinical severity and the prognosis of CDH [3–6]. CDH hypoplastic lungs are characterized by decreased bronchial branching, immature epithelium and mesenchyme, and reduced alveolar units, which results in impaired gas exchange. Pathological vascular development and remodeling of the vascular network are also present, with decreased vascular units, hyperplasia of the vessel wall, and altered vasoreactivity that causes postnatal persistent PH [7,8]. Concomitant fetal cardiac hypoplasia, especially left ventricular hypoplasia, is another crucial contributor to cardiovascular dysfunction.

Endothelial dysfunction is a central mechanism in the pathogenesis of CDH-associated PH, particularly through its impact on the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling pathway [8–15]. Experimental models showed that pulmonary endothelial NO synthase (eNOS) activity and expression are reduced in CDH, resulting in decreased endogenous NO production and impaired vasodilation, which contributes to elevated pulmonary vascular resistance and PH [8–15]. Downstream, the activity of soluble guanylate cyclase (sGC)—the enzyme that generates cGMP in response to NO—is also impaired, further limiting cGMP-mediated vasorelaxation [8–15]. Additionally, increased expression and activity of phosphodiesterase type 5 (PDE5), which degrades cGMP, further reduces the effectiveness of the NO/cGMP pathway. These abnormalities explain the limited clinical efficacy of inhaled NO in CDH-associated PH, as the pathway is compromised at multiple points, including NO synthesis, sGC activity, and cGMP degradation [8–15]. The disbalance between vasodilators (NO, prostacyclin) and vasoconstrictors (endothelin-1, thromboxane A2), favors vasoconstriction and smooth muscle proliferation, which drive pulmonary vascular remodeling and increased pulmonary vascular resistance. The loss of highly proliferative endothelial cell populations further impairs angiogenesis and vascular growth, exacerbating pulmonary hypoplasia and hypertension [8–15]. Sildenafil is a PDE-5 inhibitor that blocks cGMP degradation and contributes to maintaining vasodilation. In addition, higher cellular concentrations of cGMP stimulate the formation of cGMP-dependent protein kinase (PKG), which is implicated in smooth muscle proliferation [16]. Therefore, the inhibition of PDE5 increases intracellular cGMP levels, producing vasodilatory and anti-remodeling effects, and having synergism with inhaled NO in the treatment of PH in newborns with CDH [16–19].

Advancements in the field of prenatal care have led to significant progress in the diagnosis of CDH and the development of disease severity prediction models in affected fetuses [1,20]. These advancements have also led to the recognition of the potential for fetal interventions to improve outcomes [1,20]. The primary objective of prenatal treatment of CDH is to mitigate the severity of pulmonary hypoplasia and pulmonary hypertension. The most extensively studied and currently employed prenatal intervention is fetoscopic endoluminal tracheal occlusion (TO) [1,20]. Preclinical evidence indicates that antenatal interventions targeting PDE5 (e.g., sildenafil) can partially restore NO/cGMP signaling, improve pulmonary vascular development, and attenuate PH in animal models of CDH [21–27]. However, a systematic review and meta-analysis on the therapeutic potential of prenatal sildenafil in experimental CDH has yet to be conducted. The purpose of this systematic review was to provide a comprehensive overview of all studies using prenatal

sildenafil in animal models of CDH and to quantify, by means of a Bayesian meta-analysis, the effect of the intervention in vascular and parenchymal lung development.

2. Methods

2.1. Protocol

A protocol was registered on PROSPERO (CRD42021261102) before starting the review. We used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist for the manuscript [28].

2.2. Sources and Search Strategy

We searched Medline (via the PubMed interface) and Embase (via OVID) for original, pre-clinical studies concerning the effects of prenatal sildenafil on CDH published until 31 October 2023. An update to the search was conducted on 2 June 2025. The search strategy involved the following three components: ((sildenafil (and all synonyms) OR Phosphodiesterase 5 Inhibitors (and all synonyms)) AND congenital diaphragmatic hernia (and all synonyms) AND animals [29,30]. The complete search strategy is available in the Supplementary Material. No language or date restrictions were applied.

2.3. Inclusion Criteria and Study Selection

Studies were included in this systematic review when they met all the following criteria: (1) original in vivo animal studies using a neonatal experimental CDH model; (2) intervention was randomized, quasi-randomized or non-randomized vs. a control group; (3) tested as intervention antenatal sildenafil or other PDE-5 inhibitors; (4) reported on the primary or any of the secondary outcome measures. Non-interventional studies, studies without controls, and non-neonatal models were excluded. The neonatal period was considered to be the first 7 days of life in rats and mice [31], 5 days in lambs [32], and 10 days in rabbits [33].

Two independent reviewers (T.M.H., I.A.) screened all studies for inclusion on the basis of title and abstract using Rayyan RCI software version 1.6.3 [34]. In case of doubt, the whole publication was evaluated. Full-text copies of all publications eligible for inclusion were subsequently assessed by two independent reviewers (T.M.H., I.A.) and included when they met our pre-specified inclusion criteria. Disagreement was solved by discussion or by consulting a third investigator (E.V.).

The primary outcome was fetal survival. Secondary outcomes were lung hypoplasia, morphometric alterations in the pulmonary vasculature, airway or airspace, biomarkers, morphometric alterations in the heart, and pulmonary or cardiac functional alterations. Heart morphometry and function were not pre-registered as outcomes in the protocol. Therefore, these outcomes should be considered post hoc additions.

2.4. Data Extraction

Two reviewers (T.H., I.A.) extracted the data using a predetermined data extraction sheet and a third reviewer (E.V.) checked data for accuracy. Data were extracted for study characteristics (authors, year of publication, study location), study design (sample size for intervention, control and sham groups), intervention characteristics (timing, dose and mode of sildenafil administration) and outcome measures. Dichotomous and continuous data provided in numbers were extracted directly. If only graphs were available, Web Plot Digitizer was used to extract numerical values [35]. When a study reported a range for sample size (e.g., n = 7–9), and the exact number could not be derived from the data, the median sample size was assumed.

2.5. Risk of Bias Assessment

We used the SYRCLE Risk of Bias tool [36] to assess the risk of bias in the included studies. Two reviewers (T.H., I.A.) independently evaluated the risk of bias. Discrepancies in scoring were resolved through discussion. A 'yes' score indicates low risk of bias; a 'no' score indicates high risk of bias; and an 'unclear' score indicates unknown risk of bias.

To overcome the problem of judging too many items as 'unclear risk of bias' due to reporting of experimental details on animals, methods and materials being incomplete [37], we added three items on reporting: reporting of any measure of randomization, reporting of any measure of blinding, and reporting of a sample size calculation. For these three items, a 'yes' score indicates 'reported', and a 'no' score indicates 'unreported'.

2.6. Bayesian Meta-Analysis

Effect size of dichotomous variables was expressed as odds ratio (OR) and effect size of continuous variables was expressed using the Hedges' *g*. Values of logOR and Hedges' *g* and the corresponding standard error of each individual study were calculated using comprehensive meta-analysis (CMA) V4.0 software (Biostat Inc., Englewood, NJ, USA). The results were further pooled and analyzed by a Bayesian model-averaged (BMA) meta-analysis [38–43]. The use of BMA meta-analysis was a change from the original protocol that was registered as an amendment. We performed the BMA in JASP, which utilizes the metaBMA R package version 0.6.9 [40,41]. BMA employs Bayes factors (BFs) and Bayesian model-averaging to evaluate the likelihood of the data under the combination of models assuming the presence vs. the absence of the meta-analytic effect and heterogeneity [38–43]. The BF_{10} is the ratio of the probability of the data under H_1 over the probability of the data under H_0. The BF_{10} was interpreted using the evidence categories suggested by Lee and Wagenmakers [44]: <1/100 = extreme evidence for H_0, from 1/100 to <1/30 = very strong evidence for H_0, from 1/30 to <1/10 = strong evidence for H_0, from 1/10 to <1/3 = moderate evidence for H_0, from 1/3 to <1 weak/inconclusive evidence for H_0, from 1 to 3 = weak/inconclusive evidence for H_1, from >3 to 10 = moderate evidence for H_1, from >10 to 30 = strong evidence for H_1, from >30 to 100 = very strong evidence for H_1, and >100 extreme evidence for H_1. The BF_{rf} is the ratio of the probability of the data under the random-effects model over the probability of the data under the fixed-effects model. The categories of strength of the evidence in favor of the random effects ($BF_{rf} > 1$) or the fixed effect ($BF_{rf} < 1$) were similar to those described above for the BF_{10}.

For all the analyses, we used the neonatal-specific empirical prior distributions based on the Cochrane Database of Systematics Reviews [39,45]. Binary outcomes: logOR ~ Student-t($\mu = 0$, $\sigma = 0.29$, $\nu = 3$), tau(logRR) ~ Inverse-Gamma($k = 1.80$, $\theta = 0.42$); Continuous outcomes: Cohen's d ~ Student-t($\mu = 0$, $\sigma = 0.42$, $\nu = 5$), tau(Cohen's d) ~ Inverse-Gamma($k = 1.68$, $\theta = 0.38$) [38,45].

In order to facilitate a comparison of the Bayesian and frequentist approaches, a DerSimonian-Laird random effects meta-analysis was conducted using the CMA software.

2.7. Subgroup Analysis and Publication Bias Analysis

In the pre-registered protocol, it was specified that subgroup analysis would be conducted when there were at least 10 independent comparisons per subgroup and that publication bias analysis (using Egger's Regression Test, and Duval and Tweedie's Trim and Fill) would be conducted when there were at least 20 studies reporting a given outcome.

3. Results

The flow diagram of the systematic search is shown in Figure 1. Finally, 18 studies were included in the systematic review [21–27,46–56]. The characteristics of the included studies are summarized in Table 1. Of the 18 included studies, 17 used sildenafil [21–27,46–55] and one used tadalafil [56]. Therefore, tadalafil could not be included in a meta-analysis as only one study was available. Two of the 17 sildenafil studies included two temporal schedules (early and late sildenafil) [49,55]. As a result, a total of 19 experimental settings were included in the meta-analysis. The nitrofen model was used in 14 studies (12 in rat and 2 in mouse) [21–25,46,48–55] and the surgical CDH model was used in 4 studies (2 in rabbit and 2 in lamb) [26,27,47,56]. No studies were found on the use of sildenafil in genetic models of CDH.

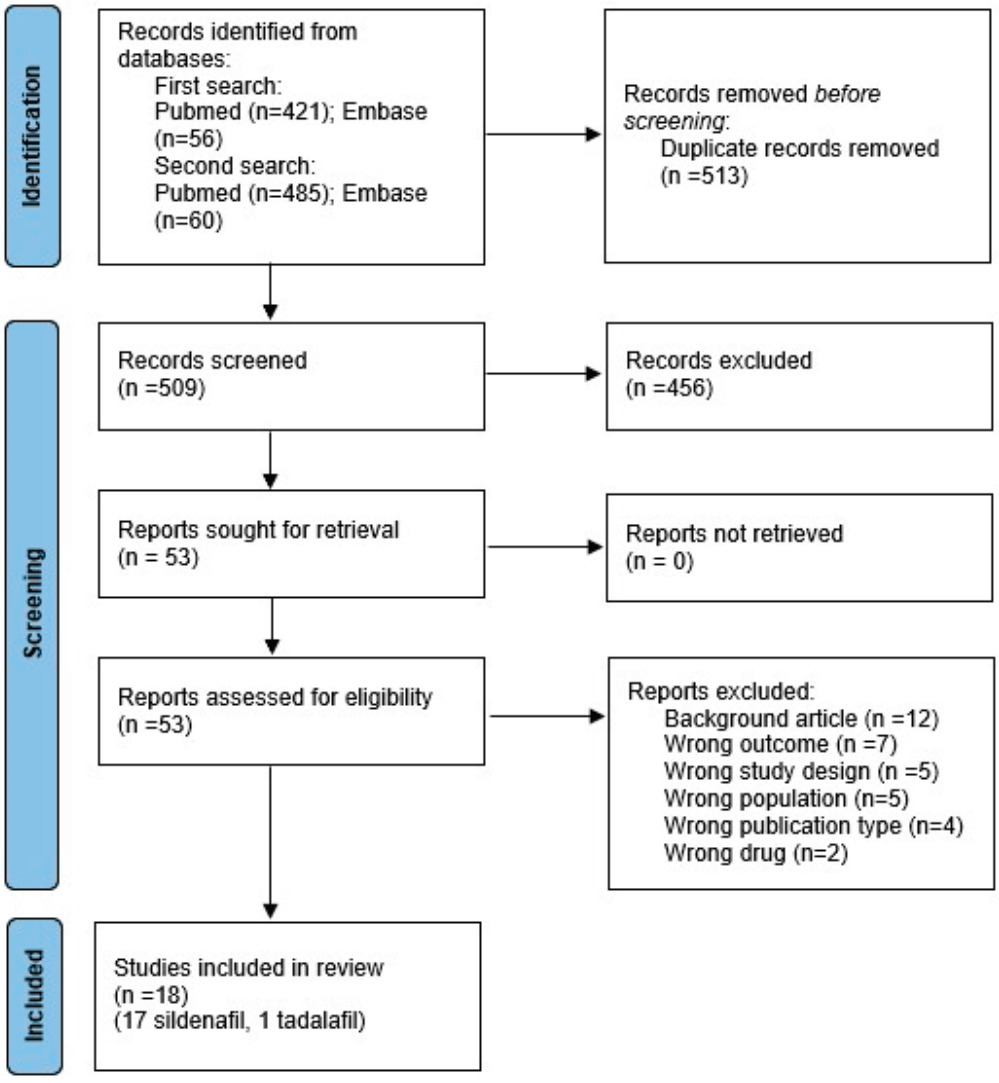


Figure 1. PRISMA 2020 flow diagram of the systematic search.

The risk of bias assessment from the 18 studies is presented in Table 2. The SYRCLE tool contains 10 entries related to selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases. None of the studies met the criteria for low risk of bias across all 10 domains. Although 12 studies reported randomizing animals to treatment, none described the method of randomization. None of the included studies described their methods of allocation concealment. Blinding was reported rarely and inconsistently depending on where in the experimental process the blinding occurred, with blinding of outcome assessment being the most frequent (7 studies, Table 1). Only two studies reported

how the sample size was calculated. The source of funding was always reported in sufficient detail to assess risk of bias. The 18 studies reported a non-industrial source of funding. Finally, 15 studies reported no conflicts of interest, one reported a potential conflict, and two were rated as unclear for not reporting whether conflicts of interest existed or not.

Table 1. Characteristics of the included studies.

First Author, Year	Model-Species	Time of Induction CDH	Day of Harvesting	Drug	Day of Administration	Dose	Route
Kashyap, 2019 [27]	Surgery-Lamb	E80	E138	Sildenafil	E105–E138	0.21 mg/kg/h	IV
Kattan, 2014 [53]	Nitrofen-Rat	E9.5	E22	Sildenafil	E11.5–E21.5	90 mg/kg	oral
Lemus-Varela, 2014 [46]	Nitrofen-Rat	E9	E21	Sildenafil	E16–E20	100 mg/kg	oral
Liao, 2020 [50]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E11.5–E21.5	100 mg/kg	oral
Luong, 2011 [21]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E11.5–E20.5	100 mg/kg	SC
Makanga, 2015 [52]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E11.5–E21.5	100 mg/kg	oral
Mesas-Burgos, 2016 [25]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E11.5–E20.5	100 mg/kg	SC
Mous, 2016 [24]	Nitrofen-Rat	E9.5	E21	Sildenafil	E17.5–E20.5	100 mg/kg	oral
Mous, 2018 [23]	Nitrofen-Rat	E9.5	E21	Sildenafil	E17.5–E20.5	100 mg/kg	oral
Okolo, 2018 [51]	Nitrofen-Mouse	E8.5	E18.5	Sildenafil	E12.5	10 μg	IA
Okolo, 2020 [55]	Nitrofen-Mouse	E8.5	P0	Sildenafil	E10.5 (early) E15.5 (late)	4 μg	IA
Russo, 2016 [26]	Surgery-Rabbit	E23	E31	Sildenafil	E24–E30	10 mg/kg	SC
Russo, 2022 [47]	Surgery-Rabbit	E23	E30	Sildenafil	E24–E30	10 mg/kg	SC
Toso, 2024 [48]	Nitrofen-Rat	E9.5	E21	Sildenafil	E14–E21	100 mg/kg	oral
Shue, 2014 [56]	Surgery-Lamb	E85–90	E-135	Tadalafil	E85–90–E135	1–2 mg/kg	oral
Yamamoto, 2014 [22]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E11.5–E20.5	100 mg/kg	SC
Yoshida, 2023 [49]	Nitrofen-Rat	E9.5	E20.5	Sildenafil	E13.5 (early) E15.5 (late)	24 μg (early) 80 μg (late)	IA
Yoshida, 2024 [54]	Nitrofen-Rat	E9.5	E21.5	Sildenafil	E19.5	80 μg	IA

E: embryonic day; IA: intra-amniotic; IV: intravenous; P: postnatal day; SC: subcutaneous.

Bayesian Meta-Analysis

The BMA analysis results are summarized in Tables 3 and 4. Results are expressed as effect sizes (OR or Hedges' *g*) and 95% credible interval (CrI). To facilitate the comparison between the frequentist and Bayesian approaches the frequentist *p*-values are also reported in the tables and the complete random-effects frequentist meta-analysis is shown in Supplementary Tables S1 and S2. The primary outcome (fetal survival) was reported in 7 studies (Figure 2, Table 3). The BMA analysis showed inconclusive evidence (BF_{10} between 0.33 and 3) for the presence or absence of an effect of sildenafil in this outcome (BF_{10} = 1.25). The evidence in favor of the presence of heterogeneity was moderate for this analysis (BF_{rf} = 3.83). Regarding the secondary outcomes, the lung to body weight ratio (LBWR), a surrogate for pulmonary hypoplasia, was assessed in 16 studies (Figure 3, Table 3). The BMA analysis showed inconclusive evidence (BF_{10} = 2.04) for the presence of an effect of sildenafil in reversing lung hypoplasia as assessed by the LBWR. The evidence in favor of the presence of heterogeneity for this analysis was extreme ($BF_{rf} > 10^6$). Since the pre-specified threshold of 10 studies was exceeded for this outcome in the nitrofen subgroup, a separate analysis of this subgroup was conducted. The evidence supporting an effect remained inconclusive (BF_{10} between 0.33 and 3) when the nitrofen subgroup was analyzed separately (13 studies, BF_{10} = 1.38) (Table 3).

Table 2. Risk of bias assessment.

Study	1. Adequate Sequence Generation	2. Baseline Characteristics Comparable	3. Allocation Concealment	4. Random Housing	5. Investigators/Caregivers Blinding	6. Random Outcome Assessment	7. Blinding Outcome Assessment	8. Incomplete Outcome Data Adequate Addressed	9. Free of Reporting Bias	10. Other Biases Absent	Randomization Mentioned?	Blinding Mentioned?	Sample Size and Calculation Mentioned?
Kashyap, 2019 [27]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	No	No	No
Kattan, 2014 [53]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	No	No	No
Lemus-Varela, 2014 [46]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Yes	Unclear	Yes	Yes	No
Liao, 2020 [50]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Yes	No	No
Luong, 2011 [21]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Yes	Yes	No	No
Makanga, 2015 [52]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Unclear	Yes	Yes	Yes	No
Mesas-Burgos, 2016 [25]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Unclear	Unclear	Yes	Yes	No
Mous, 2016 [24]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	No	No	No
Mous, 2018 [23]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	No	No	No
Okolo, 2018 [51]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	No	No	No
Okolo, 2020 [55]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	No	No	No
Russo, 2016 [26]	Yes	Yes	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Yes	Yes	Yes	Yes	Yes
Russo, 2022 [47]	Yes	Yes	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Yes	Yes	Yes	Yes	Yes
Shue, 2014 [56]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Yes	Yes	No	No
Toso, 2024 [48]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Yes	Yes	Yes	Yes	No
Yamamoto, 2014 [22]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Unclear	Yes	Yes	Yes	No
Yoshida, 2023 [49]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Yes	No	No
Yoshida, 2024 [54]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Yes	No	No

Table 3. Bayesian model averaged (BMA) meta-analysis of fetal survival, lung morphometry and function.

Outcome	Subgroup	K	Effect Size	95% CrI		Tau	95% CrI		BF_{10}	*p*-Value Effect	Evidence for Effect	BF_{rf}	*p*-Value Heterogeneity	Evidence for Heterogeneity
				Lower Limit	Upper Limit		Lower Limit	Upper Limit						
Fetal survival	All	7	1.13 [a]	0.89	1.75	1.40	1.00	2.62	1.25	0.305	undecided for	3.83	0.050	moderate for
LBWR	All	16	0.24 [b]	−0.03	0.75	0.87	0.53	1.36	2.04	0.051	undecided for	$>10^6$	<0.001	extreme for
	Nitrofen	13	0.18 [b]	−0.09	0.71	0.88	0.52	1.41	1.38	0.076	undecided for	$>10^6$	<0.001	extreme for
Distal airway complexity (MTBD)	All	3	−0.64 [b]	−1.21	−0.07	0.36	0.07	1.24	7.95	<0.001	moderate for	0.93	0.415	undecided against
Mean saccular airspace diameter (D2 score)	All	2	1.09 [b]	−0.10	1.82	0.69	0.09	2.40	7.61	<0.001	moderate for	1.72	0.906	Undecided for
Mean linear intercept	All	4	−0.01 [b]	−0.53	0.45	0.79	0.26	1.80	0.67	0.731	undecided against	287.52	<0.001	extreme for
Radial alveolar count	All	6	0.80 [b]	0.36	1.24	0.14	0.00	0.67	167.57	<0.001	very strong for	0.89	0.175	undecided against
Interalveolar septa thickness	All	4	−1.11 [b]	−1.57	−0.47	0.36	0.07	1.36	56.86	<0.001	very strong for	0.83	0.304	undecided against
Total lung capacity	All	2	0.83 [b]	−0.01	1.70	0.53	0.08	1.96	6.91	<0.001	moderate for	1.25	0.973	undecided for
Compliance	All	2	0.73 [b]	−0.07	1.56	0.49	0.08	1.76	5.19	<0.001	moderate for	1.22	0.675	undecided for
Elastance	All	2	−0.54 [b]	−1.29	0.18	0.43	0.08	1.50	2.54	0.009	undecided for	1.15	0.373	undecided for

BF_{10}: ratio of the probability of the data under H_1 over the probability of the data under H_0; BF_{rf}: ratio of the probability of the data under the random-effects model over the probability of the data under the fixed-effects model; CrI: credible interval; K: number of studies; LBWR: Lung-to-body weight ratio; MTBD: mean terminal bronchiole density. [a] Odds ratio; [b] Hedges' *g*. *p*-values for effect and heterogeneity correspond to a frequentist random-effects meta-analysis.

Table 4. Bayesian model averaged (BMA) meta-analysis of pulmonary vascular morphometry, biomarkers expression, and heart morphometry and function.

Outcome	Subgroup	K	Hedges' *g*	95% CrI		Tau	95% CrI		BF_{10}	*p*-Value Effect	Evidence for Effect	BF_{rf}	*p*-Value Heterogeneity	Evidence for Heterogeneity
				Lower Limit	Upper Limit		Lower Limit	Upper Limit						
Medial wall thickness	All	12	−1.29	−1.74	−0.74	0.66	0.36	1.15	1499	<0.001	extreme for	$>10^6$	<0.001	extreme for
	Nitrofen	10	−1.12	−1.59	−0.55	0.62	0.34	1.12	332.4	<0.001	extreme for	$>10^6$	<0.001	extreme for
Proportionate medial thickness	All	3	−1.94	−3.18	0.18	1.37	0.10	4.59	6.75	<0.001	moderate for	2.33	0.024	undecided for
Proportionate adventitial thickness	All	2	−0.46	−1.18	0.23	0.42	0.08	1.48	1.90	0.081	undecided for	1.11	0.176	undecided for
Pulmonary vascular volume	All	2	0.06	−0.61	0.74	0.33	0.07	1.09	0.74	0.867	undecided against	0.85	0.214	undecided against
VEGF expression	All	5	1.29	0.01	2.29	0.72	0.01	2.69	10.62	0.001	strong for	3.36	0.004	moderate for
eNOS expression	All	2	0.60	−0.32	2.07	1.06	0.00	3.64	2.66	0.142	undecided for	5.16	0.007	moderate for
RV/LV + S	All	4	−0.32	−0.93	0.23	0.32	0.07	0.96	1.20	0.128	undecided for	0.90	0.293	undecided against
RV wall thickness	All	2	−1.39	−3.56	0.48	2.08	0.13	6.01	2.33	0.008	undecided for	6.17	<0.001	moderate for
Doppler AT/ET	All	2	0.94	−0.05	1.83	0.61	0.08	2.34	8.30	<0.001	moderate for	1.32	0.778	undecided for

AT/ET: Acceleration/Ejection Time ratio; BF_{10}: ratio of the probability of the data under H_1 over the probability of the data under H_0; BF_{rf}: ratio of the probability of the data under the random-effects model over the probability of the data under the fixed-effects model; CrI: credible interval; K: number of studies; RV/LV + S: right ventricle to left ventricle plus septum ratio. *p*-values for effect and heterogeneity correspond to a frequentist random-effects meta-analysis.

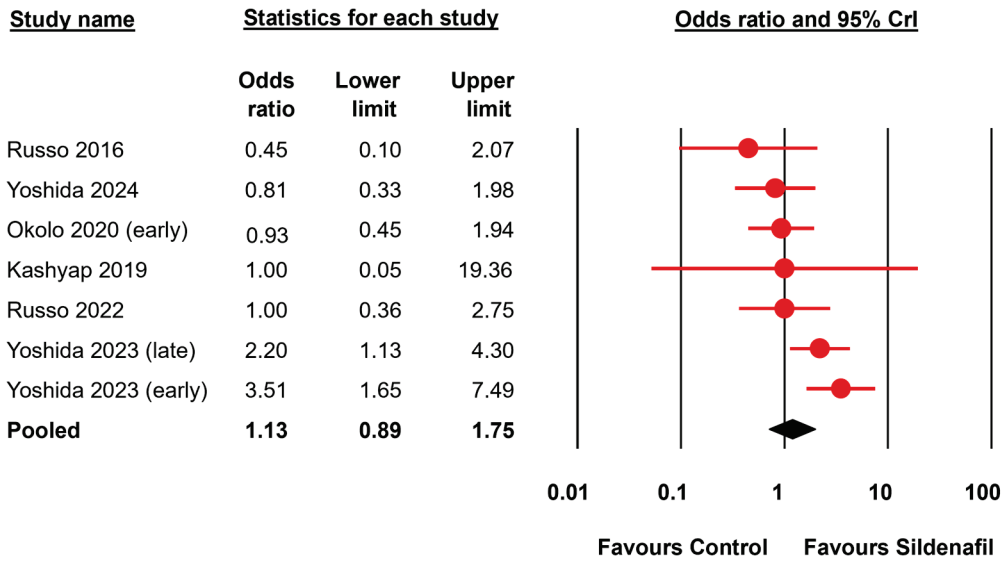


Figure 2. Bayesian model-averaged meta-analysis on the effects of sildenafil in fetal survival in experimental congenital diaphragmatic hernia [26,27,47,49,54,55]. CrI: credible interval.

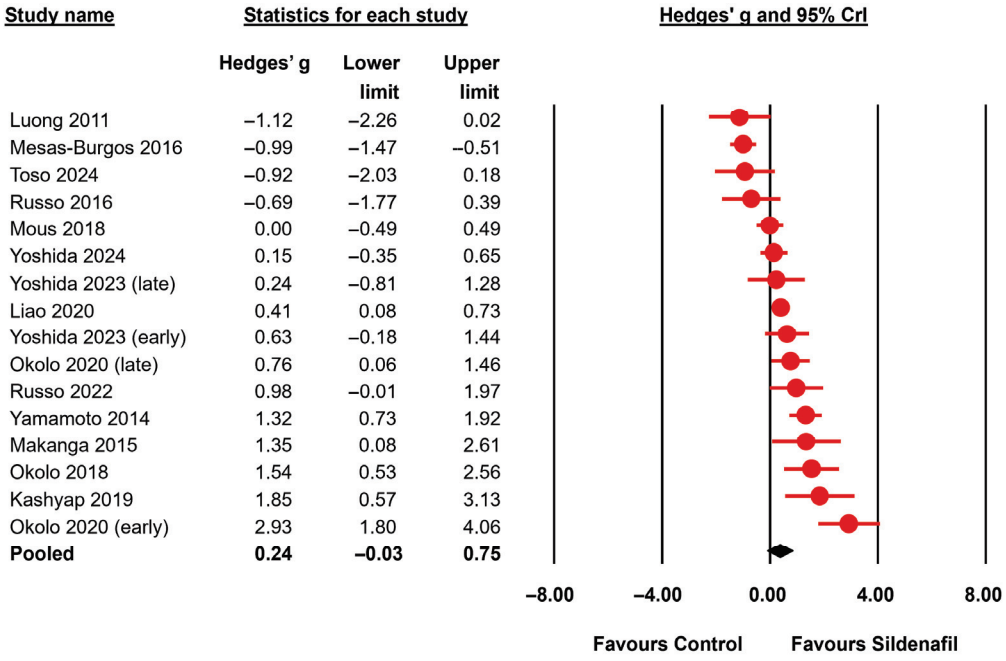


Figure 3. Bayesian model-averaged meta-analysis on the effects of sildenafil in lung hypoplasia (LBWR: lung-to-body weight ratio) in experimental congenital diaphragmatic hernia [21–23,25–27,47–52,54,55]. CrI: credible interval.

Regarding airway/airspace morphometry, the BMA analysis showed very strong evidence of a positive effect of sildenafil on radial alveolar count (6 studies, BF_{10} = 167.57, Figure 4) and interalveolar septa thickness (4 studies, BF_{10} = 56.86) and moderate evidence of a positive effect on distal airway complexity (3 studies, BF_{10} = 7.95) and mean saccular airspace diameter (2 studies, BF_{10} = 7.61) (Table 3). In addition, the BMA analysis showed moderate evidence of an improvement by sildenafil in pulmonary function as determined by total lung capacity (2 studies, BF_{10} = 6.91) and lung compliance (2 studies, BF_{10} = 5.19) (Table 3). The evidence in favor of the presence of an effect was inconclusive (BF_{10} between 0.33 and 3) for the mean linear intercept (4 studies, BF_{10} = 0.71) and the elastance (2 studies, BF_{10} = 2.54) (Table 3).

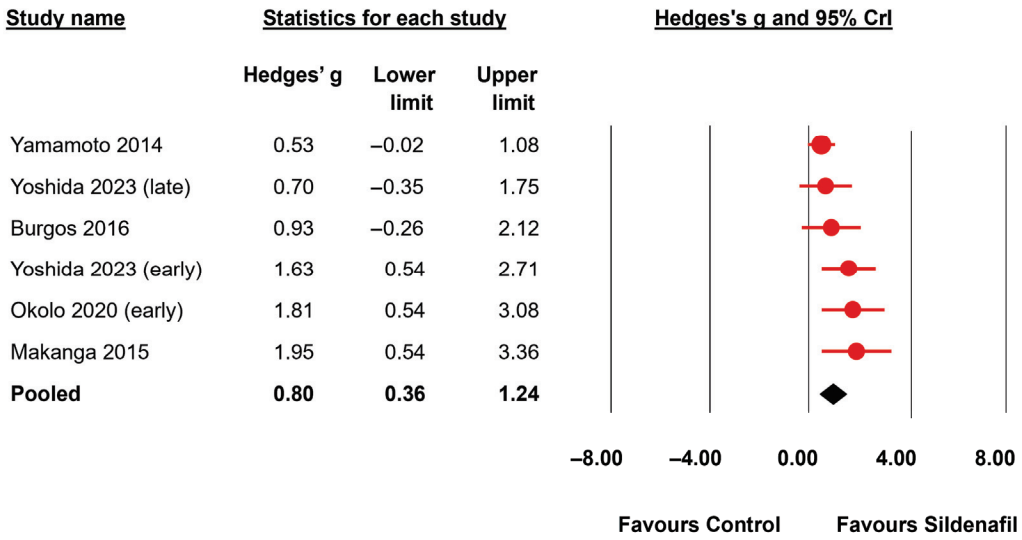


Figure 4. Bayesian model-averaged meta-analysis on the effects of sildenafil in radial alveolar count in experimental congenital diaphragmatic hernia [22,25,49,52,55]. CrI: credible interval.

Regarding the morphometry of pulmonary vessels, the BMA analysis showed extreme evidence (BF_{10} > 100) in favor of a reduction in small pulmonary arteries' medial wall thickness (MWT) in the group receiving sildenafil (12 studies, BF_{10} = 1499) (Figure 5, Table 4). The evidence in favor of heterogeneity for this meta-analysis was extreme ($BF_{rf} > 10^6$). The evidence supporting an effect remained extreme when the nitrofen subgroup was analyzed separately (10 studies, BF_{10} = 332.4) (Table 4). The BMA analysis also showed moderate evidence (BF_{10} between >3 and 10) in favor of a positive effect of sildenafil in proportionate medial thickness of small pulmonary arteries (3 studies, BF_{10} = 6.75). In contrast, the evidence was inconclusive (BF_{10} between 0.33 and 3) for proportionate adventitial thickness (2 studies, BF_{10} = 1.90) and pulmonary vascular volume (2 studies, BF_{10} = 0.74).

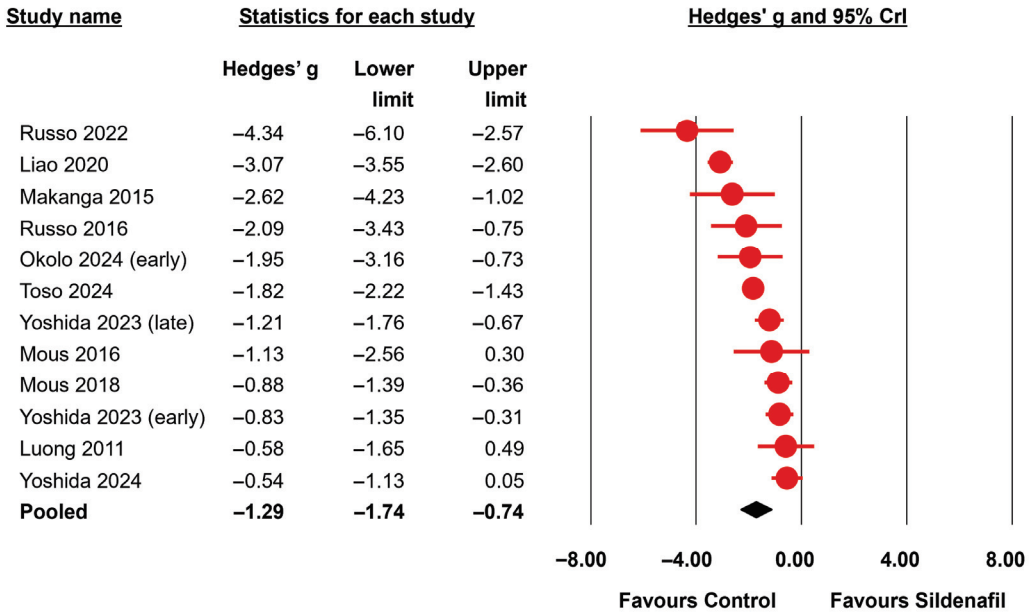


Figure 5. Bayesian model-averaged meta-analysis on the effects of sildenafil in small pulmonary arteries medial wall thickness in experimental congenital diaphragmatic hernia [21–24,26,47–50,54,55]. CrI: credible interval.

Regarding biomarkers, the BMA analysis showed strong evidence (BF_{10} between >10 and 30) of a positive effect of sildenafil in the pulmonary expression of vascular endothelial

growth factor (VEGF) (BF_{10} = 10.62) (Table 4). The pulmonary expression of eNOS was also increased by sildenafil, but the evidence in favor of this effect was weak/inconclusive (BF_{10} = 2.66).

Regarding cardiac morphometry, the evidence for or against an effect of sildenafil on decreasing right ventricle to left ventricle plus septum ratio (RV/LV + S) and right ventricle wall thickness was inconclusive (Table 4). Finally, the presence of pulmonary hypertension, as measured by the main pulmonary artery Doppler Acceleration/Ejection Time (AT/ET) ratio, was evaluated in two studies. The BMA analysis showed moderate evidence of a decrease in pulmonary hypertension in the group treated with antenatal sildenafil (BF_{10} = 8.30) (Table 4).

A meta-analysis could not be conducted for tadalafil because only one study investigated this drug [56]. That study showed that tadalafil did not induce statistically significant changes ($p > 0.05$) in either LBWR or RV/LV + S. However, tadalafil induced a significant increase in pulmonary eNOS expression in comparison to placebo ($p < 0.001$) [56].

As shown in Tables 3 and 4, when the results of the BMA analysis were compared with the frequentist meta-analysis (Supplementary Tables S1 and S2), it was observed that BF_{10} values indicative of evidence in favor of H_1 ($BF_{10} > 3$) correlated with *p*-values lower than 0.001. In addition, no conclusive evidence in favor of H_0 ($BF_{10} < 1/3$) was observed for any of the outcomes in which the frequentist *p*-value was greater than 0.05. For two (LBWR, elastance, and right ventricular wall thickness), the frequentist meta-analysis showed a *p*-value lower than 0.05, while the BMA analysis showed inconclusive evidence in favor of H_1 ($1 > BF_{10} < 3$).

Since the number of 20 studies was not reached for any of the meta-analyses, no publication bias analysis was conducted.

4. Discussion

A primary benefit of meta-analysis in preclinical research is its capacity to discern consistent patterns and potential therapeutic effects that merit further investigation in clinical trials. This approach can also highlight gaps in existing research, thereby guiding future studies to address these deficiencies [57,58]. The present Bayesian meta-analysis showed inconclusive evidence for the presence of an effect of sildenafil in fetal survival or in lung hypoplasia as assessed by the LBWR. In contrast, the evidence in favor of a positive effect of sildenafil was extreme for the reduction in small pulmonary arteries' medial wall thickness. The BMA analysis also showed evidence in favor of a positive effect of sildenafil on radial alveolar count, interalveolar septa thickness, distal airway complexity, mean saccular airspace diameter, total lung capacity, lung compliance, and pulmonary VEGF expression. These findings collectively indicate that the antenatal administration of sildenafil effectively mitigates pulmonary vascular remodeling and airway/airspace morphometric alterations in preclinical models of CDH. However, this evidence is limited by the relatively small number of studies and the substantial statistical heterogeneity observed in some of the meta-analyses.

Experimental models of CDH are crucial for understanding its pathogenesis and developing potential treatments [59–63]. However, as reviewed by Eastwood et al., there are multiple sources of heterogeneity in preclinical studies of CDH, ranging from experimental design to reported measurements [59]. According to the dual-hit hypothesis, CDH is considered a developmental embryopathy in which primary cell-specific disruption of epithelial–mesenchymal signaling first leads to aberrant lung and vascular smooth muscle development. Secondarily, these changes are exacerbated by mechanical compression of the herniated organs [7,63–65]. Surgical models are based on a surgical procedure that creates a diaphragm defect in fetal rabbits or lambs [59–63,66]. This CDH model has proven

especially useful in investigating postnatal interventional therapies. However, this model involves a diaphragmatic defect that is performed relatively late in gestation and does not provide information about the cause and early pathogenesis of lung hypoplasia [67].

The nitrofen model in rats and mice is the most widely utilized animal model for investigating CDH [59,67–69]. The administration of this herbicide during the midgestational period to pregnant dams has been demonstrated to induce developmental anomalies in the heart, lungs, diaphragm, and skeleton of the embryos, resulting in diaphragmatic hernias that resemble the human condition [59,67–69]. According to the dual-hit hypothesis, the first insult is represented by early bilateral nitrofen-induced pulmonary hypoplasia observed prior to closure of the diaphragm, probably due to a disturbance in the retinoid signaling and/or thyroid signaling pathways. The second insult is caused by herniation of the abdominal viscera into the thorax causing mechanical interference to fetal lung development [59,67–69]. Therefore, the nitrofen model is considered more appropriate for studying the pathophysiology of lung hypoplasia in CDH compared to surgical models. This is because the nitrofen model induces pulmonary hypoplasia through disruption of key developmental signaling pathways (such as FGF, BMP, Sonic Hedgehog, and retinoic acid), which are critical for early lung branching morphogenesis and alveolar differentiation, closely paralleling the molecular and cellular abnormalities observed in human CDH lungs [67–69]. However, the potential teratogenic effects of nitrofen in rodents has never been demonstrated in humans [69]. In addition, the nitrofen model has limitations related to the timing and rate of alveolarization because humans [70], lambs [71], and rabbits [72] have a longer and more gradual process that begins prenatally, while rats experience a rapid postnatal alveolarization [73]. Consequently, both the nitrofen and the surgical models are valuable for mechanistic and therapeutic studies, but their limitations must be considered when applying the findings to human CDH.

The paucity of studies has precluded the analysis of subgroups based on the experimental model (nitrofen vs. surgery) for the majority of the outcomes. Subgroup analyses with a small number of studies per subgroup are prone to low power, imprecision, and increased risk of spurious findings [74]. While Bayesian methods offer substantial advantages over frequentist techniques for subgroup analysis with few studies, they cannot fully overcome the fundamental limitations imposed by small sample sizes and data sparsity. Our prospective protocol specified that only subgroups with more than 10 studies would be analyzed. Therefore, this subgroup analysis could only be performed for two outcomes in the nitrofen subgroup: LBWR and MWT. The strength of evidence determined by the BF_{10} did not change substantially in the nitrofen subgroup for these two outcomes. The limited number of studies has also constrained the possibility of conducting additional subgroup analyses based on sildenafil dosage and administration route.

In addition to the potential heterogeneity related to the use of two different experimental models, differences in imaging protocols, tissue processing methods, and analysis techniques across different studies can introduce significant variability and bias in lung morphometry, affecting the reproducibility and accuracy of measurements. This can result in imprecision and heterogeneity in the data, which complicates the accurate combination of results from disparate studies. The enhancement of the reliability of morphometric measurements is contingent upon the implementation of standardized protocols and automated methodologies [75–77].

The translational relevance of nitrofen and surgical models is based on their ability to mimic the phenotype, but they do not address gene-specific mechanisms Both human and animal studies have established that CDH can result from a variety of genetic mechanisms, including chromosomal abnormalities, copy number variants, and single-gene mutations. In humans, pathogenic variants in genes such as *GATA4*, *ZFPM2*, *NR2F2*, and *WT1*, as well

as genes involved in transcriptional regulation and cell migration, have been implicated in both isolated and syndromic forms of CDH [78,79]. However, no published studies have reported the use of antenatal sildenafil in animal models with engineered mutations in *GATA4*, *ZFPM2*, *NR2F2*, *WT1*, or other genes directly implicated in human CDH. Thus, although antenatal sildenafil shows promise in teratogen-induced and surgical models, its efficacy in genetic models of CDH remains untested.

Both the nitrofen and surgical models of CDH are characterized by decreased distal vascular density and increased muscularization of the small arteries, resulting in increased pulmonary vascular wall thickness [8]. This vascular remodeling was partially reversed by sildenafil. In addition, sildenafil had a positive effect on distal airway complexity and alveolarization and improved lung mechanics. The notion that pulmonary vascular development passively follows airway development has been challenged in recent years. A growing body of evidence suggests that lung blood vessels actively promote normal alveolar growth during development and contribute to the maintenance of alveolar structures throughout postnatal life. Disruption of angiogenesis during lung development has been demonstrated to impede alveolarization, and the preservation of vascular growth and endothelial survival has been shown to promote lung growth and the structure of the distal airspace [80]. Within this paradigm, the beneficial effects of sildenafil in experimental CDH were associated with an increase in pulmonary VEGF expression. VEGF expression is found to be downregulated in both human and animal models of CDH [81,82]. VEGF is of paramount importance in lung development as it contributes to pulmonary angiogenesis and vascular growth with subsequent enhancement of alveolar growth and maintenance of alveolar structure [83]. It has been suggested that VEGF could serve as a viable target for therapeutic intervention in CDH, with the potential to enhance lung development and mitigate pulmonary hypoplasia [81,82]. In contrast to the effects of sildenafil on VEGF, the BMA analysis did not find conclusive evidence ($BF_{10} > 3$) that sildenafil improved pulmonary eNOS expression. However, it is important to note that the analysis of this biomarker was conducted in only two studies. Consequently, the inconclusiveness of the findings is likely attributable to the limited sample size.

In contrast to the beneficial effects of sildenafil on vascular, airway, and airspace development in experimental CDH, neither fetal survival, nor pulmonary hypoplasia, as determined by the LBWR were improved by antenatal treatment with sildenafil. The LBWR is the most frequently utilized surrogate marker of lung growth in experimental models of CDH because it is simple to obtain [59]. However, such a measure is limited in its potential to assess lung development, and its correlation with morphometry and lung function is not consistently reliable. The meta-analysis of LBWR revealed substantial heterogeneity and a paucity of conclusive evidence to support the existence of an effect. That is, the BF_{10} had a value very close to 1 ($BF_{10} = 1.25$), which does not allow a confident conclusion regarding the presence of an effect. It is noteworthy that conclusive evidence supporting the absence of an effect (i.e., $BF < 1/3$) was also not observed. Interestingly, the study of Russo et al. found that TO normalized the LBWR in a surgical model of CDH in rabbits [47]. However, TO had only a partial effect on vascular remodeling. Only when TO and antenatal sildenafil were combined, both pulmonary hypoplasia and vascular remodeling were reversed [47].

The main novelties of our study are that it is the first meta-analysis of antenatal treatment with sildenafil in experimental CDH and the use of a Bayesian statistical approach. In preclinical research, Bayesian methods are increasingly being adopted due to their ability to incorporate prior information and provide probabilistic interpretations of results [84,85]. The utilization of Bayes factors in statistical inference enables the attainment of more nuanced conclusions than the binary conclusions (significant vs. not significant) of frequentist statistics based on *p*-values [86,87]. When using frequentist statistics, for

many researchers either the results are declared significant, in which case they are likely to claim that the effect is real and important, or the results are not significant, in which case they are likely to declare that H_0 must be true [86,87]. In contrast, the BF directly quantifies the relative support for one hypothesis over another by comparing the likelihood of the data under each hypothesis. This provides a continuous measure of evidence. For example, a BF_{10} of 10 indicates that the data are 10 times more likely under the H_1 than under H_0. Furthermore, the BF helps to differentiate between absence of evidence (i.e., the data are insufficient to support either H_0 or H_1) and evidence of absence (i.e., the data provide strong support for H_0 over H_1). For example, in the case of fetal survival, the frequentist meta-analysis yielded a *p*-value of 0.305, which does not allow the rejection of H_0. Nevertheless, the inability to reject H_0 does not inherently imply its acceptance. The BF_{10} of 1.25 enables us to ascertain that the data were only 1.25 times more likely under H_1 than under H_1. Therefore, as mentioned above, the evidence provided by the data is inconclusive with regard to both H_0 and H_1 (i.e., there is an absence of evidence). In addition, we used Bayesian model-averaging, which enhances the robustness and reliability of meta-analytic conclusions by effectively handling heterogeneity and providing a flexible framework for model selection. This makes it particularly valuable in the context of preclinical studies, where small sample sizes, data variability and model uncertainty are common challenges [83,84,87].

Another limitation of our study that warrants discussion is that the assessment of the risk of bias of the primary studies was hampered by the low quality and/or incompleteness of reporting on relevant domains. The absence of comprehensive, complete, and transparent reporting of methods and results in preclinical studies hinders the precise evaluation of the risk of bias in meta-analyses, consequently impacting the reliability and validity of the synthesized evidence [88,89]. Despite recent advancements in the quality of reporting, there remains a paucity of consistent and complete reporting of methodological details, including randomization, blinding, and sample size calculations [88,89]. Of the 15 studies included in the meta-analysis, 10 mentioned a randomization method, assessment blinding was mentioned in 7 and only 2 included a sample size calculation. Consequently, there is considerable potential for enhancement to strengthen the validity of the majority of published preclinical animal studies, given the assumption that the absence of reporting may be indicative of limited conduct [88].

One limitation that is inherent to all preclinical studies is the so-termed indirectness, which is defined as how well the results translate from animals to the clinical situation [59]. The fetus is a particularly challenging population with unique responses to interventions. The difficulties associated with treating the fetus include targeting multiple dysfunctional pathways with a single treatment, the risk of side effects due to unintended targeting of other organs, the route of administration, and the differences in lung development between experimental models and human fetuses [8]. The positive preclinical results observed with sildenafil in CDH prompted the design of the first prenatal sildenafil studies in human CDH [90]. However, the study of prenatal sildenafil for human CDH has been questioned due to the potential adverse effects observed in fetuses exposed to sildenafil for other pathologies [91]. Several randomized clinical trials, including the STRIDER (Sildenafil Therapy in Dismal Prognosis Early Onset Fetal Growth Restriction) trials, have evaluated the use of sildenafil in pregnancies complicated by severe early-onset fetal growth restriction [91–94]. The findings of these studies generally indicate that sildenafil does not significantly improve perinatal outcomes, including fetal growth velocity, survival to hospital discharge, or reduction in major neonatal morbidity [95]. The Dutch STRIDER trial was halted early in July 2018 after interim analysis indicated a potential increase in neonatal pulmonary hypertension and an unlikely benefit on the primary outcome of

reducing perinatal mortality or major neonatal morbidity [91]. Similarly, the Canadian STRIDER trial was also stopped early following the safety concerns raised by the Dutch STRIDER trial [93]. Nevertheless, it has been argued that these observations in fetuses with normal lungs should not be extrapolated to CDH fetuses who have pulmonary hypoplasia and abnormal pulmonary vascular development [10,96]. Recent large-scale trials in term pregnancies found no benefit and no increase in adverse perinatal outcomes when sildenafil was administered intrapartum for potential fetal hypoxia [97]. Therefore, the safety of fetal exposure to sildenafil remains under evaluation, but current data do not support routine use in pregnancy for any indication other than maternal PH, for which it is approved [95,97].

In conclusion, notwithstanding the constraints stemming from heterogeneity and the limited number of studies, the present Bayesian meta-analysis provides substantial evidence for the beneficial effects of antenatal sildenafil treatment in experimental models of CDH. The positive effects of sildenafil encompass both the pulmonary vasculature and the lung parenchyma. Antenatal administration of sildenafil may hold promise as a therapeutic modality for CDH, functioning as either an adjuvant to tracheal occlusion or as a primary treatment in settings where tracheal occlusion is contraindicated or not available. Nevertheless, the implementation of antenatal sildenafil use has been placed on hold due to concerns regarding the potential adverse fetal effects observed in pathologies other than CDH. Consequently, the clinical translation of the present preclinical findings requires additional safety and efficacy data.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines13092274/s1, Search strategy; Table S1: Frequentist random-effects meta-analysis of fetal survival, lung morphometry and function; Table S2: Frequentist random-effects meta-analysis of pulmonary vascular morphometry, heart morphometry and function.

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Review

Persistent Pulmonary Hypertension of the Newborn: A Pragmatic Review of Pathophysiology, Diagnosis, and Advances in Management

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Abstract

Persistent pulmonary hypertension of the newborn (PPHN) results from disrupted fetal–neonatal circulatory transition, characterized by elevated pulmonary vascular resistance (PVR), right-to-left shunting, and refractory hypoxemia. Despite improved perinatal care, PPHN remains a major source of neonatal morbidity and mortality. This review details PPHN phenotypes, pathophysiology, etiology, diagnostics including echocardiography and biomarkers like B-type Natriuretic Peptide (BNP) or N-terminal pro-B-type Natriuretic Peptide (NT-proBNP), and current therapeutic modalities, from lung recruitment and surfactant to targeted vasodilator therapy (iNO, sildenafil, milrinone, bosentan) and extracorporeal membrane oxygenation (ECMO). We emphasize the role of endothelial and molecular mechanisms in precision therapy and outline guidelines for clinical decision-making in diverse care settings.

Keywords: neonatal diseases; pathophysiology; innovative therapeutic approaches; persistent pulmonary hypertension

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) is a condition resulting from a disrupted transition from fetal to neonatal circulation. It is characterized by sustained elevation of pulmonary vascular resistance (PVR) which leads to right-to-left or bi-directional shunting of blood through the foramen ovale and ductus arteriosus, and it prevents an increase in pulmonary blood flow (PBF). Therefore, newborns with PPHN suffer from persistent severe hypoxemia; this clinically presents as respiratory distress shortly after birth and often requires immediate intervention. Despite advances in perinatal care, PPHN remains a significant cause of morbidity and mortality in newborns [1,2].

The etiology of PPHN is multifactorial, and the process underlying the development of high pulmonary resistance is complex and still not fully understood. There are a range of different phenotypes of PPHN which can lead to acute pulmonary hypertension: (1) raised PVR at pre-capillary level, which is the most common phenotype; (2) increased

pulmonary blood flow and pulmonary vascular remodeling (in conditions such as left-to-right shunt lesions and arterio-venous malformations); and (3) increased pulmonary venous hypertension at the post-capillary level such as in obstructed total anomalous pulmonary venous connections or severe left ventricular disfunction (Figure 1). It is crucial to understand the phenotype and underlying pathophysiology, as these can result in different management strategies. In the past decade, significant progress has been made towards developing new therapies. There is also a growing need to develop standardized guidelines that can help clinicians in decision-making when treating patients with this inhomogeneous condition [3]. Optimizing cardiorespiratory management is the most common approach; this includes lung recruitment to optimize hypoxia treatment, surfactant therapy, conventional or high-frequency ventilation, and pulmonary vasodilators such as nitric oxide, milrinone, or sildenafil. However, despite optimizing cardiovascular support with conventional therapy (including HFOV, iNO, additional vasodilator therapy, and cardioactive medications), in some cases, extracorporeal membrane oxygenation (ECMO) is needed. A better understanding of the processes occurring in pulmonary endothelial and smooth muscle cells can help in selecting the appropriate therapy for a specific patient, as frequently employed traditional approaches may not suffice [2].

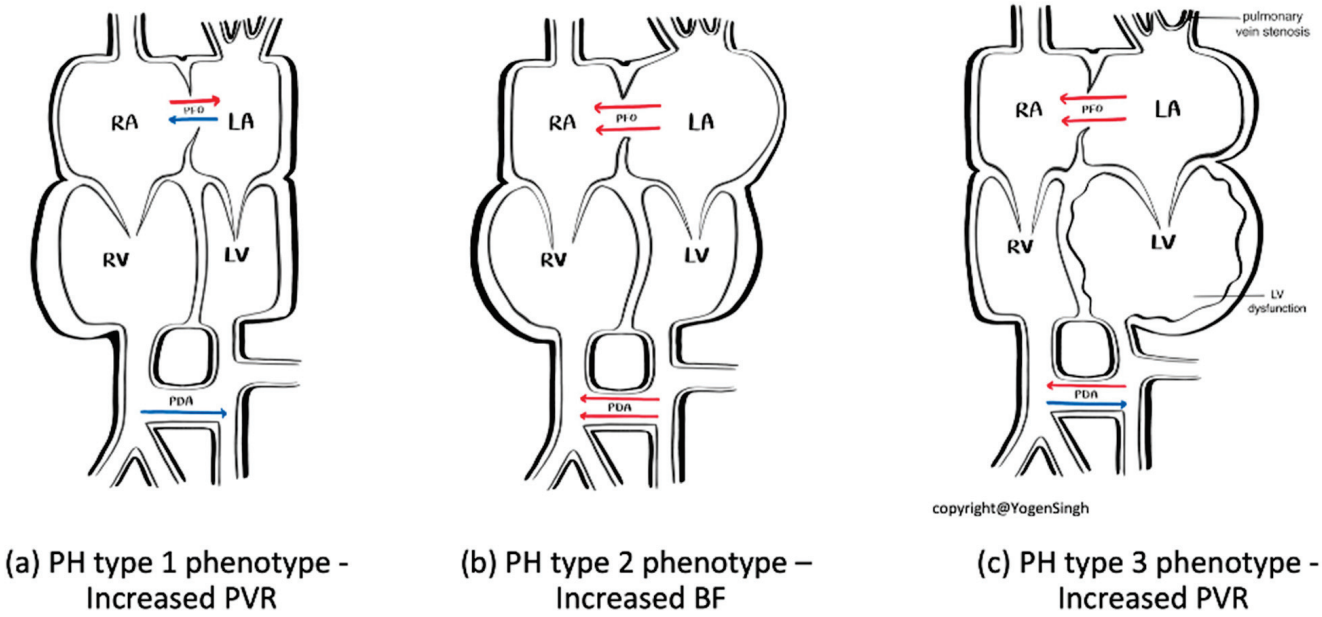


Figure 1. Phenotypes of pulmonary hypertension: Type 1—primarily increased pulmonary vascular resistance (most common type); Type 2—increased pulmonary blood flow leading to pulmonary hypertension; and Type 3—increased pulmonary wedge pressure from increased pulmonary venous pressure (least common type, such as in pulmonary vein stenosis and left ventricle dysfunction).

Beyond its immediate respiratory implications, PPHN poses additional risks to affected infants. They are more susceptible to complications such as neurological impairment, cardiovascular dysfunction, bronchopulmonary dysplasia (BPD), and long-term developmental delays [4].

The impact of PPHN on newborn health reverberates through the neonatal period and beyond, underscoring the importance of early detection, prompt intervention, and comprehensive follow-up care. This article endeavors to provide a pragmatic approach towards the management of PPHN in clinical practice, encompassing its pathophysiology, etiology, diagnostic tools, and therapeutic approaches [5].

2. Search Strategy and Selection Criteria

This narrative review on persistent pulmonary hypertension of the newborn (PPHN) is based on studies identified through PubMed/MEDLINE, Embase, and the Cochrane Library (1997–2025) to capture both cornerstone historical studies (e.g., early nitric oxide trials) and contemporary advances. Search terms included PPHN, pulmonary vasodilators, inhaled nitric oxide, ECMO, biomarkers, and neonatal pulmonary hypertension. We included RCTs, observational studies, systematic reviews, experimental work, and expert statements focused on neonates. Non-neonatal and non-English publications were excluded.

3. Etiology

The etiology of PPHN is multifactorial, involving a combination of genetic, environmental, and physiological factors [6]. Initially understood as a failure of the neonatal pulmonary circulation to transition from fetal circulation (which results in sustained high vascular resistance), the understanding of PPHN has significantly evolved.

Research in the 1980s identified the critical role of the ductus arteriosus in maintaining high PVR, pivotal for understanding the pathophysiology of PPHN [6]. PPHN can occur from primary pulmonary pathology or secondary conditions such as meconium aspiration, infections, or congenital anomalies like diaphragmatic hernia [6]. These conditions may prevent the normal postnatal decrease in PVR, leading to severe respiratory distress. Contributing factors include fetal hypoxia, acidosis, and structural immaturity of the lungs [6]. Additionally, environmental influences during pregnancy such as maternal medication use or inflammation, along with genetic predispositions, are significant in the development of PPHN.

Secondary PPHN is often linked to conditions affecting the pulmonary or cardiovascular systems of the newborn, including respiratory distress syndrome, pneumonia, and conditions like sepsis or asphyxia [6]. Interactions between genetic predispositions and environmental factors during pregnancy, such as maternal smoking or diabetes, can significantly increase the risk of developing PPHN. These factors may induce hypoxia and inflammation, leading to epigenetic modifications that affect gene expression related to pulmonary vascular tone and reactivity [7].

The introduction of inhaled nitric oxide (iNO) in the 1990s revolutionized treatment, significantly reducing pulmonary vascular resistance and enhancing oxygenation in affected neonates [8–11]. Contemporary studies have expanded to include the roles of endothelin-1, prostacyclin, and detailed genetic and molecular foundations of PPHN [8]. Such research is refining treatment strategies and exploring new pharmacological interventions, aiming to improve outcomes further.

The underlying mechanisms involve an intricate interplay between vasoactive mediators, endothelial dysfunction, and mechanical factors, including an impaired response to oxygen and vasoactive stimuli like endothelin and nitric oxide [8]. These mediators are essential for regulating vascular tone. Moreover, specific genetic variants, such as polymorphisms in the gene encoding endothelial nitric oxide synthase (eNOS), have been linked to PPHN. Deficiencies or dysfunctions in eNOS can lead to inadequate nitric oxide production, which is a crucial factor in reducing pulmonary vascular resistance immediately after birth [8–10].

As research continues to delve into these complex interactions, our understanding improves, guiding more effective and targeted therapeutic approaches. This comprehensive exploration of PPHN's causes is essential for developing novel interventions that can better manage or even prevent this challenging condition in neonates.

4. Clinical Presentation and Diagnosis

Clinically, PPHN presents with severe hypoxemia and cyanosis, which result from the under-oxygenation of arterial blood. Infants with PPHN also often show signs of respiratory distress, leading to respiratory failure. Heart auscultation may reveal flow murmurs due to excessive blood flow through the lungs, from PDA shunt, tricuspid regurgitation or sometimes from the associated underlying congenital heart defect. Additionally, these infants typically demonstrate a poor response to oxygen supplementation, where increasing oxygen delivery does not adequately improve blood oxygen levels. This condition requires immediate medical intervention to prevent long-term tissue damage caused by chronic hypoxemia and to avoid potentially fatal complications.

The diagnostic approach to PPHN is multifaceted, involving a combination of non-invasive and invasive methods tailored to assess the severity and implications of the condition effectively. Pulse oximetry and arterial blood gas analysis are crucial for monitoring oxygen saturation and assessing gas exchange capabilities [6].

The use of the oxygenation index (OI) complements these measures by quantifying the degree of respiratory support required to achieve adequate oxygenation. It serves as a vital tool for determining the severity of pulmonary dysfunction and guiding decisions regarding the escalation of care, including the potential need for ECMO when conventional therapies fail to maintain sufficient oxygen levels in the blood [10].

$$\left(\frac{MAP \times FiO_2}{PaO_2}\right) \times 100$$

MAP—Mean Airway Pressure; FiO_2—Fraction of Inspired Oxygen; PaO_2—Partial Pressure of Arterial Oxygen

The OI value provides information about the severity of hypoxemia and the effectiveness of the respiratory support being provided.

OI < 10: This range suggests mild hypoxemia. It indicates that the current respiratory support is sufficient, and the patient is responding well to the given oxygen and ventilation settings.

OI 10–25: Values within this range represent moderate hypoxemia. It suggests that the patient may require closer monitoring and possibly adjustments in respiratory support to improve oxygenation.

OI > 25: An OI greater than 25 indicates severe hypoxemia and implies a significant risk of pulmonary dysfunction. Patients with an OI above 25 often require intensive interventions, potentially including the use of ECMO to ensure adequate oxygenation and to support lung function.

Chest radiography complements these assessments by visualizing lung fields for associated pathologies such as pulmonary hypoplasia or other lung diseases impacting pulmonary pressure [12].

Although PPHN can be diagnosed clinically in most cases, echocardiography remains the investigation of choice for confirmation of diagnosis, ruling out critical congenital heart diseases and establishing severity of acute pulmonary hypertension [13]. The following structural heart defects may clinically mimic PPHN: TAPVC, transposition of great arteries (TGA), pulmonary atresia with or without ventricular septal defect (VSD), severe Fallot's tetralogy, tricuspid atresia, unguarded tricuspid orifice syndrome, severe Ebstein anomaly, and sometimes even left-sided obstructive heart disease (such as coarctation of aorta, interrupted aortic arch, and hypoplastic left heart disease) [14].

Detailed overview of echocardiographic assessments is provided in Table 1 and various echocardiographic parameters are illustrated in Figures 1–10. Chart 1 summarizes a 7-step approach to a comprehensive assessment of pulmonary hypertension.

Table 1. Overview of echocardiographic assessments for PPHN.

Aspect of Study	Description	Significance
Heart Morphology	Exclusion of structural heart defects (note on Total Anomalous Pulmonary Venous Connection, TAPVC)	Assessment for indications for other treatment such as prostaglandin E
Heart Function	On visual inspection "eyeballing" Evaluation of the size, wall thickness, and contractile function of the right ventricle	Identification of right ventricular enlargement and overload
Interventricular Septal (IVS)	Evaluation of interventricular septal flattening	Helps in estimating severity of PPHN, especially in absence of tricuspid regurgitation or PDA (Figure 4) O-shaped LV suggests normal or mildly increased pressure pulmonary artery pressure D-shaped LV with flattening of IVS indicates pulmonary artery pressure 50–100% of systemic pressure Crescent-shaped LV indicates pulmonary pressure > systemic pressure
Pulmonary Artery Pressure Measurement	Estimation based on the velocity of blood flow through regurgitant flow across the tricuspid valve using the Bernoulli equation	SPAP = 4 (TR Vmax2+ right atrial pressure (RAP) V: the velocity of regurgitation through the tricuspid valve RAP: the estimated right atrial pressure (3–10 mmHg). Pressures in the right ventricle and pulmonary artery are crucial for assessing the degree of pulmonary hypertension TV is present in approximately 60–85% of patients with PPHN. Absence of TR or minimal TR does not rule out PPHN!
Detection of Shunt	Identification and assessment of flow through the patent foramen ovale (PFO) and ductus arteriosus (PDA)	Indicates the presence and nature of pathological flow: left-to-right, bidirectional (often in moderate PPHN), or right-to-left (in very severe PPHN)
Right Ventricle Assessment	On visual inspection Movement of the tricuspid valve annulus, TAPSE (Tricuspid Annular Plane Systolic Excursion) Tei index using Tissue Doppler Imaging	Helps evaluate function of the right ventricle
Left Ventricle Assessment	On visual inspection, fraction shortening, ejection fraction, Tei index using Tissue Doppler Imaging	Helps evaluate function of the left ventricle
Assessment of Cardiac Filling	Inferior vena cava size and collapsibility	Assessment of preload
Advanced ECHO and Hemodynamic Evaluation	RV fractional area change (FAC %) Pulmonary arterial acceleration time (PAAT) and PAAT/RV ejection time ratio Speckle tracking and strain rate Estimation of left and right cardiac output and serial assessment to see the response to therapy	Helps evaluate cardiac function and response to therapy

Figures 4–7. Assessment of shunts across PDA and PFO (R to L across PFO demonstrated in figure).

Figures 9 and 10. Evaluation of PA Dopplers—shape, PAAT, and PAAT/RVET.

For cases where non-invasive methods yield inconclusive results or when precise hemodynamic measurements are required to guide management, cardiac catheterization is employed. This invasive technique allows for direct measurement of pulmonary artery pressures and assessment of vascular reactivity to vasodilators, providing definitive diagnostic data [2,15].

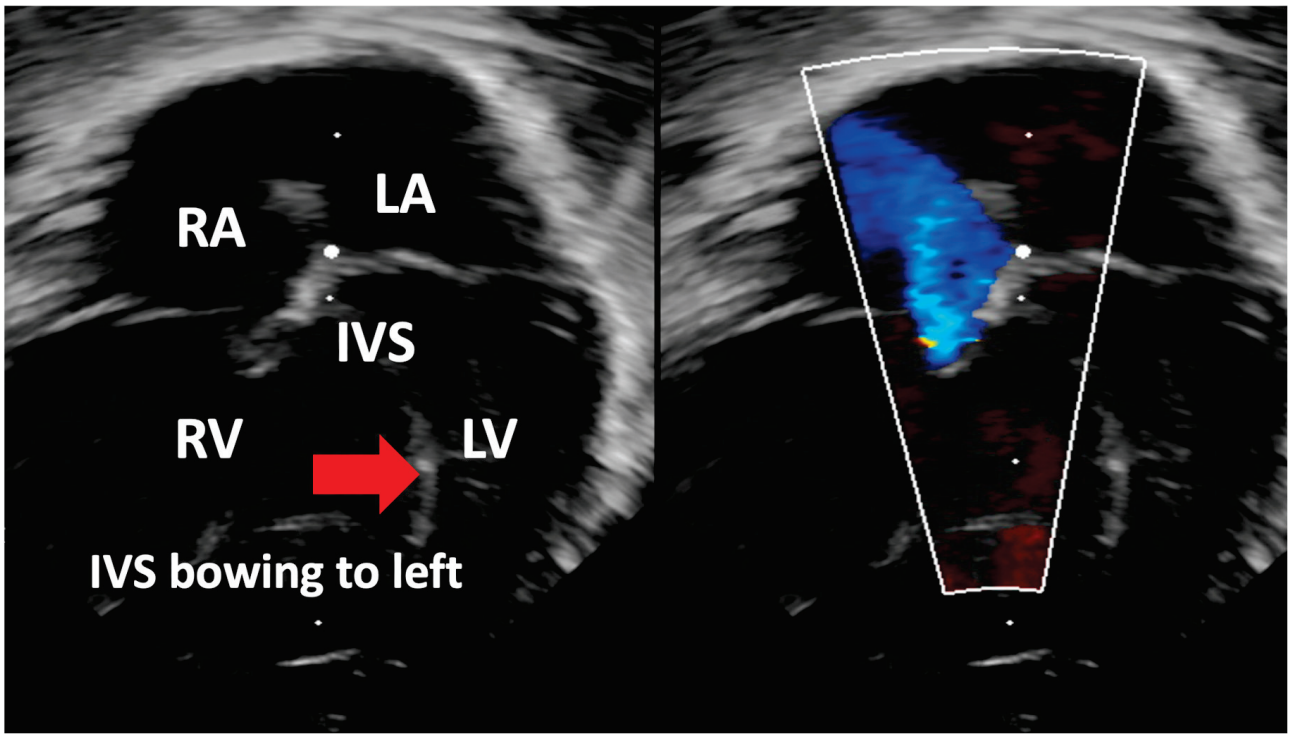


Figure 2. RV dilatation. Image showing bowing IVS to the left because of high pulmonary pressure and tricuspid regurgitation.

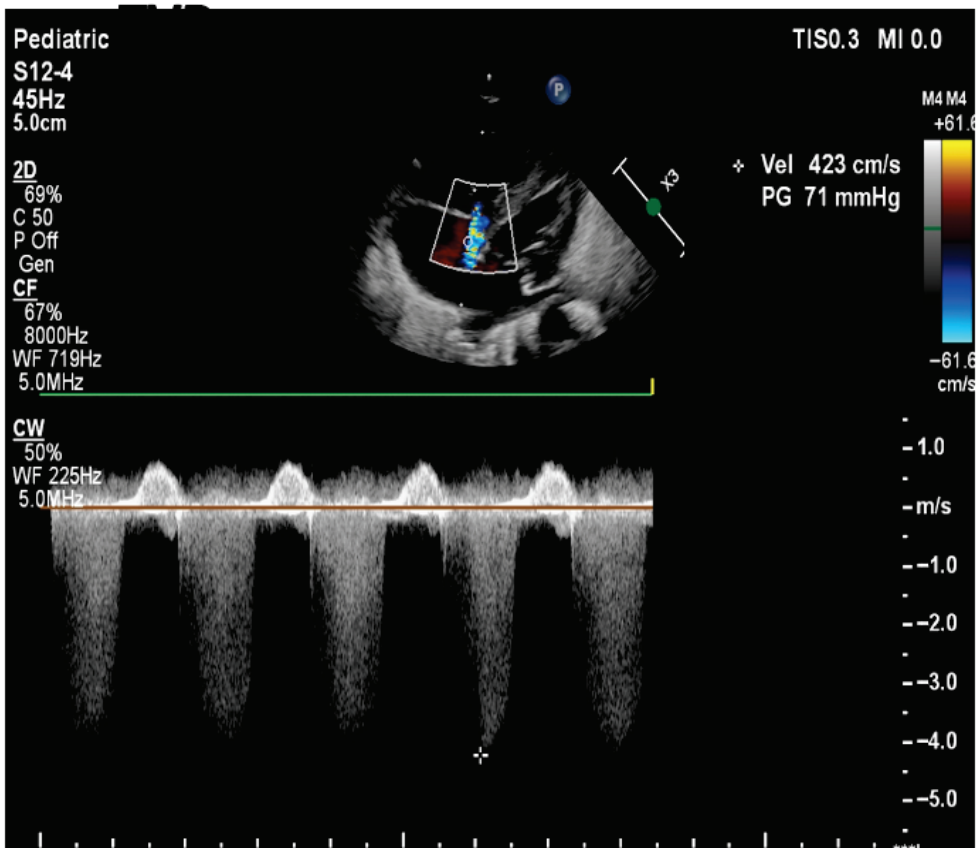


Figure 3. Estimation of pulmonary artery systolic pressure (PASP). Image showing tricuspid regurgitation (TR). Peak velocity of TR estimates pressure gradient between right ventricle and right atrium.

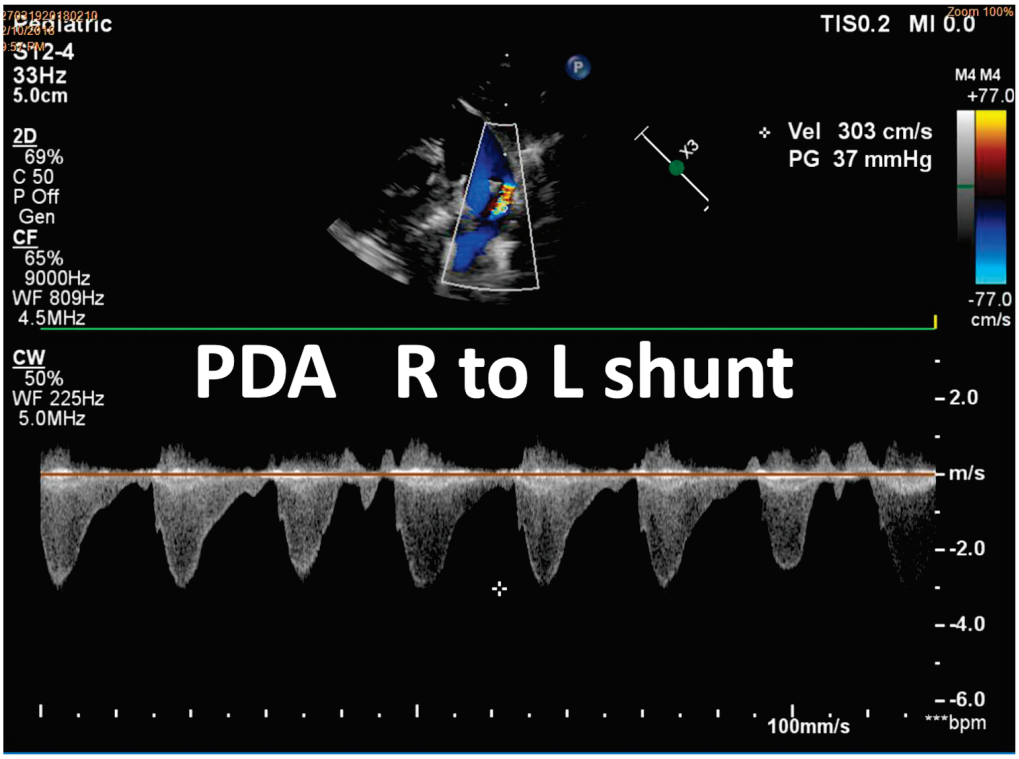


Figure 4. Image showing PDA R to L shunt.

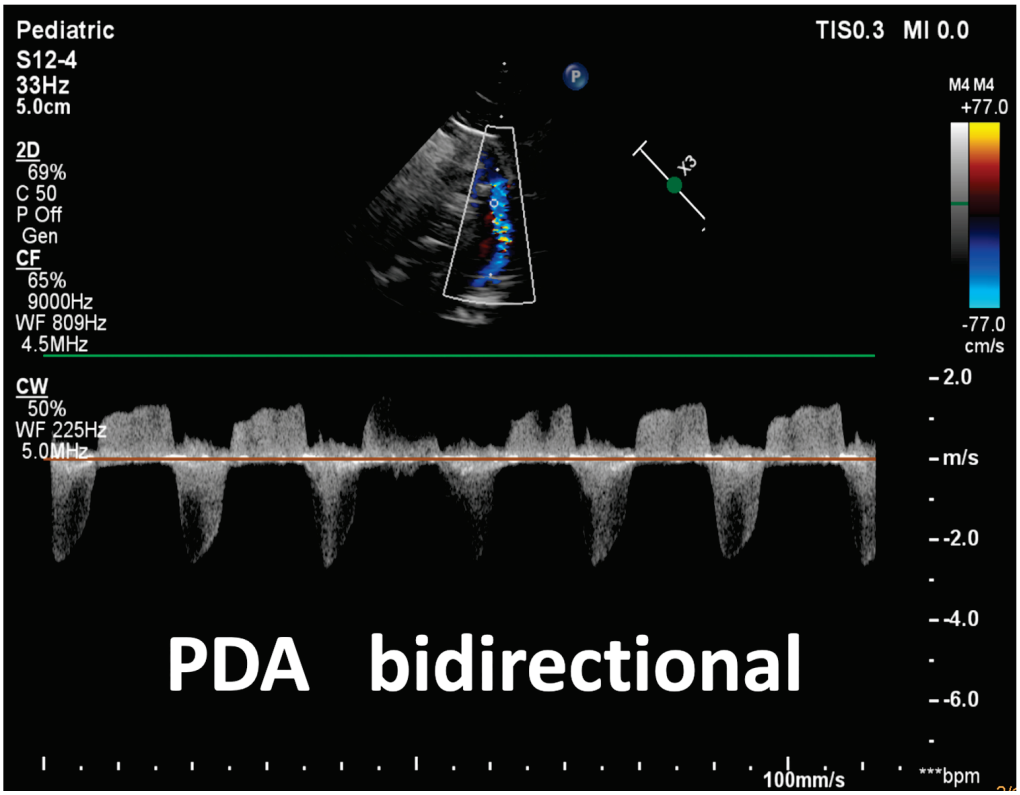


Figure 5. Image showing PDA bidirectional.

Additionally, the use of biomarkers like B-type Natriuretic Peptide (BNP) or N-terminal pro-B-type Natriuretic Peptide (NT-proBNP) plays a role in evaluating myocardial strain and cardiac stress due to high pulmonary pressures [5].

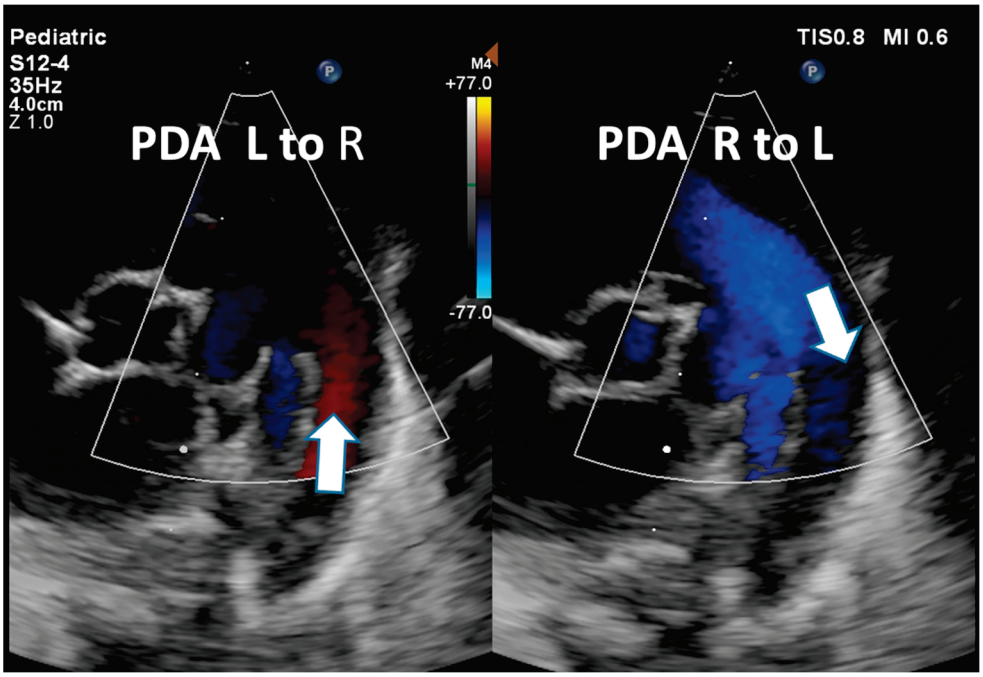


Figure 6. Image showing flow across PDA: red—L to R shunt; blue—R to L shunt.

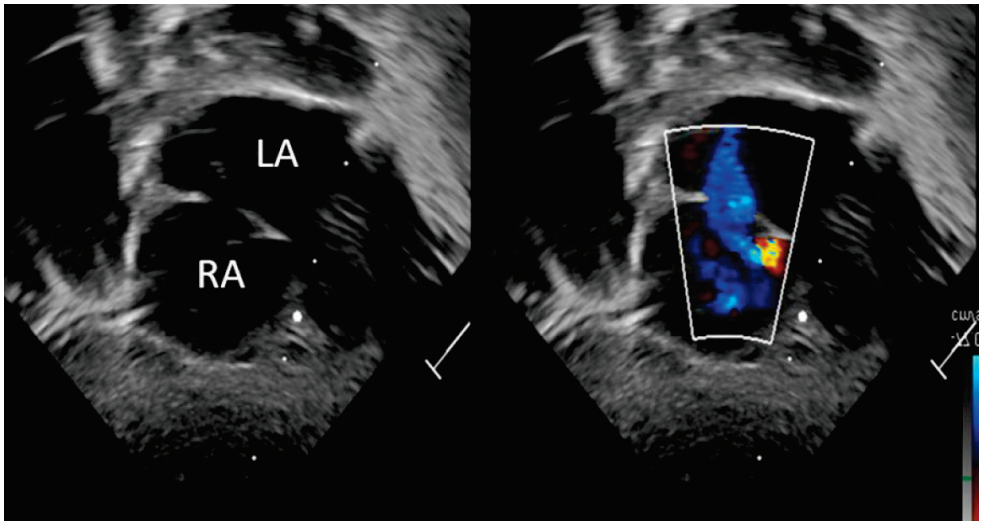


Figure 7. Image showing R to L shunt across PFO.

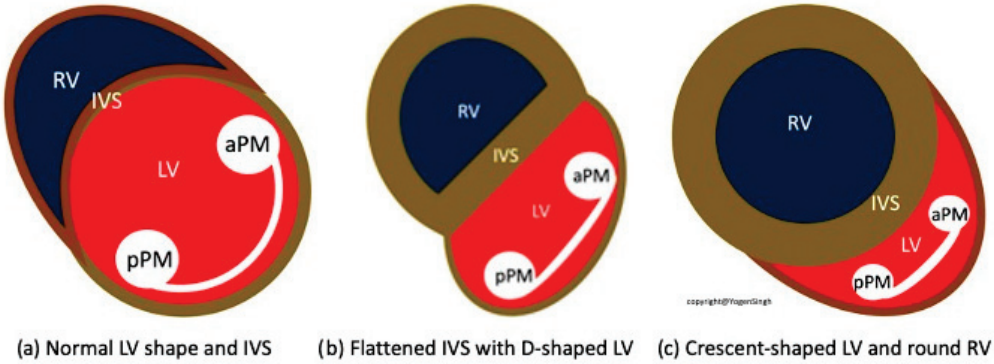


Figure 8. Assessment of inter-ventricular septum and LV shape—(**a**) mild flattening, (**b**) moderate flattening and (**c**) severe flattening of interventricular septum reflecting pulmonary hypertension severity.

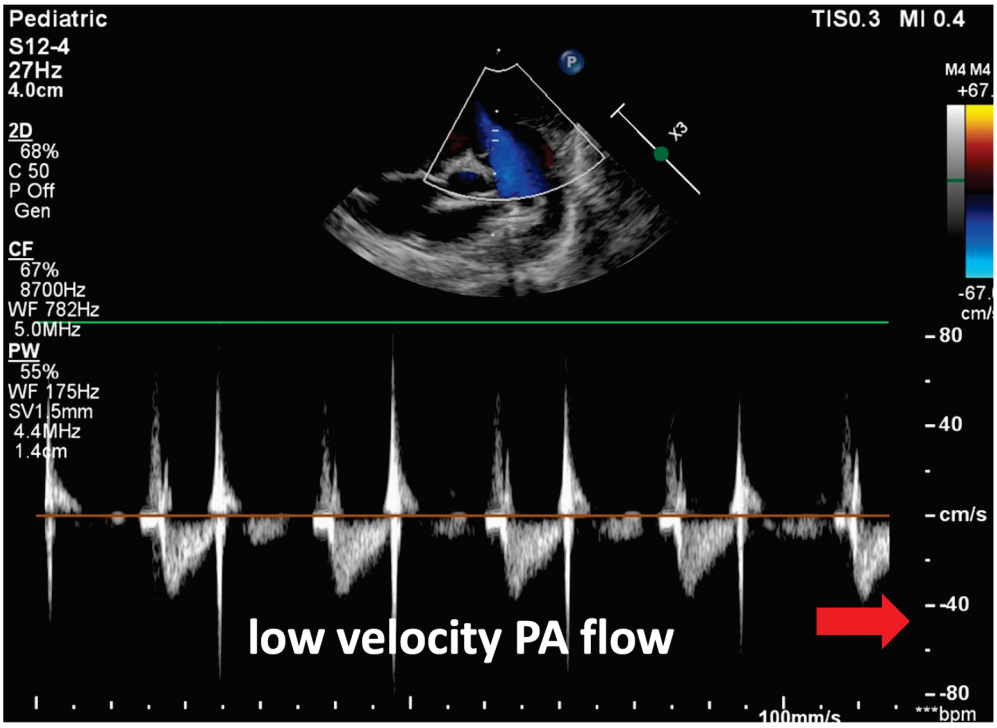


Figure 9. Image showing low velocity PA flow.

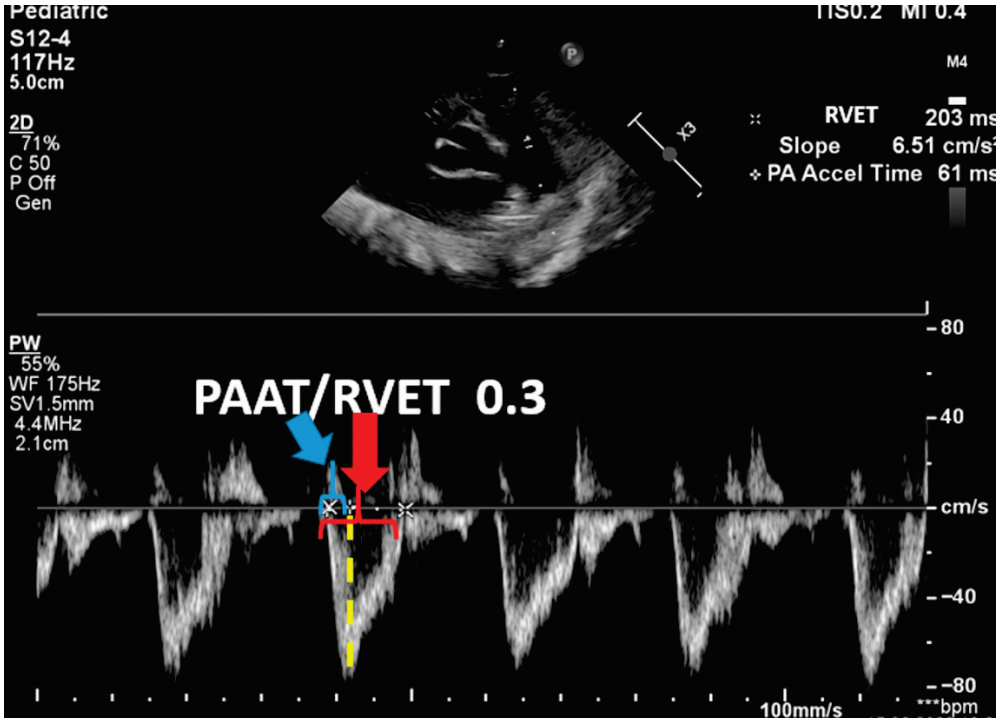


Figure 10. Image showing PAAT and PAAT/RVET measurement.

B-type Natriuretic Peptide (BNP) and its inactive cleavage product N-terminal pro-B-type Natriuretic Peptide (NT-proBNP) are released from ventricular myocardium in response to pressure or volume overload and correlate with right ventricular strain in PPHN. BNP has a short plasma half-life (~20 min), while NT-proBNP is more stable (~60–120 min), making it easier to measure reproducibly. Elevated BNP/NT-proBNP values have been consistently observed in neonates with PPHN compared to infants with other causes of hypoxemic respiratory failure. Suggested thresholds vary between studies,

but BNP values > 850 pg/mL have been shown to predict the need for inhaled nitric oxide with high sensitivity [16]. However, absolute cut-offs are not standardized and can overlap with other conditions such as sepsis, perinatal asphyxia, or prematurity.

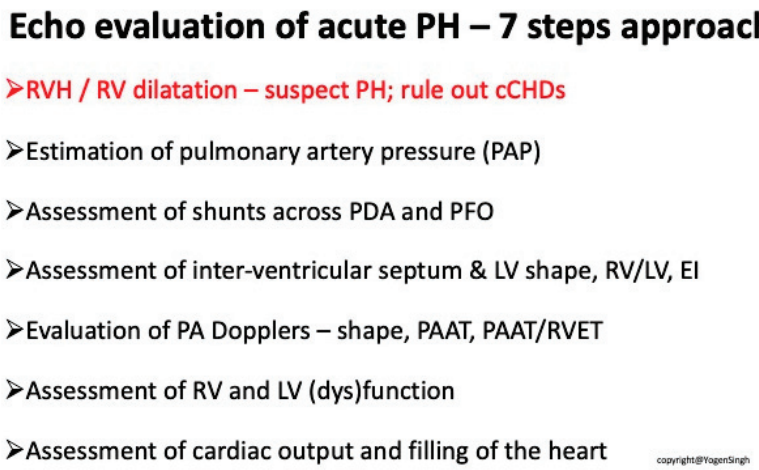


Chart 1. Summary of the 7-step approach to a comprehensive assessment of pulmonary hypertension.

The main utility of BNP/NT-proBNP in PPHN is serial monitoring: trends in concentration may help assess disease severity, response to pulmonary vasodilator therapy, and predict rebound pulmonary hypertension during weaning. Their role is supportive rather than diagnostic, and values must be interpreted in conjunction with echocardiography and clinical findings.

Emerging biomarkers under investigation include cardiac troponins (reflecting myocardial injury), endothelin-1 (linked to pulmonary vascular tone), and circulating microRNAs, which may provide additional insight into disease mechanisms and prognosis [17].

Integrating findings from these diverse diagnostic tools is crucial for forming a comprehensive understanding of an infant's cardiopulmonary status.

This holistic assessment aids in customizing therapeutic strategies, determining appropriate intervention timings, and longitudinally monitoring treatment efficacy. Screening protocols recommend specific timing for echocardiographic evaluation, especially in infants predisposed to PPHN due to factors like hypoxia or unresolved respiratory distress, ensuring timely detection and management of this critical condition [18–20].

5. Treatment

Because of its multifactorial etiology and partially unknown pathophysiological mechanisms, treatment of PPHN remains a challenge. The aim of treatment is to reduce pulmonary vascular resistance (PVR) and reverse blood flow through the shunts. Optimal ventilation after birth is the first crucial factor influencing reduction in PVR [21]. Animal models suggest that physiological cord clamping might be beneficial for children at risk of PPHN, but more research is needed in this field [22,23]. An infant with PPHN with severe respiratory failure requires mechanical ventilation. In order to prevent further lung damage, a lung protective strategy should be applied [24]. In cases where the underlying disease is associated with surfactant deficiency, according to the 2022 European Consensus Guidelines on the Management of Respiratory Distress Syndrome, surfactant administration is a key intervention and leads to improved oxygenation and reduced pulmonary vascular resistance [24].

Management steps for PPHN are shown in Tables 2 and 3.

Table 2. Management steps for PPHN.

Step in Management	Actions and Considerations
Initial Assessment and Stabilization	Assess the newborn after birth for breathing, skin color, activity, and oxygenation. Ensure airway patency, provide adequate ventilation, and optimize oxygen concentration.
Diagnostics	Pulse Oximetry: Monitor oxygen saturation. Echocardiography: Assess heart function and exclude structural abnormalities. Arterial Blood Gas Analysis: Evaluate hypoxemia and acid–base balance. Chest X-Ray: Assess lung condition and exclude other causes of respiratory distress.
Intensive Therapy	Optimized Ventilation: Use gentle mechanical ventilation to minimize barotrauma. Inhaled Nitric Oxide (iNO): First-line therapy for PPHN to decrease pulmonary vascular resistance. Surfactant Therapy: Administer if respiratory distress syndrome (RDS) is present. Sedation and Hemodynamic Support: Manage discomfort and maintain hemodynamic stability. Metabolic Correction: Address electrolyte and acid–base imbalances.
Alternative and Escalation Therapies	ECMO (Extracorporeal Membrane Oxygenation): Consider for severe PPHN unresponsive to other treatments. Vasodilator Drugs: Consider sildenafil and other vasodilators to reduce pulmonary artery pressure.

Table 3. An approach to phenotype-based diagnosis, clues to diagnosis, and approach to specific therapy.

	PH Type 1 Phenotype—Increased PVR	**PH Type 2 Phenotype—Increased BF**	**PH Type 3 Phenotype—Increased PVR**
Diagnosis clues	History of pre-disposing factors such as MAS, perinatal asphyxia, prolonged premature rupture of membranes	History of conditions leading to increased pulmonary blood flow such as persistent patent ductus arteriosus, ASD, VSD, congenital arterio-venous malformation, etc.	History of pre-disposing factors such evolving BPD, especially severe IUGR with BPD, worsening LV dysfunction
	Significant difference between pre- and post-ductal sats difference	No significant difference between pre- and post-ductal sats difference	Difference between pre- and post-ductal sats difference when PDA open but often closed
	Oligemic lung fields on chest X-ray	Plethoric lung fields on chest X-ray	Non-homogenous lung fields on chest X-ray
	Classical signs of pulmonary hypertension on echo (see text)	Volume overloading of right side of heart, specific lesions leading to significant left to right shunt	Signs of pulmonary vein stenosis or severe LV dysfunction on echo
Guide to targeted specific therapy	Optimize ventilation, pulmonary vasodilators, optimize hemodynamic support	Optimize ventilation, diuretics and treatment of specific lesion, optimize hemodynamic support	Optimize ventilation, treatment of specific lesion such pulmonary vein stenosis or improve LV function with lusitropic drugs such as milrinone

5.1. Inhaled Therapies

Oxygen is a known potent pulmonary vasodilator; however, hyperoxia also leads to an increase in PVR and the formation of free radicals which causes lung damage and thus should be avoided. In a study carried out by Lakshminrusimha et al., the authors recommend maintaining preductal oxygen saturations in low to mid-90s (93–97%) during management of infants with PPHN with PaO_2 levels between 55 and 80 mmHg [25].

To reverse blood flow through the shunts, systemic blood pressure should be maintained in proper values. Inotropic agents might often be needed, including dopamine, dobutamine, and epinephrine.

Inhaled nitric oxide (iNO) remains the only registered drug in treatment of PPHN. It works by selectively lowering pulmonary pressure without affecting systemic pressure. The initial starting dose is 20 ppm [25,26]. As reported in a review by Cookson and Kinsela, with no response, the trial of increasing to 40 or 80 ppm is acceptable. Since the half-life of iNO is 2–6 s, the response, i.e., a minimum increase of 20 mmHg, should follow after 30 min [27]. Lower starting doses of 5–10 ppm are used in premature babies.

There is inconsistent interpretation of the OI value as an indicator of both iNO and ECMO initiation. Considering the start of iNO, some authors point to the value of 15, others to one of 25; however, in most reports, the final indicator is 20. The traditional ECMO OI starting threshold was 40 for more than 4 h; however, it is reasonable to consider contacting

the ECMO center when, despite implemented therapies, OI still remains above 25, in order to prepare earlier for transportation logistics [28].

5.2. Other Pulmonary Vasodilator Therapies

Sildenafil is a PDE-5 inhibitor and, as such, leads to vasodilation. It is typically used in adult and pediatric patients with pulmonary hypertension and is increasingly being used in neonates. Although intravenous sildenafil seems more feasible in sick infants not receiving any oral feeds, it carries a higher risk of increased hypotension as compared to oral sildenafil, which is equally effective and has a significantly lower risk of hypotension [29,30]. Moreover, IV sildenafil may not be readily available in many hospitals. A recent meta-analysis showed that sildenafil used for treatment of PPHN has the potential to reduce mortality and improve oxygenation in neonates [30]. Sildenafil is commonly used as a second-line agent in iNO non-responsive patients or as a bridge therapy to wean iNO. However, in limited resource settings where iNO is not available, sildenafil is often used as a first-line pulmonary vasodilator in treating pulmonary hypotension in neonates. A promising experimental study has shown that antenatal use of sildenafil might be beneficial in congenital diaphragmatic hernia (CDH). It would be especially beneficial for those patients, as CDH is not responsive to iNO [31].

Prostanoid analogs (such as iloprost, epoprostenol, and treprostinil) act via the prostacyclin (PGI_2) pathway, stimulating cyclic adenosine monophosphate (cAMP) to induce pulmonary vasodilation and inhibit platelet aggregation. Evidence for their use in neonates with PPHN is limited to small case series and observational studies; no large randomized neonatal trials exist. Inhaled iloprost has been reported to improve oxygenation indices in iNO-non-responsive infants and is generally well tolerated. Delivery logistics are important: inhaled formulations can be administered to both ventilated and spontaneously breathing infants, whereas continuous intravenous prostacyclins (epoprostenol and treprostinil) require central vascular access and careful hemodynamic monitoring. Side effects include systemic hypotension, flushing, and gastrointestinal symptoms. Because of the limited neonatal data and practical challenges, prostanoids are usually considered as rescue therapies in severe, refractory cases of PPHN [32,33].

Milrinone, a phosphodiesterase-3 inhibitor, increases intracellular cyclic adenosine monophosphate (cAMP), leading to pulmonary vasodilation and improved myocardial contractility. When used as an adjunct to inhaled nitric oxide, it may benefit infants with PPHN who have poor response to iNO alone. Small neonatal case series and observational studies suggest that milrinone can improve oxygenation indices and echocardiographic measures of ventricular performance. However, the evidence base remains limited, with no large randomized trials. Clinicians should exercise caution as milrinone may cause systemic hypotension, particularly in preload-depleted infants; careful assessment of intravascular volume and consideration of additional inotropic support are recommended. Thus, milrinone may be considered in neonates with PPHN and concomitant ventricular dysfunction or low cardiac output, but its use should be individualized and closely monitored [34,35].

Bosentan is an oral dual endothelin-1 receptor antagonist that reduces pulmonary vascular resistance by blocking both ETA and ETB receptors. Limited neonatal experience, mostly from small cohorts and case series, suggests that bosentan can improve oxygenation and allow reduction in inhaled nitric oxide or other vasodilators. One exploratory randomized trial (FUTURE-4) provided preliminary safety and efficacy data, but robust neonatal-specific evidence is still lacking [36]. Typical dosing used in neonates is 1–2 mg/kg every 12 h, with onset of action usually observed within hours to days. Regular monitoring of liver function tests is essential because of the risk of hepatotoxicity, and hemoglobin should be followed due to occasional anemia. Given the current evidence, bosentan should

be considered as an adjunct or rescue therapy in refractory PPHN, but its use remains off-label and best guided by specialist consultation [37].

Recent scoping review concluded that the prevalence of PPHN in low- and middle-income countries (LMICs) is increased as compared to high-income medical systems [38]. However limited access to certain therapies in LMICs (iNO and ECMO) have been addressed in several papers [38,39]. Excluding iNO, PPHN is treated with off-label medications; therefore, still more data and studies are warranted to support advantages of selected medications. According to recent meta-analyses, milrinone and oral sildenafil combination results in the most effective treatment when iNO is not available [26,39].

Hence, we propose an algorithm in this clinical scenario (Charts 2 and 3). Other authors suggest different drug protocols depending on clinical constellation, i.e., normal/low pressure vs. good cardiac function/dysfunction [40]. Specific situations in developing countries regarding thiamine-responsive acute pulmonary hypertension have also been addressed in the literature [41]. Moreover, in resource-limited settings, additional medications, e.g., either inhaled or intravenous magnesium sulfate or nebulized nitroglycerin, have been proposed [42,43]. Recommendations for supportive measures and pharmacotherapy have been addressed in the updated collaborative consensus of the EPPVDN (European Pediatric Pulmonary Vascular Disease Network) [44] and also in comprehensive guidelines pointing out scientific rationale in PPHN management [45] as well as a recent review on treatment [46]. As well as pointing out detailed evidence in procedures and therapeutics, the above mentioned studies and, particularly, meta-analyses and reviews point out the need for further collaboration and planning comparative studies to optimize diagnostic and therapeutic approach to improve the low and moderate quality of the evidence [26,28,38,39].

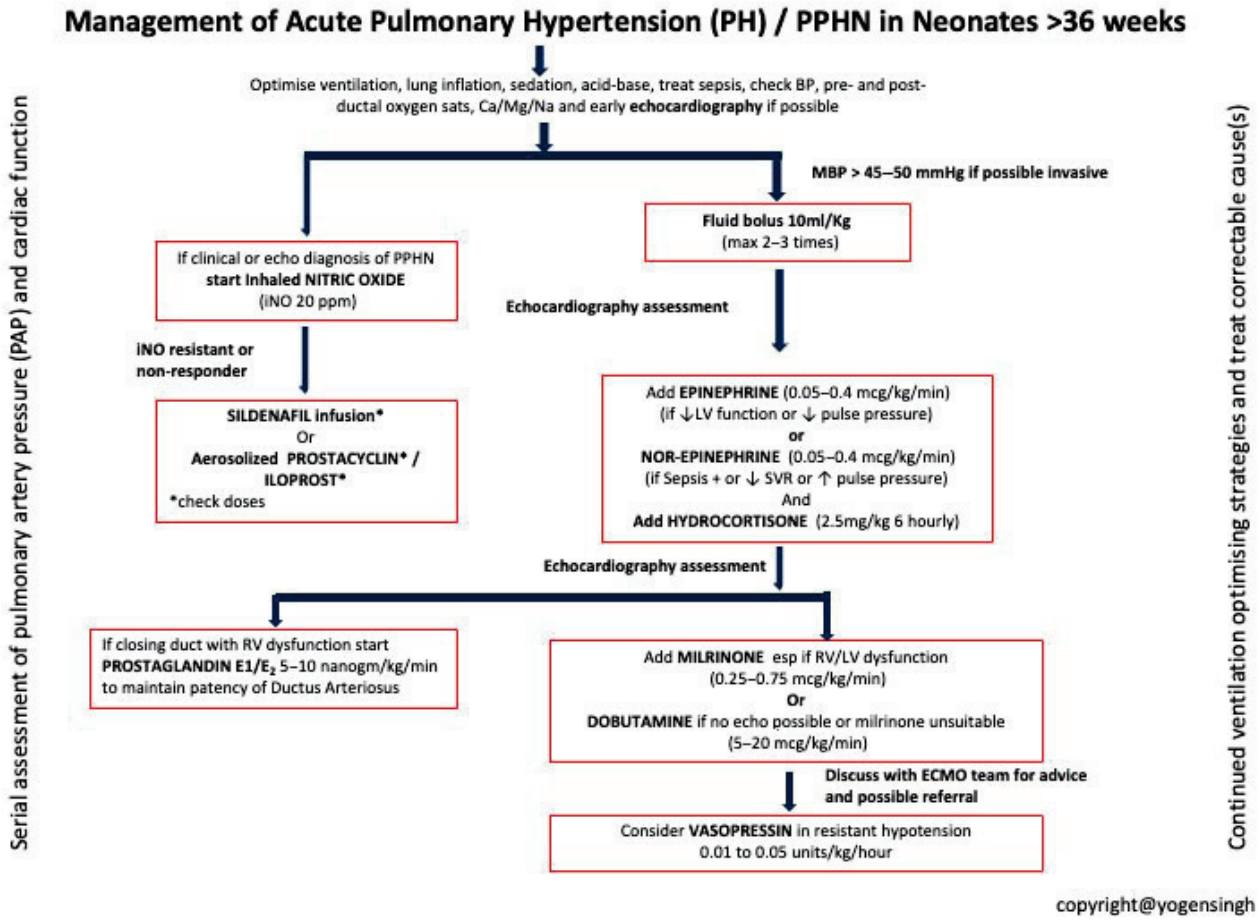


Chart 2. Management of PPHN in centers where iNO is readily available.

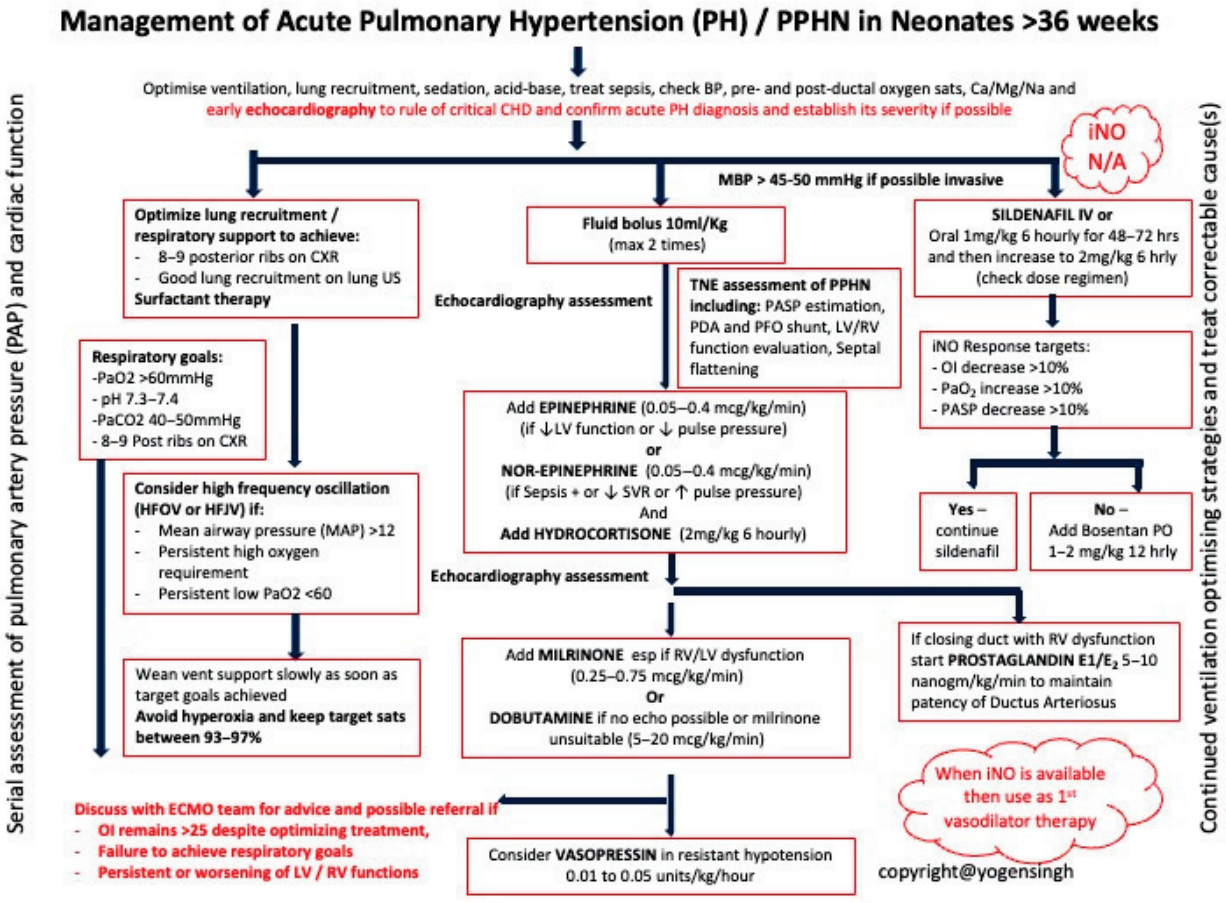


Chart 3. Management of PPHN in limited resource settings where iNO is not readily available.

PPHN is associated with a risk of long-term complications that can affect a child's development. In total, 10–30% of children who survive PPHN experience some form of neuro-psychomotor developmental disorder later in life [47]. For this reason, multidisciplinary follow-up care after hospital discharge is essential.

A detailed overview of drugs used in the treatment of PPHN is presented in Table 4. Chart 2 summarizes the management of PPHN centers where iNO is readily available, while Chart 3 summarizes the management of PPHN in limited resource settings where iNO is not readily available.

Table 4. PPHN treatment—drugs, dose, action sites, and indications.

Drug/Dose	Action Mechanism	Side Effects	Indications
First-line therapies			
Inhaled Nitric Oxide (iNO) 5–20 ppm	Activates soluble guanylate cyclase in vascular smooth muscle → selective pulmonary vasodilation	Methemoglobinemia (dose-related), decreased platelet aggregation	Hypoxemic respiratory failure (OI ≥ 25 or PaO_2 < 100 mmHg on 100% FiO_2)
Sildenafil IV: Loading 0.4 mg/kg over 3 h; Maintenance 0.067 mg/kg/h Oral: Initial 0.5 mg/kg; Maintenance 1–3 mg/kg q6h	Selective PDE5 inhibitor → increases cGMP, pulmonary vasodilation	Hypotension (more with IV), hypoxemia	Adjunct or alternative to iNO; first-line in limited-resource settings without iNO
Adjunctive therapies			
Milrinone IV: 0.2–1 µg/kg/min (continuous infusion)	PDE3 inhibitor → ↑cAMP → inotropy + vasodilation	Hypotension, arrhythmia, thrombocytopenia [ADD: caution—ensure preload, consider inotropic support]	iNO non-responders with low cardiac output; improves oxygenation and echo indices [ADD: evidence mainly from small neonatal studies]

Table 4. *Cont.*

Drug/Dose	Action Mechanism	Side Effects	Indications
Bosentan Oral: 1–2 mg/kg q12h	Endothelin-1 receptor antagonist (ETA + ETB)	Hepatotoxicity (↑LFTs), anemia	Adjunct in refractory PPHN [ADD: evidence from small cohorts + one exploratory RCT; onset hours–days; monitor LFTs/hemoglobin]
Rescue/Alternative therapies			
Prostanoids (Iloprost, Epoprostenol, Treprostinil) Iloprost: Inhaled 1–2.5 μg/kg q2–4h Epoprostenol: IV 1–2 ng/kg/min, max 50–80 ng/kg/min Treprostinil: IV/SC as above	Prostacyclin analogs → activate cAMP pathway → vasodilation, antiplatelet effects	Hypotension, flushing, diarrhea [ADD: evidence limited to small neonatal series; delivery logistics differ for ventilated vs. non-ventilated infants]	Rescue therapy in severe, refractory PPHN
Prostaglandin E1 (PGE1) IV: 5–10 ng/kg/min	Maintains ductal patency → allows right-to-left shunting and reduces RV afterload	Apnea, fever, hypotension	[DEL: RV dysfunction with closing DA maintaining ductal patency to offload the RV] [ADD: Selected PPHN cases with RV dysfunction, where ductal patency supports RV offloading and systemic circulation]

6. Conclusions

PPHN is a complex and life-threatening condition requiring early recognition and individualized management. Despite advances in neonatal care, it remains a major cause of neonatal morbidity and mortality. Although it can be diagnosed clinically, echocardiography remains the investigation of choice at the bedside. Accurate diagnosis with the use of echocardiography is needed to understand types of phenotypes, assess severity, target specific intervention and monitor response to intervention.

Inhaled nitric oxide is the mainstay therapy in the western world, but many infants require additional or alternative treatments such as sildenafil, milrinone, or ECMO. Future priorities include developing standardized guidelines, ensuring access to therapies in low-resource settings, and expanding training in neonatal echocardiography. Simultaneously, ongoing exploration of novel pharmacological therapies is essential to expand the therapeutic arsenal and to improve outcomes.

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Maternal–Fetal Infectious Risk and Early Antibiotic Treatment Patterns in Late Preterm and Term Newborns in a Romanian Secondary-Care Maternity: A Six-Month Observational Cohort

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Abstract

Background: Microbiological confirmation of early-onset neonatal infection is inconsistently available in many secondary-care maternity units, and early management often relies on maternal risk profiles and inflammatory markers. **Objectives:** This study aimed to describe maternal infectious risk factors, neonatal biomarkers, and early antibiotic treatment patterns among late preterm and term newborns evaluated for suspected maternal–fetal infection (MFI) in a Romanian secondary-care maternity, and to explore whether a simplified bedside indicator (NeoSIR) corresponded with antibiotic initiation and treatment-related outcomes. **Methods:** Observational cohort study (April–September 2024) including newborns with gestational age $\geq$ 35 weeks evaluated within 24 h for suspected MFI based on maternal risk factors and/or neonatal clinical findings. We recorded maternal infections and screening results, neonatal complete blood count and C-reactive protein (CRP) at 24 h, cultures obtained as part of routine care, antibiotic initiation, treatment duration, and neonatal intensive care unit (NICU) admission. **Results:** Among 443 newborns, empirical antibiotics were initiated in 414 (93.5%) and 62 (14.0%) required NICU admission. Maternal urinary tract infection (UTI) documented during pregnancy (recorded diagnosis and/or positive urine culture and/or treatment) was associated with antibiotic initiation ($p = 0.005$). Elevated CRP (>0.5 mg/dL) was associated with antibiotic selection ($p = 0.032$) and longer therapy (mean 8.33 vs. 6.98 days, $p < 0.001$); courses were also longer in NICU-admitted infants (mean 10.39 vs. 6.81 days, $p < 0.001$). The exploratory two-item NeoSIR indicator (maternal UTI and CRP > 0.5 mg/dL) was feasible to compute but showed limited discrimination for antibiotic initiation in this high-treatment cohort. **Conclusions:** In this selected high-risk cohort, antibiotic exposure was highly prevalent and closely associated with maternal urinary tract infection history and neonatal CRP evolution. These findings primarily describe early management patterns in a secondary-care maternity with limited microbiological confirmation and should be interpreted as hypothesis-generating. The exploratory

NeoSIR indicator is not intended as a diagnostic or decision-support tool and requires validation against culture-confirmed outcomes in more heterogeneous populations.

Keywords: suspected maternal–fetal infection; early-onset neonatal infection; maternal risk factors; antibiotic stewardship; neonatal biomarkers; Romania

1. Introduction

Early-onset neonatal sepsis (EOS), defined as a bloodstream infection within the first 72 h of life, continues to be a major contributor to neonatal morbidity and mortality worldwide [1,2]. In high-income countries, routine maternal screening and intrapartum antibiotic prophylaxis have markedly decreased the incidence of Group B Streptococcus (GBS)-related EOS. However, infections occur, particularly among late preterm and term infants, where early signs can be subtle, diagnosis remaining complex, with severe outcomes [3,4]. Early recognition and intervention are critical, as delaying treatment increases the risk of complications and death [5].

EOS most often results from vertical transmission of pathogens during labor and delivery. GBS and *Escherichia coli* (*E. coli*) are the leading pathogens, accounting for the majority of cases in near-term and term infants [6,7]. While GBS remains dominant in many settings, *E. coli* has gained increasing importance, particularly in association with maternal urinary tract infections, intrapartum fever, or prolonged rupture of membranes. Other pathogens such as *Klebsiella pneumoniae*, *Staphylococcus aureus* (including methicillin-resistant strains), and *Candida* species, appear less frequently but can cause severe infections, especially in newborns requiring intensive care [6,8–10].

Current recommendations support early initiation of empirical broad-spectrum antibiotics, usually a beta-lactam combined with an aminoglycoside, to cover the most common pathogens [3,11]. However, the diagnosis of EOS remains challenging. Early signs, such as respiratory distress, feeding intolerance, or temperature instability are often nonspecific, and laboratory markers generally available like C-reactive protein (CRP) or complete blood count (CBC) lack sensitivity and specificity during the immediate postnatal period [12,13]. Procalcitonin has been proposed as a potentially more specific biomarker for early neonatal infection assessment in some settings; however, it was not routinely available in our secondary-care unit during the study period. Consequently, CRP remained the primary inflammatory biomarker used in bedside decision-making in this cohort. Blood cultures, while considered the diagnostic gold standard, suffer from low sensitivity due to small sample volumes, frequent maternal intrapartum antibiotic exposure, and, in some contexts, limited laboratory capacity, often failing to grow a pathogen despite the presence of infection [1–3].

As a consequence, many newborns are treated empirically despite negative cultures or the absence of true infection. Although this approach prioritizes safety, it exposes large numbers of infants to unnecessary antibiotics, which can disrupt the developing microbiome, foster antimicrobial resistance, and have long-term health implications [14]. These challenges are particularly evident in low-resource settings, including areas of Eastern Europe, where access to reliable microbiological testing is variable, and clinicians often rely on maternal history and simple biomarkers to guide treatment [15].

To reduce avoidable antibiotic use without compromising newborn safety, several multivariable risk assessment tools have been proposed. The most widely used, the Kaiser Permanente Neonatal EOS Calculator, combines maternal rick factors (fever, GBS status, duration of ruptured membranes) and neonatal parameters in order to generate individ-

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ualized risk estimates [16,17]. Although such resources have helped reduce antibiotic use in high-resource environments, their implementation requires reliable maternal data and ample screening protocols, digital infrastructure, and local validation, which may not be feasible in many secondary-care settings. In such contexts, any simplified indicator should be interpreted cautiously and considered exploratory unless validated against robust clinical and microbiological endpoints.

Late preterm (35–36 weeks) and term infants (>37 weeks) represent a critical population in this context. Although their absolute risk of EOS is lower than that of very preterm neonates, they account for the majority of antibiotic exposure because of their large numbers and the overlap between early infection signs and normal postnatal adaptation [3,18]. In Romania and other Eastern European countries, secondary hospitals frequently function with limited diagnostic resources, underscoring the need for practical bedside tools which can be applied in any setting in order to identify infants at risk of infection [19].

In the EOS Calculator framework, neonatal status is categorized as well-appearing, equivocal, or clinically ill, based on objective bedside findings (e.g., respiratory distress/need for respiratory support, temperature instability, abnormal perfusion, altered responsiveness), which then modifies the management recommendation.

In this framework, maternal infection refers to clinically or microbiologically documented infectious conditions during pregnancy or labor; neonatal colonization reflects the presence of microorganisms on mucosal or skin surfaces without systemic illness; whereas true neonatal infection implies systemic inflammatory response with or without microbiological confirmation. In secondary-care settings where cultures are inconsistently available, these entities often overlap in clinical reasoning and may contribute to precautionary empirical antibiotic exposure.

In this context, the primary objective of this prospective observational cohort study was to characterize maternal infectious risk factors, neonatal biomarkers, and early antibiotic treatment pathways among newborns evaluated for suspected MFI in a secondary-care maternity setting. A secondary, exploratory objective was to assess whether a simplified bedside indicator (NeoSIR) corresponded with antibiotic initiation and treatment-related outcomes. NeoSIR was not intended as a validated diagnostic or decision-support tool but as a hypothesis-generating indicator reflecting real-world practice in settings with limited microbiological confirmation.

2. Material and Methods

2.1. Study Design and Setting

This was a prospective observational study conducted in a secondary-level maternity over a six-month period, between April and September 2024. All newborns were followed during their routine hospitalization, and no interventions outside standard care were performed. The primary purpose of the study was to evaluate maternal risk factors, neonatal biomarkers, and clinical outcomes in order to optimize antibiotic use in newborns at risk of infection.

2.2. Patients

Consecutive newborns with gestational age $\geq$35 weeks were eligible if they were evaluated within the first 24 h of life for suspected maternal–fetal infection (MFI), defined pragmatically as the presence of maternal infectious risk factors and/or neonatal clinical signs prompting laboratory testing and/or empirical antibiotic consideration. Neonatal clinical signs prompting infection evaluation included temperature instability, respiratory distress, feeding intolerance, tachycardia, lethargy, hypotonia, poor perfusion, or metabolic instability documented within the first 24 h of life. Maternal risk factors included

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documented urinary tract infection (UTI) during pregnancy, clinically abnormal vaginal discharge or positive genital cultures, prolonged rupture of membranes (>12 h), maternal intrapartum fever or suspected chorioamnionitis, meconium-stained or foul-smelling amniotic fluid, and incomplete prenatal surveillance compared to national recommendations (at least 8 prenatal visits with three key ultrasounds; blood typing and anemia assessment; screening for hepatitis B, HIV and syphilis; bacteriological screening including GBS testing; and screening for preeclampsia and gestational diabetes). Prenatal monitoring was classified as incomplete when one or more of the above benchmark elements were not documented in the maternal record.

Eligibility could be triggered by: (i) maternal infectious risk factors in the absence of neonatal symptoms, (ii) neonatal clinical signs suggestive of infection without documented maternal risk factors, or (iii) both. Neonatal clinical signs prompting evaluation included temperature instability, respiratory distress, feeding intolerance, tachycardia, lethargy, hypotonia, or metabolic instability recorded in the first 24 h.

When gestational age was recorded as an interval (e.g., 34/35), the upper bound was used for eligibility classification and descriptive reporting.

When Ballard-based gestational age was available, the higher of last-menstrual-period and Ballard estimates was used for eligibility classification, to minimize misclassification in records with incomplete obstetric dating.

Exclusion criteria were gestational age <35 weeks, stillbirths, newborns transferred immediately after delivery to tertiary centers, and infants born after uneventful pregnancies with complete recommended obstetric monitoring and no recorded infectious risk factors (low-risk population not targeted by this cohort).

2.3. Ethical Considerations

The protocol was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (approval no. 127/15 June 2023). Written informed consent was obtained from the mothers of all newborns enrolled.

2.4. Maternal and Neonatal Assessments

For mothers, routine infection screening and obstetric microbiology were performed according to local practice, and vaginal secretions and amniotic fluid samples were collected during labor or cesarean section when clinically indicated. For newborns, the standard early evaluation included gastric aspirate and peripheral swabs (umbilical, skin, and ear) obtained as part of routine care, alongside a complete blood count (CBC) and C-reactive protein (CRP) measurement at 24 h of life; CBC and CRP were repeated after 72 h when antibiotic therapy continued. Blood cultures (and cerebrospinal fluid analysis when clinically indicated) were performed at the treating clinician's discretion but were not consistently available due to limited laboratory capacity. In this cohort, blood cultures were obtained in 20 newborns, with no microbiological confirmation. Culture results were therefore treated as descriptive supportive information rather than as a diagnostic reference standard. Given these constraints, culture results were treated as descriptive supportive information rather than as a diagnostic reference standard, and treatment decisions were primarily driven by maternal history, neonatal clinical presentation, and inflammatory markers. Where prenatal cultures were performed, the exact gestational age and interval to delivery were not consistently documented, so microbiological findings were treated as qualitative screening indicators rather than time-updated exposures.

2.5. Outcomes

The primary endpoint was prolonged antibiotic exposure, defined a priori as $\geq$10 antibiotic-days (days of therapy, DOT). We selected $\geq$10 antibiotic-days as a prag-

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matic threshold to distinguish short empirical courses (typically reassessed at 48–72 h) from extended treatment trajectories reflecting ongoing clinical concern, biomarker persistence, NICU-level monitoring, or escalation decisions. This operational cut-off was chosen to capture prolonged exposure relevant to antibiotic stewardship within the constraints of the available dataset. Secondary endpoints were (i) broad-spectrum escalation (use of any third-generation cephalosporin, carbapenem, fluoroquinolone, colistin, or linezolid during hospitalization) and (ii) neonatal intensive care unit (NICU) admission. Antibiotic initiation (yes/no) was reported descriptively as an early management indicator.

2.6. Antibiotic Management

Empirical antibiotic therapy was initiated according to institutional protocols when infection was suspected, (within the first 24 h) based on maternal history, neonatal clinical status, and age-specific laboratory thresholds (e.g., leukocytosis > $20 \times 10^3/\mu L$, neutrophilia > $12 \times 10^3/\mu L$, or CRP > 0.5 mg/dL at 24 h). First-line regimens were typically beta-lactam-based (e.g., ampicillin), with aminoglycosides or broader-spectrum agents added or substituted at the clinician's discretion in the presence of clinical deterioration, rising inflammatory markers, or microbiological findings suggestive of pathogenic colonization. Clinical reassessment was routinely performed at 48–72 h to decide on continuation, narrowing, or discontinuation of therapy.

2.7. Data Handling

The variables collected included various maternal variables in order to track the maternal compliance to the national prenatal monitoring guidelines (infection history, bacteriological findings, number of checkups and ultrasounds), as well as multiple neonatal characteristics including but not limited to sex, gestational age, birth weight, NICU admission, and laboratory parameters (CRP levels, CBC results, bacteriological findings). Data were reviewed for duplicates and errors.

2.8. Statistical Analysis

All analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Categorical variables were compared using the Chi-squared test or Fisher's exact test, as appropriate. Continuous variables were assessed for normality using the Shapiro–Wilk test; normally distributed data were analyzed with Student's *t*-test (two groups) or one-way ANOVA ($\geq$3 groups), while non-normally distributed data were compared using the Mann–Whitney U or Kruskal–Wallis test. Spearman's rank correlation was used for monotonic associations between continuous variables. Results are reported as *n* (%) and as mean $\pm$ SD or median (IQR), as appropriate. Given the exploratory nature of this work, *p*-values are interpreted descriptively (two-sided $\alpha = 0.05$).

Antibiotic exposure was summarized as antibiotic-days (i.e., the sum of days across all administered agents), which reflects total antimicrobial exposure when combination therapy is used. In addition to univariable comparisons, multivariable logistic regression models were fitted to identify independent predictors of (i) NICU admission and (ii) prolonged antibiotic exposure, defined a priori as $\geq$10 antibiotic-days (corresponding to $\geq$5 days of dual ampicillin-gentamicin therapy). Candidate covariates were selected for clinical relevance and data availability (gestational age, birth weight, sex, prolonged rupture of membranes >18 h, maternal UTI and cervical culture findings, neonatal resuscitation at birth, and early inflammatory markers including CRP, WBC, and platelet count). Continuous predictors were z-standardized; missing maternal microbiology results were handled using a missing-indicator approach. This was used because microbiology testing was not performed uniformly in routine care; thus, missingness was partly structural ('not performed') rather than random. A complete-case approach would have reduced the effec-

tive sample size and could introduce selection bias. Results are reported as adjusted odds ratios (aORs) with 95% confidence intervals. These regression models were constructed to estimate associations with antibiotic-related outcomes and NICU admission in this cohort and should not be interpreted as predictive or risk-stratification models. In parallel, length of therapy (LOT) was defined as the maximum number of days across administered agents, reflecting the duration of any antibiotic treatment regardless of combination use.

Covariate selection and model specification: For antibiotic-related endpoints (prolonged antibiotic exposure and broad-spectrum escalation), covariates were pre-specified a priori based on temporality and clinical plausibility: gestational age, birth weight, sex, rupture of membranes duration (>18 h), maternal urinary tract infection status, maternal screening cultures (cervical/vaginal and urine cultures; positive/negative; tests not performed coded using a missing-indicator), neonatal resuscitation at birth, and 24 h inflammatory markers (CRP2, WBC2, and PLT2). To minimize collinearity, Apgar-5 was not entered simultaneously with resuscitation; the primary models used resuscitation, with Apgar-5 explored in sensitivity analyses. Model 1 excluded NICU admission to avoid adjustment for a potential intermediate; Model 2 additionally included NICU admission as an early marker of illness severity and care intensity. Model 1 denotes the univariable (unadjusted) logistic regression model. Model 2 denotes the multivariable logistic regression model adjusted for predefined covariates, specified a priori based on clinical plausibility and data availability. Continuous predictors were modeled per 1 SD increase; rupture of membranes duration was log-transformed where skewness was substantial. Multicollinearity was assessed using variance inflation factors (VIF), and all retained predictors had VIF < 5. Because CRP at 24 h was measured selectively, the broad-spectrum escalation models used baseline laboratory values (CRP1, WBC1, PLT1) to maximize completeness.

2.9. Development of the NeoSIR Score

Because the Kaiser Permanente EOS calculator could not be applied consistently in this cohort (e.g., incomplete maternal GBS status and limited availability of key intrapartum parameters), we explored a simplified bedside indicator: the Neonatal Sepsis Inflammatory Risk (NeoSIR) score. Two binary components were specified a priori based on local availability and clinical relevance: elevated neonatal CRP (>0.5 mg/dL at 24 h) and documented maternal UTI during pregnancy. Each component contributed one point (range 0–2). Although leukocytosis and neutrophilia were recorded and examined in univariable analyses, neonatal leukocyte indices show substantial physiological variability during the first 24 h of life, which may limit their discriminative utility for early management decisions. Erythrocyte sedimentation rate (ESR) was not routinely used for immediate neonatal infection assessment in our unit and was not systematically available. NeoSIR was therefore evaluated as a secondary, exploratory indicator to contextualize early clinical management (antibiotic initiation and NICU admission) and should not be interpreted as a validated diagnostic or decision-support tool for culture-confirmed EOS.

2.10. Sample Size Considerations

The minimum required sample size was estimated using G*Power 3.1.9.7 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany) for the χ^2 test family (test of independence), assuming a two-sided significance level of $\alpha = 0.05$, statistical power of $(1 - \beta) = 0.95$, and a medium effect size (Cohen's w = 0.25). For comparisons involving three categorical levels (df = 2), the resulting minimum sample size was 248 participants. As this was an observational cohort with consecutive inclusion over a six-month period, all eligible newborns were included consecutively, resulting in an enrolled cohort of $n = 443$.

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This exceeds the estimated minimum and supports adequate power to detect at least medium associations in the main categorical analyses.

3. Results

3.1. Maternal Characteristics and Monitoring Practices

The study included 438 mothers, corresponding to the 443 newborns analyzed in this cohort. The mean maternal age was 27.6 ± 6.5 years (95% CI: 26.96–28.18), with a median of 27 years. Ages ranged from 14 to 50 years. The 5% trimmed mean (27.5 years) was close to the raw mean, suggesting a symmetrical distribution.

Maternal age was significantly associated with pregnancy monitoring ($p < 0.001$, Chi-square), but was not independently associated with neonatal antibiotic initiation. Women aged 20–34 years most frequently had complete antenatal monitoring (61.1%), followed by women ≥ 35 years (12.0%). Partial monitoring was reported in 13.1% of women aged 20–34 and in 1.8% ≥ 35 years. Non-monitoring was rare (0.7%) and observed only among mothers < 35 years. Spearman's correlation confirmed a positive association between maternal age and compliance with antenatal care (r = 0.286, $p < 0.05$).

Gynecological visits followed the same trend ($p < 0.001$). In the 20–34 group, 38.5% attended more than nine prenatal visits, while only 1.4% of adolescents reached this level. Spearman's correlation (r = 0.268, $p < 0.05$) confirmed the association. Ultrasound examinations also correlated with age ($p < 0.001$): 35.5% of women aged 20–34 had more than nine ultrasounds, compared with 7.2% of those ≥ 35 years and 0.5% of those < 20 years.

Compliance with infectious disease screening varied: TORCH testing was most frequent in the 20–34 age group (50.5% immunized), followed by those ≥ 35 (9.7%). Only one case of active toxoplasmosis was identified (20–34 group). Syphilis testing was generally high, though 10.9% of women aged 20–34 and 5.4% of adolescents were untested. HIV testing showed a similar pattern: highest in 20–34 years (64.0%), with non-testing more common in adolescents (5.7%) and younger adults (10.9%), but rare in ≥35 years (1.6%). TB screening showed the same distribution (r = 0.232, $p < 0.05$).

Hepatitis B and C testing was significantly associated with age ($p < 0.001$). Negative results were most common in the 20–34 group (63.1%). HBs antigen positivity was rare (0.7% in 20–34; 0.9% in ≥35). Anti-HCV antibodies were detected in one case (0.2%).

Urinary tract infections (UTIs) during pregnancy were significantly associated with age ($p < 0.001$). Positive urine cultures were most frequent in the 20–34 group (28.7%), followed by ≥35 years (5.7%) and <20 years (2.7%). Treatment rates mirrored prevalence (28.5% in 20–34; 5.7% ≥ 35). Correlation analysis did not confirm an association between maternal age and treatment initiation (r = 0.083, $p > 0.05$).

Cervical cultures were positive most often in the 20–34 group (26.9%). Treatment following positive culture was significantly associated with maternal age ($p = 0.001$; r = 0.157, $p < 0.05$).

At admission, pathological vaginal discharge was not significantly associated with age ($p = 0.433$; r = −0.030, $p > 0.05$). Vaginal cultures identified a variety of pathogens, including *Candida* spp., *E. coli*, *Klebsiella* spp., *Staphylococcus aureus*, and GBS, though more than half were sterile ($n = 219$). Amniotic fluid cultures were also sterile in most cases, with no significant variation by maternal age ($p = 0.292$). Urine cultures collected at admission were predominantly sterile, with no association between age and positivity ($p = 0.373$). The few positive findings (mostly *E. coli*) were clinically relevant and contributed to maternal infection diagnosis.

Duration of membrane rupture showed a significant association with maternal age ($p = 0.001$). Antibiotic initiation decisions were primarily associated with documented maternal infection and neonatal laboratory findings rather than maternal age. Intact

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membranes were documented in 54.1% of women aged 20–34. By contrast, the appearance of amniotic fluid did not differ significantly across groups ($p = 0.926$), with clear fluid'predominating.

3.2. Neonatal Characteristics and Outcomes

During the six-month study period, 443 newborns met the predefined eligibility criteria and were included in the final analysis. The cohort had a near-equal sex distribution, with 218/443 (49.2%) females and 225/443 (50.8%) males. Birth weight analysis showed that most infants (83.5%, $n = 369$) were within the normal weight range. A smaller proportion were underweight (14.0%, $n = 62$), while only 1.8% ($n = 8$) had high birth weight.

When evaluating the relationship between maternal infectious status and neonatal surface or gastric colonization, no statistically significant association was identified. Specifically, maternal vaginal discharge at delivery did not correlate with positive neonatal gastric aspirate cultures ($p = 0.77$, Chi-square test). This finding suggests that isolated maternal vaginal colonization had limited predictive value for neonatal therapeutic decisions. In line with the study design, neonatal gastric aspirates and peripheral swabs were considered observational markers of colonization rather than diagnostic indicators of infection.

3.3. Statistical Associations Between Maternal Infectious Status, Neonatal Antibiotic Therapy, and NICU Admission

Analysis of maternal and neonatal infectious parameters revealed several significant associations with antibiotic initiation, as seen in Table 1. Elevated neonatal C-reactive protein (CRP > 0.5 mg/dL) was strongly associated with the start of antibiotic therapy ($p = 0.032$, Chi-square test). Similarly, a history of maternal urinary tract infection (UTI) during pregnancy significantly increased the likelihood of neonatal antibiotic treatment ($p = 0.005$).

Table 1. Associations between maternal and neonatal infectious status, laboratory findings and antibiotics initiation.

Association	*p*
WBC vs. Gastric culture	0.26 *
RBC vs. Gastric culture	0.23 *
CRP vs. Gastric culture	0.18 *
Categorical CRP vs. Antibiotics	**0.032** **
Categorical CRP vs. NICU	0.29 **
Maternal UTI vs. Antibiotics	**0.005** **
Cervical culture vs. Antibiotics	0.95 **
Vaginal secretion on admission vs. Antibiotics	0.99 **
Pregnancy monitoring vs. Antibiotics	0.97 **

* Kruskal–Wallis test; ** Chi-square test. Bolded values indicate statistical significance at $\alpha = 0.05$.

In contrast, other maternal bacteriological findings (including positive cervical or vaginal cultures) did not show a significant association with antibiotic initiation ($p > 0.95$). Likewise, the presence of vaginal secretions at admission and the overall degree of pregnancy monitoring were not predictive of antibiotic therapy ($p = 0.99$ and $p = 0.97$, respectively).

These results indicate that while neonatal inflammatory markers (particularly CRP) and maternal UTIs were influential in guiding therapeutic decisions, maternal colonization findings and pregnancy monitoring alone did not significantly influence antibiotic initia-

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tion. Neonatal swabs and gastric aspirates were evaluated as observational indicators of colonization and did not show predictive value for clinical management.

Detailed analysis showed that among neonates with elevated CRP (≥0.5 mg/dL), 102/107 (95.3%) received ampicillin, and very few required broader-spectrum antibiotics (Table 2). The occasional use of ceftriaxone or imipenem–cilastatin reflects selective escalation in infants with clinical deterioration, rising inflammatory markers, or concern for resistant pathogens, rather than routine first-line neonatal therapy. These exposures highlight practice variability under uncertainty in settings with limited microbiological confirmation and reinforce the stewardship need for structured reassessment and escalation criteria.

Table 2. CRP and antibiotic type.

CRP at 24 h	*n*	Ampicillin	Cefoperazone/ Sulbactam	Cefotaxime	Ceftriaxone	Ciprofloxacin	Imipenem/ Cilastatin	Meropenem	No Antibiotics
Normal (<0.5 mg/dL)	335	281 (83.9%)	2 (0.6%)	1 (0.3%)	4 (1.2%)	3 (0.9%)	2 (0.6%)	13 (3.9%)	29 (8.7%)
Elevated (≥0.5 mg/dL)	107	102 (95.3%)	0 (0.0%)	0 (0.0%)	3 (2.8%)	0 (0.0%)	0 (0.0%)	2 (1.9%)	0 (0.0%)

Data are presented as *n* (%). CRP refers to the value measured at ~24 h of life. Broad-spectrum agents (e.g., ceftriaxone, imipenem–cilastatin, meropenem) reflect selective escalation in a minority of cases and were not used as routine first-line therapy.

3.4. Correlation of Maternal Infection with Antibiotic Initiation

Maternal urinary tract infection (UTI) during pregnancy remained one of the most influential maternal factors associated with neonatal antibiotic therapy (Table 3). A significant correlation was identified between documented maternal UTI and the initiation of antibiotics in newborns ($p < 0.005$). Clinicians consistently regarded confirmed maternal UTIs as a notable risk indicator, particularly in a setting where early neonatal diagnostics rely on simple laboratory markers.

Table 3. Maternal infection screening indicators and outcomes (univariable associations).

Maternal Variable	Outcome	*p* Value
Maternal UTI	Antibiotic initiation	**0.005**
Vaginal secretions during hospitalization	Antibiotic initiation	0.99
Cervical culture status	Antibiotic initiation	0.95
Maternal UTI	NICU admission	0.91
Vaginal secretions during hospitalization	NICU admission	0.93
Cervical culture status	NICU admission	0.97

Data are presented as *p* values from Chi-square tests; *p* values are descriptive. Bolded values indicate statistical significance at $\alpha = 0.05$.

Among the maternal urine cultures, *Escherichia coli* was the most frequently isolated organism, detected in 110 cases (24.9%). Of these, 102 newborns (92.7%) received early antibiotic treatment. Thirteen of the infants born to mothers with *E. coli* UTIs required NICU admission, representing 19.7% of all NICU admissions recorded during the study period. These figures illustrate the strong influence of maternal UTIs, especially those caused by *E. coli*, on clinical management immediately after birth.

Polymicrobial UTIs were less common but clinically relevant. Most involved *E. coli* in combination with *Enterococcus*, *Klebsiella*, *Staphylococcus*, or *Proteus* species. Despite their lower prevalence, these mixed infections were also followed by neonatal antibiotic initiation, underscoring that any maternal UTI with recognized pathogenic potential guided a cautious therapeutic approach.

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Maternal vaginal secretion findings at admission and cervical culture results also contributed to the assessment of maternal infectious status; however, neither showed a statistically significant association with neonatal antibiotic initiation ($p > 0.95$) or with NICU admission ($p > 0.90$). Although microorganisms such as *Candida* spp., *E. coli*, *Klebsiella* spp., *Staphylococcus aureus*, and GBS were identified, these findings did not consistently alter neonatal therapeutic decisions. This pattern suggests that, compared with UTIs, lower-genital-tract infections were seen as less predictive of early neonatal complications in this cohort.

In addition, six mothers (1.4%) had documented SARS-CoV-2 infection during pregnancy. Two of these pregnancies also had positive cervical cultures, three had UTIs, and all six newborns received empirical antibiotics.

Neonatal inflammatory markers influenced treatment decisions in parallel with maternal risk factors. In addition to elevated CRP, increased total leukocyte count and high neutrophil proportion also contributed to the decision to initiate antibiotic therapy, even though these associations were not the strongest statistically. This reflects the practical reality of a resource-limited setting, where decisions rely mostly on accessible laboratory parameters, while highlighting the challenge of balancing early intervention with antibiotic stewardship in environments where diagnostic certainty is difficult to achieve.

3.5. Influence of Antenatal Monitoring on Neonatal Therapy

Antenatal monitoring varied widely among the mothers included in the study, yet these differences did not appear to meaningfully influence neonatal antibiotic initiation. Descriptive analysis showed that 71.9% of neonates born to mothers with complete prenatal monitoring received antibiotics, compared with 93.6% of those whose mothers had incomplete monitoring. Antibiotic initiation was most frequent among newborns from pregnancies with few or no documented prenatal visits or ultrasounds.

Despite these descriptive trends, statistical testing did not identify a significant association between the extent of antenatal monitoring and neonatal antibiotic therapy ($p > 0.84$ for all parameters). This indicates that, although incomplete monitoring was common among mothers whose newborns eventually received antibiotics, it did not independently predict treatment when considered alongside maternal infection and neonatal biomarkers.

These results highlight the distinct role of maternal infections (particularly UTIs) in shaping neonatal management. In contrast, the degree of prenatal monitoring did not influence whether antibiotics were started, despite the higher descriptive rates seen in incompletely monitored pregnancies. Clinicians appeared to base therapeutic decisions primarily on objective indicators present at birth, such as maternal infection status and neonatal laboratory abnormalities, rather than on the extent of maternal follow-up during pregnancy.

This approach reflects the practical realities in some areas, where immediate and accessible clinical information carries more weight than prenatal documentation when evaluating the risk of early neonatal infection.

3.6. Multivariable Associations with Antibiotic Exposure, NICU Admission, and Escalation

The length of antibiotic treatment (expressed as antibiotic-days) varied according to the newborns' clinical and laboratory profiles. Elevated C-reactive protein (CRP > 0.5 mg/dL) at 24 h of life was associated with a longer treatment course, with infants in this group receiving antibiotics for an average of 8.33 days, compared with 6.98 days in those with normal CRP values ($p < 0.001$, Mann–Whitney U test). This difference reflects the strong influence of inflammatory markers on decisions to continue treatment beyond the initial empirical phase.

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Among the 443 eligible newborns, empirical antibiotics were initiated in 414 (93.5%), and 62 (14.0%) required NICU admission. The most common initial regimen was ampicillin plus gentamicin (379/414, 91.5%). Total antimicrobial exposure, expressed as days of therapy (DOT; antibiotic-days), had a mean of 7.83 ± 2.87 and a median of 6 (IQR 6–10). The corresponding length of therapy (LOT) had a mean of 3.94 ± 1.35 and a median of 3 (IQR 3–5). Broad-spectrum escalation occurred in 81/414 infants (19.6%), including carbapenem exposure in 60/414 (14.5%). Compared with non-NICU infants, NICU-admitted newborns had longer DOT (median 10 (IQR 8–11.8) vs. 6 (IQR 6–10); $p < 0.001$) and longer LOT (median 5 (IQR 5–5) vs. 3 (IQR 3–5); $p < 0.001$) and were more likely to receive broad-spectrum agents (45.2% vs. 15.1%; $p < 0.001$).

Multivariable predictors of broad-spectrum escalation are presented in Table S1.

NICU admission was another factor associated with prolonged therapy. Newborns requiring intensive care received antibiotics for an average of 10.39 days, significantly longer than those managed on the postnatal ward (mean 6.81 days, $p < 0.001$). The extended duration likely reflects the need for closer monitoring and the greater clinical instability typically seen in infants admitted to NICU.

By contrast, the extent of maternal prenatal monitoring did not significantly affect antibiotic duration ($p = 0.313$). Whether mothers had comprehensive, partial, or absent antenatal care, treatment length in their newborns was determined mainly by neonatal findings, rather than by maternal follow-up history.

These patterns underscore the central role of early neonatal laboratory and clinical indicators in guiding treatment decisions. In a setting where definitive microbiological confirmation is often unavailable, clinicians relied on dynamic markers and the infant's overall clinical course to judge the appropriate duration of therapy.

In multivariable analysis of prolonged antibiotic exposure (≥10 antibiotic-days), NICU admission remained the strongest predictor (aOR 6.04, 95% CI 3.08–11.87; $p < 0.001$). Higher CRP (aOR 1.74 per SD, 95% CI 1.36–2.23; $p < 0.001$) and WBC (aOR 1.39 per SD, 95% CI 1.11–1.76; $p = 0.005$), as well as lower platelet count (aOR 0.77 per SD, 95% CI 0.61–0.97; $p = 0.026$), were independently associated with prolonged exposure, whereas maternal bacteriological findings and PROM > 18 h were not (Table 4). Continuous predictors are expressed per 1 SD increase, and maternal microbiology tests not performed were handled using a missing-indicator approach.

Table 4. Multivariable predictors of prolonged antibiotic exposure (≥10 antibiotic-days).

Predictor	Adjusted OR (95% CI)	*p*-Value
NICU admission (yes vs. no)	6.04 (3.08–11.87)	<0.001
Neonatal CRP at 24 h (per SD)	1.74 (1.36–2.23)	<0.001
Neonatal WBC at 24 h (per SD)	1.39 (1.11–1.76)	0.005
Neonatal platelets at 24 h (per SD)	0.77 (0.61–0.97)	0.026
Gestational age (per SD)	1.05 (0.78–1.43)	0.732
Birth weight (per SD)	0.91 (0.70–1.19)	0.492
Male sex (vs. female)	0.88 (0.55–1.40)	0.582
PROM > 18 h (yes vs. no)	1.78 (0.40–7.99)	0.451

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Table 4. *Cont.*

Predictor	Adjusted OR (95% CI)	*p*-Value
Maternal UTI during pregnancy (yes vs. no)	1.28 (0.76–2.15)	0.348
Positive cervical culture (yes vs. no)	1.34 (0.79–2.26)	0.282
Neonatal resuscitation at birth (yes vs. no)	0.83 (0.51–1.37)	0.470
EOS risk at birth (log, per SD)	1.11 (0.84–1.47)	0.470

Adjusted odds ratios (aORs) from logistic regression; outcome defined as ≥10 antibiotic-days. Continuous predictors are expressed per 1 SD increase; EOS risk at birth was log-transformed. Maternal microbiology tests not performed were handled using a missing-indicator approach.

3.7. The NeoSIR Score for Risk Stratification in Suspected Maternal–Fetal Infection

NeoSIR (Neonatal Sepsis Inflammatory Risk) was explored as a simple bedside indicator combining two routinely available parameters: maternal UTI during pregnancy and neonatal CRP > 0.5 mg/dL at 24 h. Each component contributed one point (range 0–2).

Antibiotic initiation was high across NeoSIR strata: 191/206 (92.7%) for score 0, 187/201 (93.0%) for score 1, and 35/35 (100%) for score 2 (Table 5). The high antibiotic initiation rate even in the NeoSIR = 0 group reflects the selected high-risk cohort and a low threshold for empirical treatment in the absence of consistently available culture confirmation; clinical signs and other laboratory abnormalities frequently prompted therapy despite a normal CRP at 24 h.

Table 5. NeoSIR score and antibiotic therapy.

Score NeoSIR	Antibiotherapy (*n*, %)	
	YES	NO
0	191 (92.7%)	15 (7.3%)
1	187 (93.0%)	14 (7.0%)
2	35 (100.0%)	0 (0.0%)

The overall association between NeoSIR category and antibiotic initiation was not statistically significant (Chi-squared test, $p = 0.26$), and discrimination for antibiotic initiation was limited (AUC = 0.55). Accordingly, we do not propose NeoSIR for clinical cut-offs or treatment initiation decisions in this dataset; it is presented strictly as hypothesis-generating. A NeoSIR score of 2 acted as a specific rule-in flag (specificity 100%) but had low sensitivity (8.5%).

Overall, this two-item composite was feasible to apply in routine care but showed limited ability to separate lower-risk strata under the prevailing low threshold for empirical treatment. Further refinement and validation against culture-confirmed outcomes are required before NeoSIR can be considered a predictive tool.

Accordingly, NeoSIR is presented here as an exploratory indicator intended to support structured reassessment rather than to replace established sepsis calculators or guideline-based observation pathways.

4. Discussion

In this selected cohort of late preterm and term newborns evaluated for suspected maternal–fetal infection (MFI), maternal infectious conditions—particularly UTIs and abnormal vaginal secretions—were common and were associated with early clinical management decisions. The very high rate of empirical antibiotic initiation suggests a low

threshold for treatment in the absence of consistently available microbiological confirmation, highlighting the tension between timely therapy and antibiotic stewardship.

Screening for TORCH infections, HIV, syphilis, hepatitis B/C, and tuberculosis was more consistently completed among older mothers, reflecting a trend described in recent European analyses showing higher antenatal engagement among women aged 20–34 years compared with adolescents or young adults. Younger mothers, particularly adolescents, exhibited the lowest compliance, increasing the likelihood of undiagnosed infections or missed opportunities for timely treatment [19,20].

Given that COVID-19 during pregnancy may alter inflammatory pathways and predispose to gestational complications), evaluating maternal SARS-CoV-2 history was considered part of the maternal infection assessment [21,22].

Maternal UTIs were strongly associated with neonatal antibiotic therapy initiation and NICU admission in our cohort. One explanation may be that maternal inflammatory states, also described in conditions such as preeclampsia, influence neonatal vulnerability [23].

Recent studies have reaffirmed the relevance of maternal genitourinary infections as predictors of neonatal infectious morbidity, even in late-preterm and term populations [15]. Despite the relatively limited microbiological confirmation available, antibiotic exposure remained high because the cohort was selected as clinically high-risk and local practice maintained a low threshold for empirical treatment under diagnostic uncertainty. Similar observations have been reported in recent perinatal studies, where clinical decision-making increasingly prioritizes maternal infection history and neonatal biomarkers over isolated maternal colonization [24–26].

The frequent absence of microbiological confirmation in our population does not exclude clinically relevant inflammatory risk, as antenatal low-grade fetal inflammatory responses have been shown to increase susceptibility to EOS even when intrauterine infection is not microbiologically detectable [27].

The pattern of neonatal outcomes in this cohort reflects the broader challenges faced in secondary-care environments with limited access to blood cultures. Elevated C-reactive protein (CRP) at 24 h of life and NICU admission were strongly associated with prolonged antibiotic therapy, consistent with recent findings that CRP trajectories and clinical instability remain influential predictors of neonatal infection severity when microbiological confirmation is lacking [27–29].

Our data also showed that leukocytosis and high neutrophil counts contributed to the decision to initiate antibiotic therapy. Although these hematological markers are not highly specific, they remain widely used as adjunctive tools, particularly in settings where access to more advanced biomarkers (e.g., procalcitonin or presepsin) is limited [30–32].

However, the degree of prenatal monitoring did not statistically influence antibiotic initiation or treatment duration once neonatal laboratory abnormalities and clinical symptoms were considered. This pattern is consistent with recent reports that emphasize the dominant role of postnatal indicators in shaping real-time therapeutic decisions in resource-limited settings [15,33].

These findings illustrate the persistent trade-off between ensuring early treatment and avoiding unnecessary antibiotic exposure. Notably, empirical antibiotics were initiated in 306/335 (91.3%) newborns with normal CRP at 24 h (<0.5 mg/dL), underscoring that clinical and maternal-context factors frequently outweighed early biomarker reassurance in routine practice.

Recent European initiatives emphasize minimizing empiric antibiotic use in well-appearing infants with isolated risk factors, primarily through structured observation pathways and improved access to maternal intrapartum data [25,26]. Translating these recommendations to secondary-care settings like ours requires the availability of dependable

maternal screening and consistent neonatal monitoring, conditions that remain variable in many parts of Eastern Europe [34].

The NeoSIR indicator was proposed to address the reality that comprehensive risk calculators cannot always be applied when key intrapartum data are missing or prenatal screening is incomplete. NeoSIR represents a simplified exploratory indicator derived from routinely available information; however, its limited discriminatory performance in this cohort underscores the need for cautious interpretation and external validation.

While more advanced tools such as the Kaiser Permanente EOS calculator have demonstrated the ability to reduce unnecessary antibiotic use, their applicability in similar settings is limited, with recent studies underscoring the value of adapted risk scores in environments where access to culture-based diagnostics is limited [16,33].

However, in this cohort—characterized by near-universal empirical treatment—NeoSIR showed limited discrimination for antibiotic initiation (AUC = 0.55) and did not materially separate lower-risk strata. NeoSIR should therefore be viewed as exploratory, and further refinement and external validation against culture-confirmed outcomes are required before considering any clinical decision support role. The selection of NeoSIR components reflects pragmatic availability and early clinical relevance in our setting rather than an attempt to build a comprehensive laboratory-based diagnostic score.

Because antibiotic duration and NICU admission reflect clinical severity and physician judgment, the observed associations are susceptible to confounding by indication: infants who appeared clinically sicker were more likely to be admitted to NICU and to receive prolonged or escalated therapy. Therefore, our findings should be interpreted as describing management-linked associations rather than causal effects.

This study has several limitations. First, blood cultures and other microbiological reference standards were not consistently captured, preventing classification of culture-confirmed early-onset sepsis and limiting inference about diagnostic accuracy. Second, the cohort represents a selected high-risk population rather than an unselected birth cohort, which restricts generalizability and inflates the baseline probability of empirical treatment. This context likely compressed between-group differences and reduced discrimination metrics, including NeoSIR performance, which may not reflect its behavior in more heterogeneous populations. Third, as an observational single-center study, the analyses are vulnerable to confounding by indication and by unmeasured intrapartum variables (e.g., timing of prophylaxis), and we did not fit multivariable predictive models. Finally, outcomes beyond the early hospitalization period were not assessed. In addition, CRP was analyzed using a clinically used threshold because continuous values were not uniformly recorded, limiting assessment of dose–response patterns.

An additional limitation is the restricted ability to classify true neonatal infection using objective confirmation, because microbiological testing was not consistently available and advanced diagnostic modalities (beyond routine cultures) were not systematically performed. Where complete-case modeling was feasible (e.g., the escalation models reported in Table S1), estimates were directionally consistent, supporting the robustness of the main interpretation; however, systematic restriction to tested-only dyads could introduce selection bias in this setting.

The findings highlight the importance of improving prenatal care, particularly universal screening for maternal infections and consistent management of maternal conditions. Increasing antenatal compliance among younger mothers may reduce undiagnosed infections and improve neonatal outcomes [34].

Evidence linking neonatal infection and inflammatory exposure to long-term neurodevelopmental outcomes further supports a cautious approach to early neonatal illness,

where clinical and inflammatory indicators may justify empirical treatment despite negative cultures when maternal risk factors are present [24,35].

In the neonatal period, structured risk assessment using simple, widely available indicators could help clinicians balance timely treatment with the principles of antibiotic stewardship. Recent studies show that even modest improvements in risk stratification can significantly reduce unnecessary antibiotic exposure without compromising safety [25,29,36].

Adapting neonatal infection management strategies to the realities of low-resource hospitals will require both improved maternal screening and updated neonatal observation protocols. The approach tested in this study may represent a feasible step toward more consistent and targeted antibiotic use, especially in settings where limited staffing and high patient turnover constrain the practicality of prolonged structured clinical observation [37].

International guidance increasingly supports probability-based management and serial clinical examinations to reduce unnecessary antibiotic exposure in well-appearing infants, while still ensuring prompt treatment for those with clinical deterioration or high risk. Examples include risk calculators and national probability-based protocols, as well as WHO recommendations tailored to resource-limited settings. Adapting these approaches locally will require more consistent capture of intrapartum data and improved access to microbiology to enable timely discontinuation when infection is unlikely [26,29,31,38–41].

5. Conclusions

In a secondary-care setting with limited microbiological resources, the evaluation of suspected maternal–fetal infection relies mainly on maternal history, basic neonatal inflammatory markers, and early clinical signs. Maternal urinary tract infections and CRP elevation at 24 h of life were the most significant factors guiding antibiotic initiation, while leukocytosis, neutrophilia, and NICU admission influenced treatment duration. These patterns reflect the realities of neonatal care in many countries, where incomplete prenatal screening and restricted access to neonatal cultures remain inconsistently available.

Our exploratory NeoSIR indicator may help structure documentation and prompt reassessment in settings where comprehensive sepsis calculators are difficult to apply; however, it showed limited discrimination for antibiotic initiation in this high-treatment cohort and should not be interpreted as a diagnostic or treatment-guiding tool. Multicenter studies incorporating more consistent microbiological confirmation and standardized reassessment protocols are required before any simplified indicator could be considered for clinical decision support.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines14030538/s1, Table S1. Multivariable logistic regression models for broad-spectrum escalation (expanded coverage) among antibiotic-treated eligible newborns (complete-case n = 380; events = 77).

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Data Availability Statement: Data supporting reported results can be found at anca.vulcanescu@umfcv.ro, mirela.siminel@umfcv.ro; due to the need to uphold intellectual property rights and confidentiality agreements, ensure the integrity and accuracy of ongoing analyses, and comply with ethical and regulatory guidelines that govern data dissemination before formal publication.

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