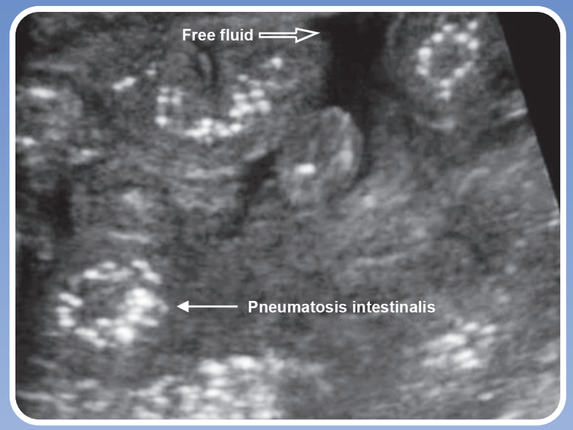
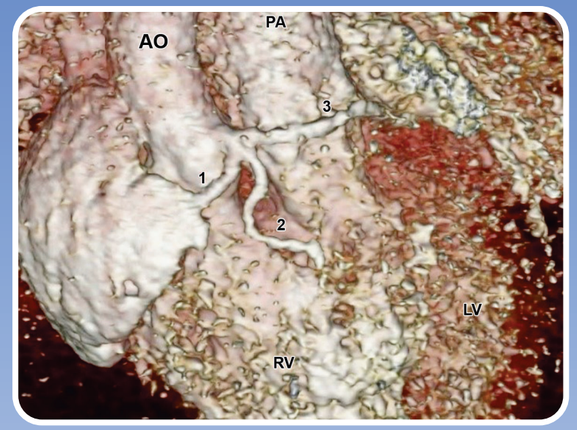
January–March 2022
Volume 1
Issue 1
ISSN: 2769-514X

newborn

Official Journal of the Global Newborn Society



Sonographic diagnosis of neonatal necrotizing enterocolitis



Advanced cardiac imaging in an infant with d-transposition of the great arteries. There is a single coronary artery arising from the aorta. Ao: Aorta. PA: Pul Artery

Other highlights:

Intestinal resection is more likely to be effective in necrotizing enterocolitis extending to colon than in disease limited to the small intestine

Accretion rates of fat-and fat-free mass in infants at 30 to 45 weeks's postmenstrual age

Also available online at
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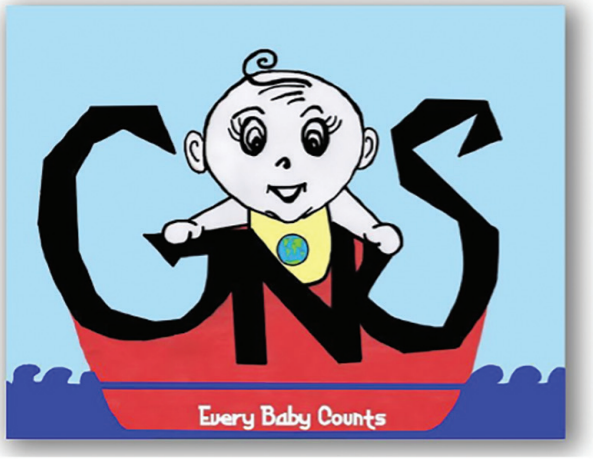
January–March 2022 Volume 1 Issue 1 newborn Official Journal of the Global Newborn Society ISSN: 2769-514X



Global Newborn Society

Each time we lose an infant, we lose an entire life and its potential!

Newborn is the official journal of the Global Newborn Society (GNS), a globally-active, non-profit organization that is registered as a 501(c)(3) non-profit formation in the United States and is currently being listed as an analogous charity in many other nations. The aim is to enhance research in newborn medicine, understand epidemiology (risk-factors) of disease, train healthcare workers, and promote social engagement. The GNS was needed because despite all improvements in medical care, infants remain a high-risk patient population with mortality rates similar to 60-year-olds. We need to remind ourselves that *Every Baby Counts*, and that *Each Time We Lose an Infant, We Lose an Entire Life and its Potential*.

Our logo above, a hand-drawn painting, graphically summarizes our thought-process. There is a lovable little young infant exuding innocent, genuine happiness. The curly hair, shape of the eyes, long eye-lashes, and the absence of skin color emphasize that infants need care all over the world, irrespective of ethnicity, race, and gender. On the bib, the yellow background reflects happiness, hope, and spontaneity; the globe symbolizes well-coordinated, world-wide efforts. The age-related vulnerability of an infant, with all the limitations in verbal expression, is seen in being alone in the boat.

The unexpressed loneliness that many infants endure is seen in the rough waters and the surrounding large, featureless sky. However, the shades of blue indicate that the hope of peace and tranquility is not completely lost. The acronym letters, GNS, on the starboard are made of casted metal and are pillars of strength. However, the angular rough edges need continued polishing to ascertain adequacy and progress. The red color of the boat symbolizes our affection. The expression "*Every Baby Counts*" seen on the boat's draft below the waterline indicates our commitment to philanthropy, and if needed, to altruism that does not always need to be visible. The shadow behind the picture shows that it has been glued on a solid wall, one built out of our adoption and commitment.

Design of the Journal Cover

The blue color on the journal cover was a careful choice. Blue is the color of flowing water, and symbolizes the abnormalities of blood vascular flow that are seen in many neonatal illnesses. There is a gradual transition in the shades of blue from the top of the cover downwards. The deeper shades of blue on the top emphasize the depth, expertise, and stability, which the renowned authors bring. Light blue is associated with health, healing, tranquility, understanding, and softness, which their studies bring. The small letter "*n*" in the title of the journal, *newborn*, was chosen to emphasize the little size of a newborn baby. The issue editors chose four articles to be specifically highlighted; the two pictures and two titles below reflects an order suggested by them.

Instructions to Authors

The journal welcomes original articles and review articles. We also welcome consensus statements, guidelines, trials methodology, and core outcomes relevant to fetuses/young infants in the first 1000 days. A detailed set of instructions to authors can be seen online at https://www.globalnewbornsociety.org/intructions-for-authors. The manuscripts can be submitted via the online manuscript submission system.

Issue Information

Volume 1, Issue 1; January–March 2022

ISSN: 2769-514X

Published: GNS, LLC; 6114 Lily Garden, Clarksville, MD, USA; Ph +1 708 910 8729

Printed: Jaypee Brothers Medical Publishers
4838/24, Ansari Road, Darya Ganj, Delhi - 110 002
Phone: +91 11 4357 4357, Fax: +91 11 4357 4314

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PREFACE

10.5005/newborn-1-1-v

This is the inaugural issue of the journal *Newborn*, which aims to present current scientific activity relevant to the fetus during gestation, the newborn infant, and various problems that may be seen in the first 1000 days after birth.

This issue presents scientific articles and reviews focused on a wide variety of medical/surgical issues seen in these patients. The editorial board and the organization (the GNS) are grateful to the issue editors, Drs. Rachana Singh and Robert D. Christensen, two globally-renowned leaders in the field. We are also grateful to all our reviewers, the publishers, the GNS, LLC. and Jaypee Brothers; and all the members of the editorial board for their continuous support, encouragement, and expertise.

With sincere regards on behalf of the Global Newborn Society.

Akhil Maheshwari MD
Editor-in-Chief

ORIGINAL RESEARCH

Low-lying Umbilical Venous Catheters are not Always Associated with Increased Complications

Sunil Joghee[1], Majeeda Kamaluddeen[2], Amuchou Soraisham[3]

Abstract

Introduction: Umbilical venous catheters (UVCs) are frequently used for clinical care in neonatal intensive care units (NICUs). Umbilical venous catheters cannot always be positioned perfectly in the inferior vena cava, and low catheters have to be used until a more stable peripherally inserted central catheter can be placed after ruling-out early onset sepsis. There are concerns that low UVCs may be associated with complications such as infection, extravasation, and thrombosis.

Objectives: To determine whether UVC complications were associated with (1) low positioning of the catheter tip and (2) the postnatal age at insertion.

Methods: We examined a retrospective cohort of infants with UVCs in a tertiary NICU. Neonates with major congenital anomalies, hydrops fetalis, prenatally diagnosed cardiac arrhythmias, pericardial effusion, or ascites were excluded. The position of UVCs is considered as optimum if its tip is seen on radiographs at the level between 8th and 10th thoracic vertebrae (T8–T10), to be low if below T10, and high if above T8. The primary outcome was UVC-related complications resulting in early removal of catheter. We compared the rates of UVC-related complications resulting in removal of UVCs with tips in normal (T8–T10) vs low-lying (below T10) positions at the time of insertion. We also examined the impact of postnatal age, before or after 12 hours, and the frequency of the UVC-related complication.

Results: Of the 919 eligible infants, UVC tips were located optimally in 433 (47%) and were low in 415 (45%). The UVC was positioned at an abnormally high position in 71 (8%) infants. Of the 919 infants, UVC-related complication was seen in 54 (5.9%) infants. Low-lying UVCs were removed due to complications in 27 of 415 (6.5%) compared with 20 of 433 (4.6%) optimally position catheters [adjusted odds ratio (aOR) = 1.16; 95% confidence interval (CI): (0.62–2.17)]. High-placed UVCs were associated with a higher rate of cardiac complications (aOR = 6.09, 95% CI [2.03–18.28]) compared with optimally position UVCs. There was also no difference in UVC-related complications between early and late insertion of UVC (6.3% vs 4.7%, $p = 0.34$).

Conclusions: The frequency of complications and consequent need for removal did not differ in UVCs with a tip position traditionally perceived to be optimal or low or by the time of insertion after birth.

Keywords: Complications, Newborn, Umbilical venous catheter.

Newborn (2022): 10.5005/jp-journals-11002-0004

Introduction

Umbilical venous catheters (UVCs) are commonly inserted for vascular access in critically ill or extremely preterm infants. About 20% of all infants admitted to neonatal intensive care unit (NICU) and more than 50% of very low birth weight infants had UVC during hospital stay.[1–3] The ideal position of UVC tip is just outside the heart at the junction of the right atrium and inferior vena cava (IVC).[4]

Adverse events may occur both during UVC insertion and dwell time. Malposition of the UVC directly after insertion has been reported in 11.5 to 56% of neonates, although the definition varies widely.[5–9] Umbilical venous catheterization is associated with various complications including bloodstream infections, cardiac complications such as arrhythmias, pericardial effusion, cardiac tamponade, hepatic complications (including liver hematoma, thrombosis, abscess, ascites), and mechanical complications such as occlusion, breakage, and migration of fragmented catheter.[9–18] Malposition and low-lying UVCs have been noted to be more frequently associated with complications.[1–3,14]

The standard practice in NICU is to retract the UVC if catheter tip is below the optimal position and reposition the tip below the contour of the liver as seen on radiograph. However, there is no evidence in the literature to support the safety of using a low-lying UVC other than for neonatal resuscitation. It is common practice in certain NICU that low-lying UVC was not used for clinical care due to fear of complications. Attempting UVC insertion after the umbilical cord has dried is difficult and is associated with low success rate. Anecdotally, we notice that when UVC was inserted after dried umbilical cord, there was difficulty in advancing the catheter, and it tends to be in the low-lying position. Very few studies examined the association between the position of the UVC tip and the incidence of UVC-related complications in neonates.[2,3] The association between age at UVC insertion and the incidence of complications has not been studied in the past. The objective of our study is to compare

[1]Child Trust Hospital, Department of Pediatrics, Coimbatore, Tamil Nadu, India

[2,3]Department of Pediatrics, Alberta Children's Hospital Research Institute, University of Calgary, Calgary, Alberta, Canada

Corresponding Author: Amuchou Soraisham, Department of Pediatrics, Alberta Children's Hospital Research Institute, University of Calgary, Calgary, Alberta, Canada, Phone: 403-944-8101, e-mail: amuchou.soraisham@albertahealthservices.ca

How to cite this article: Joghee S, Kamaluddeen M, Soraisham A. Low-lying Umbilical Venous Catheters are not Always Associated with Increased Complications. Newborn 2022;1(1):1–6.

Source of support: Nil

Conflict of interest: None

UVC-related complications based on the catheter tip position as well as timing of catheter insertion.

Methods

Study Design, Setting, and Population

This retrospective cohort study was conducted in a 39-bed tertiary NICU in Western Canada. We included infants admitted to the NICU between January 2016 and December 2018, in whom the position of the UVC placed during hospital stay was adjusted and confirmed by radiograph. Infants with major congenital malformations, hydrops fetalis, prenatally diagnosed arrhythmias, pericardial effusion, ascites, and those who did not have radiographic confirmation after reposition of the catheter were excluded. The institutional research ethics board approved the study.

In our unit, UVC is inserted by trained staff and position of the catheter tip is confirmed by thoracoabdominal radiograph (TAR). In addition, bedside ultrasound is done to confirm the position of UVC tip in some patients depending on the availability of trained personnel to perform bedside ultrasound. The final position of the UVC tip in relation to the vertebral body, cardiac silhouette, and diaphragmatic level on TAR is documented. The decision to use low-lying UVC is at the discretion of the medical team based on the infant's condition and number of attempts at intravenous line insertion. Peripherally inserted central catheter is inserted if the infant needs longer duration of intravenous access.

We reviewed electronic medical records and charts of eligible infants. We collected infant demographics (including gestational age, birth weight, and sex), age at UVC insertion, position of UVC tip on TAR, the duration of catheter, and UVC-related complications resulting in nonelective catheter removal. Two authors independently reviewed all radiographs including the final radiograph to assess the position of catheter tip after adjustment. Based on the radiographic finding, we defined optimal position if the UVC tip was between the upper border of the eighth thoracic vertebral body (T8) and lower border of T10 on the anteroposterior TAR. When the catheter tip was below the lower border of T10, it was classified as a low-lying UVC, and those with catheter tip above the upper border of T8 was classified as high UVC.

Outcomes

The primary outcome of this study was UVC-related complications resulting in catheter removal. We defined UVC-related complications as any new onset complication associated with UVC such as (1) cardiac-arrhythmias, pericardial effusion, tamponade, and intracardiac thrombus; (2) hepatic complications such as liver hematoma, thrombosis of portal vein, ascites, and liver abscess; (3) catheter-related bloodstream infection(CR-BSI) defined as a primary bloodstream infection in a patient showing signs of infection 2 days after of UVC placement or within 48 hours of catheter removal, without another identifiable infection source;[19] and (d) mechanical complications including occlusion, catheter leakage, or breakage resulting in removal of catheter.

Statistical Analysis

Descriptive statistics were performed to compare infants who had catheter tip in optimal position, low and high position at the time of insertion. Pearson Chi-square or Fisher's exact test for categorical variables and student's *t*-test or analysis of variance (ANOVA) *F*-test for continuous variables was used for the analyses. Multivariable logistic regression analyses were performed to adjust for other potential confounding factors that may have an independent effect on UVC-related complications. Confounding variables adjusted for in the multivariate analyses include gestational age and duration of catheter. Statistical analyses were performed using Stata 14.0 (StataCorp LLC, College Station, Texas, USA). A p value of <0.05 was considered statistically significant for all analyses.

Results

During the study period, 3339 infants were admitted to the NICU, and UVC was inserted in 979 (29.3%) infants. Sixty infants were excluded, and remaining 919 infants were included for analysis. Of the 919 infants, 433 (47.1%) had UVC tip in the optimal position, 415 (45.2%) had low-lying UVC, and 71 (7.7%) had high UVC position (Flowchart 1). A total of 665 (72%) infants had UVC inserted within 12 hours. Majority (522) infants had UVC within 2 hours of admission to NICU. The mean gestational age and birth weight of the study cohort was 31 ± 5 weeks and 1737 ± 1014 g, respectively. The baseline characteristics were comparable between the groups (Table 1).

Overall, 54 (5.9%) infants developed UVC-related complications resulting in early catheter removal. There were no significant differences in the overall complication rates resulting in catheter removal between the three groups (Table 2). However, cardiac complications were significantly higher in high-position group compared with optimal position group (7% vs 0.23%, $p = 0.001$). Of the five infants with cardiac complications in high-position

Flowchart 1: Flow diagram of study population

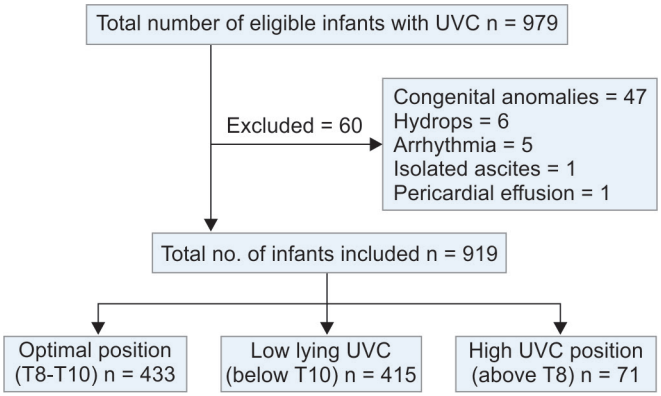

Table 1: Comparison of characteristics between three UVC positions

Characteristics	*Optimal position (n = 433)*	*Low-lying UVC (n = 415)*	*High position (n = 71)*
Gestational age, mean (SD), wk	31.09 (5.1)	31.03 (4.8)	30.6 (5.6)
Birth weight, mean (SD), g	1746 (1071)	1732 (989)	1713 (1227)
Male, *n* (%)	252 (58.2)	237 (57.1)	33 (46.4)
Duration of catheter days; mean (SD)	5.35 ± 2.37	4.31 ± 2.17[1]	5.21 ± 2.27
Duration of UVC days; median (IQR)	5 (4,7)	4 (3,6)[1]	5 (3,7)
Total UVC days	2,313	1,780	370

IQR, interquartile range; SD, standard deviation; UVC, umbilical venous catheter; [1]p <0.05, optimal position vs low-lying; [2]p <0.05, optimal vs high position

group, three infants had supraventricular tachycardia (SVT), one had pericardial effusion, and one infant had right atrial thrombus. Cardiac arrhythmias recovered after pulling the catheter to low position. Only one infant had thrombus extending from the IVC into the right atrium in optimal position.

UVC-associated hepatic complications were not different between the optimal position and low-lying UVC. In optimum position group, three infants had hepatic hematoma and one had portal vein thrombosis. In the low-lying UVC group, two infants had liver hematoma, two had cystic fluid collections (attributed to total parenteral nutrition) in the liver, and one had portal vein thrombosis and ascites.

The CR-BSI was not significantly different between the three groups (Table 2). The CR-BSI rate was 4.3/1000 catheter days in the optimal UVC position group, 5.02/1000 catheter days in the low-lying position and 5.4/1000 catheter days in the high-position group. The difference was not statistically significant. The common organisms isolated from the blood include *Coagulase Negative Staphylococcus* (*CoNS*) in 14 infants, followed by *Escherichia coli* in five infants, *Enterococcus fecalis* and *Pseudomonas spp*. in one infant each.

Mechanical complications were more frequent in the low-lying UVC as compared with optimal position group, but the difference was not statistically significant (Table 2). Of the 13 mechanical complications in low-lying UVC position, seven had leakage from catheter site, four had catheter dislodgement, one infant had scrotal and abdominal wall edema, and one infant had red streaking over the abdomen resulting in catheter removal. In optimal position group, catheter was dislodged in four infants, and one had leakage from catheter site resulting in catheter removal.

Table 3 shows the multivariate logistic regression analysis adjusting for gestational age and duration of catheter. Low-lying UVC were not associated with increased risk of catheter-related complication resulting in early removal as compared with optimal position [adjusted odds ratio (aOR) = 1.16; 95% confidence interval (CI): (0.62–2.17)]. High-placed UVCs were associated with a higher rate of cardiac complications (aOR = 6.09, 95% CI [2.03–18.28]) compared with optimally position UVCs.

Timing of UVC Insertion

Among 919 neonates, 665 (72.4%) had early UVC insertion (<12 hours), and 254 (27.6%) had late insertion (≥12 hours) of UVC. The success rate of achieving optimal UVC position was significantly higher in the early insertion group compared with the late insertion group (52% vs 34.2%, p <0.001) (Table 4). However, there was no difference in UVC-related complications between early and late insertion of UVC (6.3 % vs 4.7%, p = 0.34).

Discussion

Umbilical venous catheter insertion is a very common and essential procedure in the NICU. The length of insertion of the UVC is usually determined either by Dunn shoulder–umbilicus length method or Shukla–Ferrara formula based on birth weight.[20,21] These formulae have not been validated in extremely preterm and very low birth weight neonates. Studies have shown that Dunn nomogram and Shukla formula were accurate in only 38 to 45% and 20 to 53% of subjects, respectively.[22–24] Few studies have evaluated the UVC-associated complications in relation to catheter tip position. In this study, we examine the catheter-related complications based on the catheter tip position and timing of insertion. The overall UVC-related complication rate in our cohort was 5.9%. The UVC-related complications were lowest in optimal position (4.6%), followed by low-lying UVC (6.5%) and highest in the high UVC position (9.8%). Low-lying UVC was not associated with increased risk of catheter-related complication resulting in early removal as compared with

Table 2: Comparison of UVC-related complications based on catheter tip position

Complications	*Optimal position (N = 433)*	*Low-lying UVC (N = 415)*	*High position (N = 71)*	*p value*
Any complication resulting in early catheter removal, *n* (%)	20 (4.6)	27 (6.5)	7 (9.8)	0.16
Catheter-related bloodstream infection, *n* (%)	10 (2.3)	9 (2.1)	2 (2.8)	0.83
Cardiac complications, *n* (%)	1 (0.23)	0	5 (7.0)	0.001
Hepatic complications, *n* (%)	4 (0.92)	5 (1.2)	0	0.89
Mechanical complications, *n* (%)	5 (1.1)	13 (3.1)	0	0.07

UVC, umbilical venous catheter

Table 3: Adjusted outcomes

	Reference	*High position (aOR, 95% CI)*	*Low-lying UVC (aOR, 95% CI)*
Any complication resulting in early removal of UVC	Optimal position	1.45 (0.91–2.29)	1.16 (0.62–2.17)
Cardiac complication	Optimal position	6.09 (2.03–18.28)	–
Catheter-related blood-stream infection	Optimal position	0.98 (0.44–2.16)	1.14 (0.44–2.92)
Hepatic complications	Optimal position	–	1.36 (0.34–5.38)
Mechanical complications	Optimal position	–	1.19 (0.38–3.70)

Adjusted for gestational age and duration of catheter. aOR, adjusted odds ratio; CI, confidence interval; UVC, umbilical venous catheter

Table 4: Timing of UVC insertion, position of catheter tip and complications

	Timing of UVC insertion		
Characteristics	*Insertion <12 hr, N = 665*	*≥12 hr, n = 254*	*p value*
Catheter position			
Optimal position, *n* (%)	346 (52)	87 (34.2)	<0.001
Low position, *n* (%)	264 (39.7)	151 (59.4)	
High position, *n* (%)	55 (8.3)	16 (6.3)	
Any complication resulting in early removal of UVC, *n* (%)	42 (6.3)	12 (4.7)	0.34
Catheter related bloodstream infection, *n* (%)	17 (2.56)	4 (1.57)	0.46
Cardiac complications, *n* (%)	4 (0.6)	2 (0.79)	0.67
Hepatic complications, *n* (%)	9 (1.35)	1 (0.39)	0.30
Mechanical complications, *n* (%)	13 (1.95)	5 (1.97)	0.98

UVC, umbilical venous catheter

optimal position. High-placed UVCs were associated with a sixfold higher risk of cardiac complications compared with optimally position UVCs. We did not observed any significant difference in complication rate based on the timing of UVC insertion; however, success rate of positioning in optimum position was lower in late insertion.

In contrast to our finding, Mutlu et al. noticed UVC-related complications in 20.3% of infants in their retrospective study.[1] However, complications other than malposition were noted in only 1.2% of infants with UVCs.[1] In a retrospective study of 2011 infants, El Ters et al. reported that the rate of clinically significant complications for central UVCs was 0.5 per 1,000 catheter days, whereas for low-lying UVCs, the rate of complications was 1.5 per 1,000 catheter days. Although the complication rate was higher in low-lying UVC compared with those with central UVC, the difference was not statistically significant (OR = 2.1, 95% CI: [0.5–8.6]). Levit et al. reported that 13.3% of UVC-related complications among 2017 infants. However, complications other than malposition were noted in 1.8% of infants with UVCs.[8]

Thoracoabdominal radiograph is the most commonly used method for identification of UVC tip position. The optimal position of UVC tip (at the junction of right atrium and IVC) outside the heart as determined by ultrasound or echocardiography was observed in only about 15 to 57% of subjects who were labeled UVC tip at optimal position on radiograph.[5,6,24,25]

Comparison of UVC-related complication between the published studies is difficult due to differences in the classification of UVC position based on radiograph. For example, Mutlu et al. defined ideal UVC position as catheter tip between T9 and T10 vertebral levels on radiograph.[1] El Ters et al. defined that "central UVC position" if the catheter tip at or above the right hemidiaphragm based on radiograph and "low-lying" if the catheter tip is below the right hemidiaphragm or below the bottom of the T9 vertebral body (overlying the liver or below the liver border on the radiogram).[2] Shahroor et al. defined "proper position" when UVC tip was between the upper border of T9 and the lower border of T10 (at the level of diaphragm) and "low position" if the UVC tip is below T10.[3] Levit et al. defined ideal position as 0.5 to 1 cm above the right hemidiaphragm and reported 88.5% infants had UVC tip in ideal position.[8]

We observed higher cardiac complications including arrhythmias and pericardial effusion in the high UVC group in our study. This finding is not surprising. El Ters et al. reported that two infants with central UVC who developed SVT and one infant with centrally positioned UVC who developed cardiac tamponade.[2] The propose mechanism for central line associated arrhythmia include intracardiac central line disposition or atrial triggering to develop a reentrant pathway.[9,10] The presence of a catheter deep inside the heart with direct contact to the endocardium may predispose the patient to have premature atrial beats that may lead to SVT in presence of an accessory pathway.[9] Also, there is the risk of migration of the ideally placed catheter inside the heart with time[26] and can present with cardiac arrhythmias.[10]

Hepatic complications were not significantly different between the optimal- and low-position groups in our cohort. This finding is similar to El Ters et al. study.[2] However, some studies reported that significantly higher incidence of UVC extravasation with low-lying UVC.[3,9,27] Catheter malposition, hypertonic parenteral solutions, dopamine infusion through an inappropriately placed UVC and using of long duration of UVC have been incriminated in the development of hepatic injury.[28] The incidence of hepatic complications in optimally placed UVC in our study may be secondary to injury at the time of insertion or migration of the UVC tip with time. It may also be secondary to the fact that many times UVC tip seen to be in ideal position on TAR may actually be positioned lower as seen in many studies.

The incidence of CR-BSI was 2.3% in our cohort. The reported rate of CR-BSI varies from 0.4 to 7.1% of neonates with UVC.[1,2,8,18] We did not observed significant difference in the CR-BSI between the three groups. Our finding is similar to El Ters et al. who reported no significant difference in infection between the central and low-lying UVC.[2] In contrast, Sharoor et al. reported that low-lying UVC was associated with higher infection rate and extravasation among preterm infants.[3] They reported that the incidence of UVC associated BSI was higher with increased duration of the indwelling UVC, regardless of the UVC tip position. Leveillee et al. also reported a higher incidence of infection rate in low UVC as compared with high UVC group (17.31/1000 catheter days vs 11.49/1000 catheter days).[29] The suggested theories for increase infection with increasing dwell time included catheter hub being the main portal of entry for infectious organisms, intraluminal colonization, and growth of microorganisms especially for catheter with prolonged dwell time. The other explanation for increase in UVC related BSI in the low-lying UVCs is related to the shorter distance between the umbilical stump (high potential of colonization) and the tip of the catheter, or proximity of the umbilicus to the groin and genital area (high potential of colonization).[3]

In our study, the success rate of achieving optimal UVC position was significantly higher in the early insertion group compared with the late insertion group. Shahroor et al. reported that about 67% of UVCs were placed in good position when attempted on first day of life in preterm infants ≤32 weeks gestation.[3] However, the complication rates were not significantly different between the early insertion and late insertion groups.[3] There are many reasons for failure to achieve optimal position during UVC insertion. The umbilical vein has a direct course from the umbilicus to the portal sinus of the liver from where the portal veins and ductus venosus arise and the ductus venosus opens into the IVC.[30,31] The anatomy of the umbilical vein and ductus venosus may predispose to malposition of the UVC tip in the umbilical vein, the right or left portal vein, the hepatic parenchyma or the splenic vein. The timing

of closure of ductus venosus vary from Day 2 to Day 18 after birth, and most studies report it is closed by Day 10 of life.[32,33] The early closure of ductus venosus may predispose to malposition of UVC in the portal venous system when attempted late.

The strengths of our study include large sample size of very low birth weight infant who are born in tertiary care center, providing homogeneity in standard UVC placement and practice. Our study is the first study that evaluated timing of UVC insertion, success and complication rate. The limitations of the study include retrospective nature of the study. UVC can migrate over time from their initial position. Being a retrospective study, we assigned the groups based on catheter tip position at the initial insertion, and we did not have the actual position of catheter at the time of catheter removal due to complications. It is not ethical or clinically practical to perform X-ray prior to removal of the catheter and expose infants to radiation. Due to retrospective nature, not all babies had ultrasound assessment to assess other complications such as thrombosis.

Conclusions

In our study, we did not observed a statistically significant difference in complications between optimal position, low-lying UVC, and high position UVC. However, cardiac complications were higher in the high UVC group. Early insertion resulted in greater success in the optimal positioning of the UVC without any difference in complications between the early and late UVC group.

Orcid

Amuchou Soraisham https://orcid.org/0000-0002-9851-8759

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ORIGINAL RESEARCH

Accretion Rates of Fat and Fat-free Mass in Infants at 30–45 weeks' Postmenstrual Age

Sreekanth Viswanathan[1], Kera M McNelis[2], Akhil Maheshwari[3], Zaineh Aja'Nini[4], Stephanie Merlino[5], Marissa Culver[6], Marc Collin[7], Darlene Calhoun[8], Sharon Grow-Wargo[9]

Abstract

Background: Body composition assessment using noninvasive air displacement plethysmography (ADP) can help determine the quality of postnatal growth in infants. The accretion rates of fat mass (FM) and fat-free mass (FFM), both are known to change in various clinicopathological situations in a discordant fashion, can also help evaluate the short-term impacts of nutritional interventions on body composition.

Objectives: To determine the FM and FFM accretion rates from 30 to 45 weeks' postmenstrual age (PMA) and whether these rates are different between male and female infants.

Methods: We used previously published normative data with median FM and FFM values for infants at 30–45 weeks' PMA (Norris et al., 2019). Weekly gains in FM and FFM in g/week and g/kg/week were calculated using early one-point and average two-point methods.

Results: FM and FFM accretion rates calculated by the early one-point method were higher than the average two-point method. Male and female infants had similar FM and FFM accretion rates. Constant accretion rates of FM and FFM were not aligned with individual weekly accretion rates, which showed a twofold–fourfold change. A composite index (FFM/FM accretion rate ratio), which we named the "body composition accretion ratio" (BCAR), was more sensitive than the individual weekly accretion rates and showed a ninefold change during the study period.

Conclusions: Weekly FM and FFM accretion rates can help assess quality of postnatal growth in young infants, but BCAR can be a more useful, sensitive index for early identification of body composition changes and may possibly guide nutritional interventions.

Keywords: Body composition, Postnatal growth, Preterm infants.

Newborn (2022): 10.5005/jp-journals-11002-0018

Introduction

The quality and quantity of early postnatal growth can impact the survival, neurodevelopment, and later metabolic function in preterm infants.[1–3] Body composition studies in preterm infants have observed that early body composition, especially with a gain in fat-free mass (FFM), is associated with better long-term neurodevelopmental outcomes.[4–6] Current nutritional practices in neonatal intensive care units (NICUs) that attempt to grow preterm infants along the *in utero* growth curves using weight-based calorie intake goals are often associated with deficit in fat-free mass (FFM) and increased fat mass (FM) at term-corrected age compared to term-born peers.[7–11] Such disproportionate growth in FM and FFM has raised concerns about weight-based nutritional interventions. With the availability of safe, reliable, noninvasive infant body composition assessment using air displacement plethysmography (ADP, trade name "PEA POD"—an infant version of the ADP device), body composition assessment is increasingly clinically used to determine the quality of postnatal growth in preterm infants.[12,13] Also, normative, sex-specific reference charts for body composition for the first 6 months of life to monitor growth are now available (Norris body composition charts).[14]

To calculate the short-term growth in infants being treated in the NICUs, growth velocity calculated over a defined time period (e.g., weekly) is often used and a weight gain of 15–20 g/kg/day is often considered acceptable.[15–17] This growth velocity rate is usually expected to be similar in both male and female infants, and a sex-specific growth velocity rate for weight is not usually used.[16] However, unlike weight gain, there is no readily available data for the FM and FFM accretion rate over a defined time period. These data are important for clinicians to assess the short-term impact on the body composition of various nutritional interventions aimed to optimize the postnatal growth of preterm infants. Also, previous data suggest that male infants have higher FFM, lower FM, and lower body fat% compared to female infants.[14] This difference in body composition between male and female infants may be best evaluated with sex-specific FM and FFM accretion rates. The objective of this study was to determine the

[1,6,8]Division of Neonatology, Department of Pediatrics, Nemours Children's Hospital, University of Central Florida College of Medicine, Orlando, Florida, United States of America

[2]Division of Neonatology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH, United States of America

[3]Global Newborn Society, Baltimore, Maryland, United States of America

[4]Division of Neonatology, Department of Pediatrics, Mercy Kids Children's Hospital, University of Missouri School of Medicine, Springfield, MO, United States of America

[5,7,9]Division of Neonatology, Department of Pediatrics, MetroHealth Medical Center, Cleveland, OH, United States of America

Corresponding Author: Sreekanth Viswanathan, Division of Neonatology, Department of Pediatrics, Nemours Children's Hospital, University of Central Florida College of Medicine, Orlando, Florida, United States of America, Phone: +2163152327, e-mail: sreekanth.viswanathan@nemours.org

How to cite this article: Viswanathan S, McNelis KM, Maheshwari A, *et al*. Accretion Rates of Fat and Fat-free Mass in Infants at 30–45 weeks' Postmenstrual Age. Newborn 2022;1(1):7–13.

Source of support: Nil

Conflict of interest: None

expected sex-specific weekly FM and FFM accretion rates for infants across the postmenstrual ages (PMAs) 30–45 weeks calculated based on the Norris body composition charts, and we hypothesized that these accretion rates would be different between male and female infants.

Methods

This study is a secondary analysis of the previously published Norris body composition charts that were created using pooled data from four studies comprising both term and preterm infants ($n \sim 1{,}450$ which included 222 preterm infants) from three high-income countries (Ireland, Italy, and the United States).[14] Institutional review board approval was not needed for this secondary analysis of data that are available in the public domain. The infants included in these studies were born singleton, medically stable, and without significant perinatal morbidity. Using the combined data, sex-specific centiles for FM (kg), FFM (kg), and body fat percentage were estimated using the lambda-mu-sigma (LMS) method. For each sex and measure (e.g., FM), these charts also include postconceptional charts, ranging from 30.3 to 67.3 weeks' PMA, which enable serial postnatal monitoring of body composition in preterm infants.

To determine weight growth velocity, the three common calculation methods used are early one-point method, average two-point method, and exponential two-point method.[16,17] The exponential model was not suitable in this study because of the assumption that growth occurred at a constant fraction of the previous weight over time; existing data show that FM accumulation usually increases rapidly toward term gestation. As defined earlier, the early one-point method was calculated as [(W2 – W1)/(W1 ÷ 1000]/number of days. The average two-point method was computed as (W2 – W1)/[(W1 + W2) ÷2 ÷ 1000]/number of days.[17] First, we calculated the FM and FFM absolute values (g) corresponding to the median (50th percentile) for 30–45 weeks' PMA according to the Norris body composition charts for male and female infants. Then, the weekly gain in FM and FFM in g/week and g/kg/week (by early one-point method and average two-point method) was calculated. For example, for FM, we used the following formulas for early one-point method [FM week 2–FM week 1/weight at week 1] × 1,000 and average two-point method [FM week 2–FM week 1/(weight week 1 + weight week 2)/2] × 1000.

Statistics

Student *t*-test was used for parametric continuous variables (FM and FFM gain and weekly accretion rates) to identify the unadjusted differences between male and female infants. All quantitative data were expressed as the mean ± standard deviation (SD). A $p \leq 0.05$ was considered statistically significant. The statistical software IBM SPSS Statistics version 24 (SPSS, Chicago, Illinois) was used for the statistical analysis of the data.

Results

The reference median rates of FM and FFM accretion across 30–45 weeks' PMA for male and female infants are shown in Tables 1 and 2. Both FM gain and FFM gain (g/week) increase with advancing PMA in both male and female infants (Tables 1 and 2, Figs 1A and D). FM and FFM median weekly accretion rates (g/kg/week) by early one-point method were higher than those by the average two-point method in both male and female infants throughout the gestational age ranges studied (Tables 1 and 2, Figs 1B to F). The FM accretion rates increased gradually with advancing PMA, while FFM accretion rates decreased with advancing PMA, suggesting a relatively higher FM accretion toward later gestational weeks in both male and female infants.

The cumulative average FM and FFM gain and accretion rates in males and female infants are given in Table 3. The cumulative average FM accretion rate (g/kg/week) across the whole 30–45 weeks' PMA for males and females was 16.1 and 15.8 by early one-point and 10.4 and 10.3 each by average two-point method, respectively. Similarly, the cumulative average FFM accretion rate (g/kg/week) across the whole 30–45 weeks' PMA for males and

Table 1: Reference weekly fat mass (FM) and fat-free mass (FFM) median accretion rate (50th percentile) in male infants from 30 to 45 weeks' postmenstrual age (PMA) according to the calculation method based on the Norris body composition chart

		Males				
PMA, weeks	*FM (g) gain/week*	*FM (g) gain/kg/week—early one-point method*	*FM (g) gain/kg/week—average two-point method*	*FFM (g) gain/week*	*FFM (g) gain/kg/week—early one-point method*	*FFM (g) gain/kg/week—average two-point method*
30–31	7.5	5.0	3.3	76.3	51.1	33.4
31–32	14.8	9.4	6.1	143.2	90.8	58.6
32–33	17.2	9.9	6.4	154.6	89.1	57.5
33–34	19.9	10.4	6.7	165.5	86.8	56.0
34–35	22.5	10.8	7.0	173.0	82.7	53.5
35–36	25.4	11.1	7.2	176.4	77.1	49.9
36–37	28.1	11.3	7.3	175.9	70.6	45.8
37–38	32.0	11.9	7.7	170.7	63.4	41.2
38–39	37.8	13.1	8.5	164.3	56.7	37.0
39–40	46.9	15.1	9.9	158.7	51.2	33.4
40–41	60.1	18.2	11.9	156.0	47.2	30.8
41–42	64.2	18.2	11.9	160.3	45.5	29.7
42–43	116.2	31.0	20.2	164.4	43.9	28.6
43–44	113.9	28.3	18.4	166.0	41.2	26.9
44–45	119.8	27.8	18.2	164.0	38.1	24.8
45–46	116.2	25.3	16.6	159.0	34.6	22.6

Table 2: Reference weekly fat mass (FM) and fat-free mass (FFM) median accretion rate (50th percentile) in female infants from 30 to 45 weeks' postmenstrual age (PMA) according to the calculation method based on the Norris body composition chart

	Females					
PMA, weeks	*FM (g) gain/week*	*FM (g) gain/kg/week—early one-point method*	*FM (g) gain/kg/week—average two-point method*	*FFM (g) gain/week*	*FFM (g) gain/kg/week—early one-point method*	*FFM (g) gain/kg/week—average two-point method*
30–31	8.2	6.0	6.0	72.5	52.8	34.5
31–32	17.1	11.7	11.7	142.5	98.0	63.0
32–33	19.7	12.2	12.2	156.0	96.6	62.2
33–34	22.2	12.4	12.4	167.0	93.3	60.1
34–35	24.8	12.5	12.5	175.5	88.7	57.2
35–36	27.5	12.6	12.6	179.0	82.1	53.1
36–37	30.2	12.7	12.7	177.0	74.2	48.1
37–38	33.5	12.9	12.9	170.0	65.6	42.6
38–39	38.3	13.7	13.7	160.0	57.2	37.3
39–40	45.0	15.0	15.0	146.9	49.0	32.0
40–41	54.4	17.1	17.1	137.1	43.0	28.1
41–42	67.6	20.0	20.0	132.5	39.2	25.6
42–43	80.4	22.5	22.5	132.5	37.0	24.2
43–44	91.3	24.1	24.1	133.8	35.3	23.1
44–45	98.1	24.4	24.4	134.0	33.4	21.8
45–46	99.9	23.5	23.5	132.2	31.1	20.4

females was 60.6 and 61.0 by early one-point and 39.4 and 39.6 by average two-point method, respectively (Table 3). The similar trend in FM and FFM accretion rates in male and female infants is shown in Figure 2.

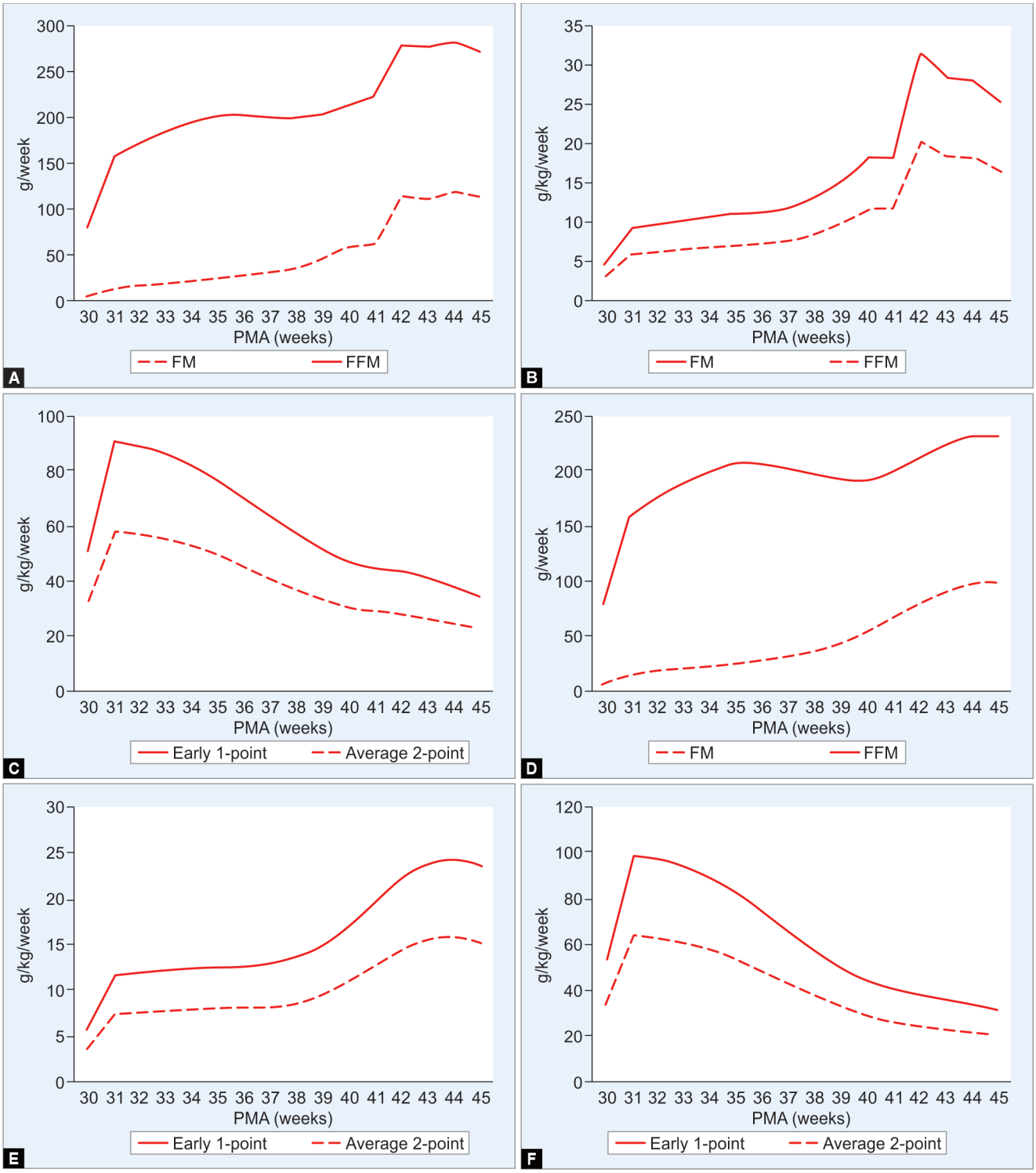
Since there were no significant differences in FM and FFM accretion rates between male and female infants, an average of these was taken to calculate the constant FM accretion rate for the 30–45 weeks' PMA by early one-point (16.0) and average two-point (10.4) and constant FFM accretion rate by early one-point (61.0) and average two-point (39.5), respectively. The constant FM and FFM rates were then superimposed over the weekly reference FM and FFM accretion rates to see the overlap (Fig. 3). The concordance for reference and constant accretion rates for both FM and FFM were limited to a short range of PMA weeks (Fig. 3). For FM, the constant average two-point rate (10.4) was more aligned with the reference before 37-week PMA, and the constant early one-point rate (16.0) after 37 weeks' PMA. On the contrary, for FFM, the constant early one-point rate (61.4) was more aligned with the reference before 37 weeks' PMA, and the constant average two-point rate (39.4) after 37 weeks' PMA.

We did not find the constant accretion rates to simulate the reference accretion rates. Hence, we also computed individual weekly normative FM and FFM accretion rates by averaging the median accretion rates of male and female infants for 30–45 weeks' PMA (Table 4). We also calculated a composite index comprised of the ratio of weekly median FFM/FM accretion rates, which we named as the "body composition accretion ratios" (BCAR) based on early-1 point and average two-point methods (Table 4). Irrespective of the calculation method used, BCAR were similar across the studied PMA. The BCAR ratios trended downwards with advancing gestation suggesting a relatively higher proportional FM accretion, compared to FFM (Fig. 4). Compared to the individual weekly accretion rates which showed a 2–4-fold change during the study period, the BCAR showed a 9-fold change, suggesting that it is a more sensitive metric for weekly body composition changes.

Discussion

There is increasing emphasis on improving the quality of postnatal growth in preterm infants as cumulative data suggest that the trajectory of postnatal growth in terms of FM and FFM accretion rate and proportion can influence short-term and long-term clinical outcomes.[5,6,13,18–20] Noninvasive body composition assessment using ADP is a validated technique in infants, and many NICUs are using this tool for quality improvement initiatives as well as an objective way of assessing the impact of nutritional interventions in routine NICU clinical practice.[12] Our data reported here on the expected weekly changes in FM and FFM accretion rates and BCAR across the gestational age range of 30–45 weeks' PMA can be a clinically meaningful, readily available practical guide to monitor and compare the quality of postnatal growth and nutrition in NICU infants.

Body composition data during the early postnatal weeks in preterm infants suggest that absolute body weight, FM, and FFM increase with advancing gestation.[14] However, our data show that FM and FFM accretion rates follow a contrasting trend. FM accretion rates increases, while FFM accretion rate decreases with advancing postnatal age because of the relatively higher FM deposition resulting in a higher percentage of body fat. The progressively higher fat deposition with advancing gestation in the first 3–4 months of life is secondary to the physiological adaptation process to extrauterine life from the age-related changes in total body water and its compartments—as the total body water decreases with gradual contraction of extracellular

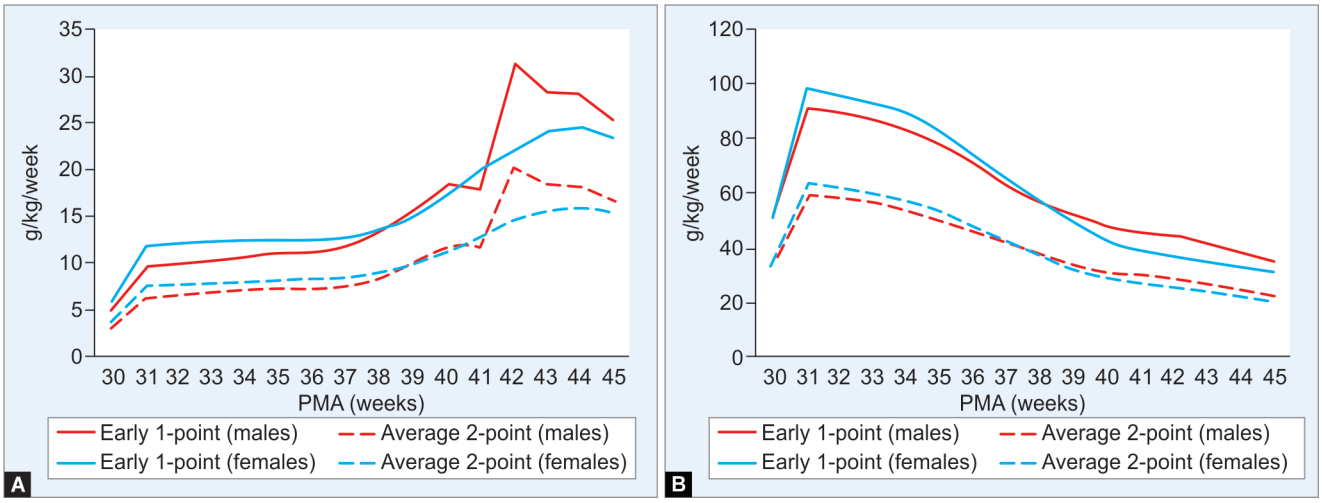


Figs 1A to F: Postnatal fat accretion during 30-45 weeks' postmenstrual age, compared by gender. (Panels A, D), Total FM and FFM gain/week (data from males in A, females in D); (Panels B, E), FM median accretion rate per unit weight (g/kg/week; data from males in B, females in E); and (Panels C, F), FFM median accretion rate per unit weight (g/kg/week; data from males in C, females in F). The figures show the temporal evolution of these indices in the Norris body composition charts. FM, fat mass; FFM, fat-free mass; PMA, postmenstrual age

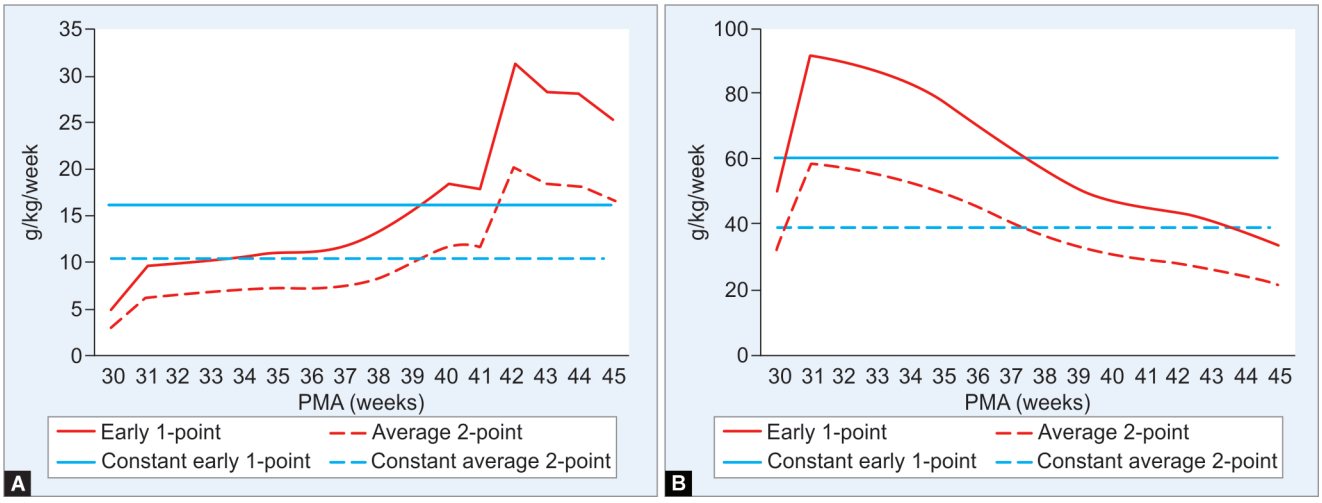
fluid, the body fat proportionately increases.[21] Recent data suggest that energy intake, appetite, and energy expenditure in young infants are strongly associated with metabolically active, energy-using tissues in FFM, but not FM.[22] However, the status quo, as it pertains to nutritional practices in the NICU, is guided by weight gain trends and not based on the quality of growth (i.e., changes in body composition). Our attempts to meet the *one-size-fits-all* conventional weight-based feeding volume targets (150–160 mL/kg/day) do not account for the possible variations in underlying body composition. It is possible that feeding and milk intake volume

Table 3: The cumulative average weight, fat mass (FM), and fat-free mass (FFM) gain and weekly accretion rates in males and female infants across 30–45 weeks' postmenstrual age (PMA) according to the calculation method based on the Norris body composition chart

Characteristics	*Males*	*Females*	*p value*
Weight (g)	3070.7 ± 1036.5	2904.4 ± 960.8	0.64
FM (g)	360.7 ± 262.5	379.3 ± 234.9	0.83
FFM (g)	2710.0 ± 786.2	2525.0 ± 735.9	0.50
FM gain (g)/week	52.7 ± 41.0	47.4 ± 30.7	0.68
FM (g) gain/kg/week—early one-point method	16.1 ± 7.9	15.8 ± 5.5	0.93
FM (g) gain/kg/week—average two-point method	10.4 ± 5.2	10.3 ± 3.6	0.93
FFM gain (g)/week	158.0 ± 23.4	146.8 ± 26.5	0.21
FFM (g) gain/kg/week—early one-point method	60.6 ± 19.5	61.0 ± 24.5	0.96
FFM (g) gain/kg/week—average two-point method	39.4 ± 12.5	39.6 ± 15.6	0.97



Figs 2A and B: Weekly FM (A) and FFM (B) Median accretion rate (g/kg/week) across the 30–45 weeks' PMA calculated based on early one-point and average two-point method compared between male and female infants. FM, fat mass; FFM, fat-free mass; PMA, postmenstrual age



Figs 3A and B: Weekly FM (A) and FFM (B) Median accretion rate (g/kg/week) across the 30–45 weeks' PMA calculated based on early one-point and average two-point method compared to the constant rate. FM, fat mass; FFM, fat-free mass; PMA, postmenstrual age

Table 4: Normative weekly FM and FFM median accretion rate (50th percentile) and FFM/FM weekly accretion rate ratio (BCAR) in infants from 30 to 45 weeks' PMA according to the calculation method based on the Norris body composition chart

PMA, weeks	*FM (g) gain/kg/ week—early one-point method*	*FM (g) gain/kg/ week—average two-point method*	*FFM (g) gain/kg/ week—early one-point method*	*FFM (g) gain/kg/ week—average two-point method*	*FFM/FM accretion rate ratio—early one-point method (BCAR)*	*FFM/FM accretion rate ratio—average two-point method (BCAR)*
30–31	5.5	3.6	51.9	34.0	9.4	9.4
31–32	10.6	6.8	94.4	60.8	8.9	8.9
32–33	11.0	7.1	92.9	59.8	8.4	8.4
33–34	11.4	7.4	90.0	58.1	7.9	7.9
34–35	11.6	7.5	85.7	55.3	7.4	7.4
35–36	11.9	7.7	79.6	51.5	6.7	6.7
36–37	12.0	7.8	72.4	47.0	6.0	6.0
37–38	12.4	8.1	64.5	41.9	5.2	5.2
38–39	13.4	8.7	57.0	37.1	4.3	4.3
39–40	15.1	9.8	50.1	32.7	3.3	3.3
40–41	17.6	11.5	45.1	29.5	2.6	2.6
41–42	19.1	12.5	42.4	27.7	2.2	2.2
42–43	26.7	17.4	40.5	26.4	1.5	1.5
43–44	26.2	17.1	38.3	25.0	1.5	1.5
44–45	26.1	17.1	35.7	23.3	1.4	1.4
45–46	24.4	16.0	32.9	21.5	1.3	1.3

FM, fat mass; FFM, fat-free mass; BCAR, body composition accretion ratio; PMA, postmenstrual age

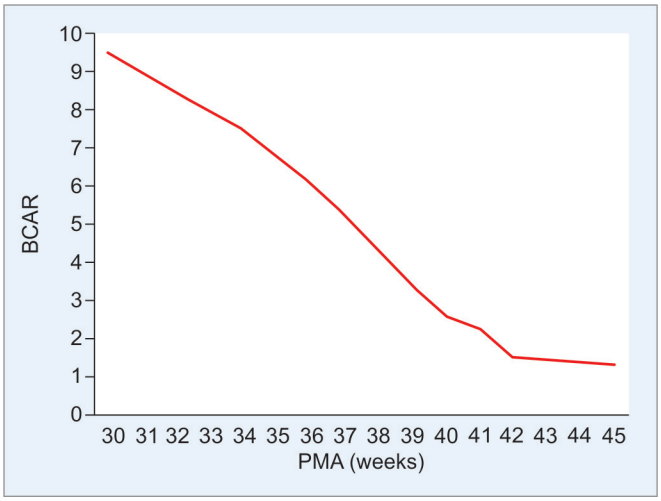


Fig. 4: Weekly FFM/FM accretion rate ratio (body composition accretion ratio, BCAR) across the 30–45 weeks' PMA

in convalescent NICU infants may be determined by FFM volume and proportion. Further studies are needed to test the hypothesis that whether optimizing milk intake volume and nutrition based on serial body composition will improve the growth and clinical outcomes in NICU infants.

Because of FM and FFM accretion rates are similar in both male and female infants across the gestational weeks studied, it is reasonable to use a common FM and FFM accretion rates for both sexes that is calculated by averaging the median accretion rates for male and female infants for each individual week (Table 4). The sex difference in body composition is appreciated even in early life with probable contribution from sex steroids (testosterone and estrogen).[23] Longitudinal body composition studies from birth to 5 months have shown that male infants accumulate 17 g/week additional FFM, compared to female infants.[23] For practical purposes, a common FM and FFM accretion rates for male and females during the 30–45 weeks' PMA is reasonable, but it is possible that the difference in accretion rates may become significant with further period of growth, such that sex-specific accretion rates may become necessary.

Compared to the constant weight velocity of 15–20 g/kg/day, the constant FM and FFM accretion rates were covering only a limited PMA ranges, suggesting that FM growth and FFM growth are not constant during this period of growth. Therefore, for more accurate results, it is better to use the individual weekly FM and FFM accretion rates as given in Table 4. We believe that the early one-point accretion rate can be used to predict the expected gain in FM and FFM (g) over the next week when at least one ADP measurement is available, while the average two-point method can be used to compare the FM and FFM accretion rate (g/kg/week) when ADP data are available at two time points. Similar to the growth velocity calculation for weight, we apply caution while using early one-point method to summarize the FM and FFM accretion rate of more than one week duration because of the use of smaller denominator (using early body weight), thereby producing a larger estimate than average two-point method.[16] When trying to calculate the FM and FFM accretion rate over a longer period (e.g., 2–4 weeks apart), we suggest using the constant average two-point method accretion rate by averaging the weekly accretion rate of each individual week included in the defined time period.

Most conventional indices in FM and FFM showed twofold–fourfold changes over the 30–45 weeks' PMA study period. Because the accretion rates of FM and FFM changed in different trajectories during the studied period of postmenstrual age, we sought to determine whether a composite index of the ratio of FFM/FM accretion rates could be useful. We named this index the body composition accretion ratio (BCAR; Table 4). Interestingly, this index showed a

ninefold change during the study period, indicating that the BCAR could be a much more sensitive measure of the accretion of lean body mass than the direct measurements of FFM and FM. Clearly, these observations suggest a need for further investigation in this area. Because the accretion of FFM is related to better clinical outcomes, a higher BCAR may be preferable and could be a useful measure of the accretion efficiency of nutrition being currently provided.

We acknowledge that our study has both strengths and limitations. The ADP technique is sensitive enough to detect a 30–45 g change in FFM in measurements taken one week apart and was therefore appropriate for these studies.[20] One limitation of this, and all studies focused on the utility of nutritional interventions, is in the limitations of the normative reference charts. The Norris reference charts included only 222 preterm infants, which is a considerably smaller cohort than the ones used to construct the anthropometric reference growth charts commonly used by clinicians.[14,24,25] This may limit the applicability at the extremes of growth, below the 10th percentile and greater than the 90th percentile. Additionally, the limited cohort of preterm infants in the Norris reference who were recruited in the US cohort included may not represent a more global population. There may be other population-based factors that alter the growth potential including genetic and ethnic differences. Similar to the Fenton growth charts, the Norris growth charts are a reference intrauterine growth chart instead of a "prescriptive" postnatal curve. Still, despite all shortcomings, these findings suggest that the ADP technique may be a useful bedside tool for clinicians to interpret body composition trajectory in NICU infants and merit further evaluation of these findings in a larger, multicentric cohort.[26]

Abbreviations

ADP: Air displacement plethysmography
FM: Fat mass
FFM: Fat-free mass
NICU: Neonatal intensive care unit
PMA: Postmenstrual age

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ORIGINAL RESEARCH

Intestinal Resection is More Likely to be Effective in Necrotizing Enterocolitis Extending to Colon than in Disease Limited to the Small Intestine

Parvesh M Garg[1], Jaslyn L Paschal[2], Katherine Lett[3], Charles Middleton[4], Neha Varshney[5], Akhil Maheshwari[6]

Abstract

Background: The prognosis in surgical necrotizing enterocolitis (NEC) has focused on the total length of the resected bowel; the relative impact of small intestinal vs colonic resection is not well studied.

Objective: We hypothesized that intestinal resections may reduce mortality and length of hospital stay (LOS) more likely in infants who have NEC extending into the colon than in those with disease limited to the small intestine. We also investigated the relationship between gestational maturation and NEC-related mortality.

Methods: A retrospective study of 153 patients compared demographic, clinical, and histopathological information in infants who had NEC limited to the small intestine vs disease with colonic involvement.

Results: Our 153 infants had a mean (±standard deviation) gestational age of 27.4 ± 3.4 weeks and a birth weight of 987 ± 505 g. NEC was limited to the small intestine in 103 (67.3%) infants and extended into the colon in 50 (32.7%). Infants with small intestinal NEC needed shorter bowel resections of 28 ± 31.9 cm than 42.2 ± 40.7 cm in those with colonic involvement ($p = 0.02$). The LOS was longer in NEC limited to the small intestine than in disease with colonic lesions (96 ± 88.1 vs 69.7 ± 19.1 days; $p < 0.05$). In small intestinal NEC, mortality decreased to <50% beyond a gestational age (GA) >37 weeks. In contrast, infants with NEC that involved the colon had mortality <50% mortality beyond 27.3 weeks' GA ($p = 0.008$).

Conclusions: Bowel resections may be more likely associated with shorter LOS in surgical NEC that involves both the small bowel and colon, even when longer segments of the gastrointestinal tract are removed, than in disease limited to the small intestine.

Keywords: Necrotizing enterocolitis, Neonatology, Newborn.

Newborn (2022): 10.5005/jp-journals-11002-0024

Introduction

Necrotizing enterocolitis (NEC) is an inflammatory bowel necrosis seen in premature and critically ill neonates,[1] particularly in those born at gestational ages of 22–28 weeks with birth weights less than 1500 g.[1,2] The incidence of NEC has shown some encouraging reduction in recent years.[3,4] However, in affected infants, it remains a life-threatening illness requiring close monitoring and intensive care.[5] About half of all infants with confirmed NEC need surgical intervention. Regardless of gestational age and birth weight, infants with advanced disease typically have extended hospital stays and at least 30% mortality.[6]

The pathogenesis of NEC is unclear. Early diagnosis is difficult and rests on suggestive clinical and radiological signs in at-risk infants. At onset, the clinical features are nonspecific and may include abdominal distension, gastric stasis or emesis, and gastrointestinal bleeding.[7] The pathognomonic sign, *pneumatosis intestinalis*, is the radiological (or intraoperative) observation of intramural cysts that are known to contain gaseous products of bacterial fermentation, and is seen in about half of all patients.[4,7] NEC is treated with bowel rest, antibiotics, and in advanced disease, with surgical resection of the diseased bowel and anastomosis or exteriorization of the healthier parts of the intestine.[5,8–10] The typical histopathological findings of NEC are coagulative necrosis, inflammation, interstitial hemorrhages, pneumatosis, and in some foci, reparative changes.[2–6] The postoperative period may be marked by feeding difficulties, surgical wound complications, infections, cholestasis, and in some infants, short bowel syndrome.[3,8,9,11–14]

In infants with surgical NEC, increasing length of the diseased/resected intestine is usually viewed with a pessimistic outlook, as associated with a lower likelihood of postoperative rehabilitation

[1–3]Department of Pediatrics/Neonatology, University of Mississippi Medical Center, Jackson, Mississippi, United States of America

[4,5]Department of Pathology, University of Mississippi Medical Center, Jackson, Mississippi, United States of America

[6]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Parvesh M Garg, Department of Pediatrics/Neonatology, University of Mississippi Medical Center, Jackson, Mississippi, United States of America, e-mail: gargparvesh@hotmail.com

How to cite this article: Garg PM, Paschal JL, Lett K, *et al.* Intestinal Resection is More Likely to be Effective in Necrotizing Enterocolitis Extending to Colon than in Disease Limited to the Small Intestine. Newborn 2022;1(1):14–26.

Source of support: Dr Parvesh M Garg is partially supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number U54GM115428. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest: None

and survival.[10,15,16] However, most studies of prognosis in surgical NEC have focused on the total length of the resected bowel; the relative impact of small intestinal vs colonic resection is not well documented. We hypothesized that surgical resection for NEC that extends into the colon may be more effective in reducing mortality and the length of hospital stay (LOS) than in disease limited to the small intestine. We posited that bowel resections extending into the colon, even if longer, might not be as harmful because of potentially greater reductions in the total load and transmural translocation of luminal bacteria, and consequently, in intestinal and systemic inflammation.

We performed this study using a retrospective design but made an extensive effort to record all possible clinical information. A major difficulty in studying surgical NEC is to find accurate data from an optimum number of patients. We are fortunate that the incidence of NEC is relatively limited at 3–5% of very-low-birth-weight (VLBW) infants, but these low numbers also bring challenges in finding a statistically adequate cohort for clinical studies. Among infants with confirmed NEC, 30% need surgery, and 30% of these patients may have postoperative mortality. Therefore, to detect a 50% difference in mortality due to surgical NEC involving the colon vs disease limited to the small intestine, we needed at least 150 patients. Even though University of Mississippi Medical Center (UMMC) is a major regional facility for surgical treatment of NEC, prospective enrollment of this large number of eligible patients was difficult within a reasonable period. A multicenter format was an alternative, but there were major differences in medical records and in the thresholds for surgical intervention at other regional centers. Again, considering the relatively low incidence of surgical NEC, a retrospective study format seemed reasonable provided we could carefully record information from all possible medical, surgical, and nursing records. We have recently described 90 infants who were treated at UMMC for surgical NEC during the years 2000–2015,[5,17] and an extension of the study period to 2018 provided an adequate number of patients (as described in methods). As may be evident in the tables, we reviewed clinical records and histopathology sections from all infants, and gross pathology specimens from most patients.

Methods

This retrospective study was conducted at the University of Mississippi Medical Center (UMMC) at Jackson, Mississippi, which is a regional referral center for surgical NEC, after approval by the Institutional Review Board. A detailed review of medical records identified 193 patients who had undergone exploratory laparotomy and other surgical procedures for advanced NEC during the period between January 2000 and December 2018. After excluding 15 infants who were missing clinical data and 25 for confounding congenital anomalies involving the gastrointestinal tract or multiple systems, we identified 153 eligible infants (Flowchart 1).

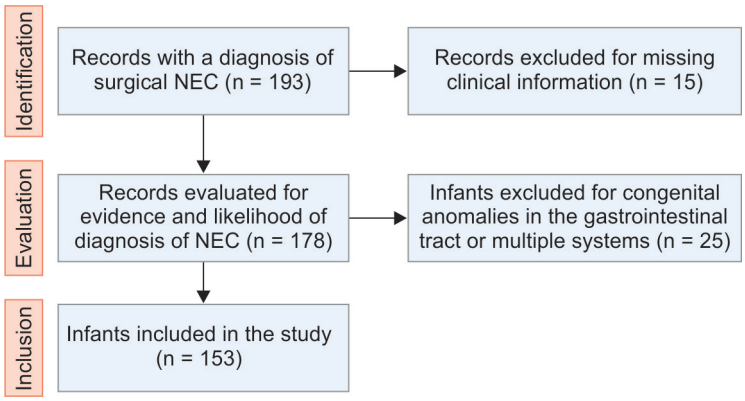
Clinical Information

We recorded prenatal factors such as pregnancy-induced hypertension (PIH), chorioamnionitis, and antenatal steroids. Demographic data included gestational age, birth weight, small-for-gestational age (SGA) status, ethnicity, gender, and outborn status. Clinical information included Apgar scores ≤6 at 5 minutes, age at initiation of feedings, culture-proven sepsis prior to NEC, central line use, assisted ventilation, patent ductus arteriosus (PDA), and its medical/surgical treatment. The age at onset of NEC, its clinical presentation (abdominal distension, bloody stools, and emesis), and length of the resected intestine were noted. Our two primary clinical outcomes of NEC were mortality and the LOS. Other postoperative data included hemodynamic instability, duration of paralytic ileus, need for pressor support for ≥24 hours after surgery, days of antibiotic treatment, days on parenteral nutrition, and the number of days to reach full enteral feedings (120 mL/kg/day). We also recorded late-onset sepsis, surgical wound dehiscence or infection, strictures, and short bowel syndrome (SBS; need for parenteral nutrition ≥3 months following bowel resection).[18]

Pathology

Two blinded pathologists evaluated hematoxylin and eosin (H&E)-stained intestinal sections for histopathological evidence of NEC, including coagulative necrosis, inflammation, hemorrhages, and reparative changes. Coagulative necrosis was defined by the loss of nuclear staining and diminished eosinophilic staining of the cytoplasm, but with relatively preserved "ghost-like" crypt-villus histoarchitecture. Inflammation was marked by leukocyte infiltration; these white blood cells were also enumerated per high-power field (HPF). Hemorrhages were noted in various intestinal regions and layers. The severity of NEC was assessed by the depth to which these histopathological changes were seen; grade I was limited to the mucosa, grade II extended to the submucosa, grade III to the *muscularis*, and grade IV was transmural. Reparative changes included neovascularization, epithelial regeneration, and increase in fibroblasts and/or myofibroblasts.

Flowchart 1: Enrollment of patients in the study



Statistical Information

Descriptive, categorical data were summarized as frequencies (absolute and relative) and tested for differences using Chi-square tests. Continuous data, when symmetric, were recorded as averages, with both standard deviations (SD) and standard error of the mean (SEM) as indices of variability. In studies of premature infants, concomitant presentation of SDs and SEMs is useful;[5] SDs are familiar, accurate descriptors of variability, whereas SEMs can convey the precision with which inferences drawn from the relatively limited, disease-specific cohorts of preterm infants can be extrapolated to the larger universe of these infants.[19] If data were skewed, we used medians (with ranges) for presentation. *T*-values were used to compute the size of the difference relative to the variation in the sample data. Data were evaluated for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. Two or multiple groups with continuous, parametric data were compared using a Student's *t* or analysis of variance, respectively. For nonparametric data, we used the Mann-Whitney *U* or Kruskal-Wallis *H* tests. Kaplan-Meier survival curves were plotted with 95% confidence intervals against gestational age, birth weights, postnatal age, and lengths of the small intestine and the colon. These curves were compared using the log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.[20] Statistical analysis was done using the software programs STATA 15 (Stata Software, College Station, Texas) and GraphPad Prism (San Diego, California). Statistical significance was accepted at *p*-values below 0.05.

Results

Clinical Information

We reviewed the medical records of 153 infants with surgical NEC (Flowchart 1) for their demographic data, clinical course, and outcomes (Table 1). In this group, 90 infants have been previously described.[17] The 153 infants included in this study had an average gestational age (mean ± SD; ± SEM) of 27.4 ± 3.4 weeks (±0.3 weeks SEM) and a birth weight of 987 ± 505 g (±40.8 g SEM). Eighty-seven (56.8%) were outborn. There were 96 (62.7%) males and 57 (37.3%) females. One hundred and nineteen (77.8%) infants were African American, 28 (18.3%) were Caucasian, 3 (2%) were Latino, and 3 (2%) were of mixed ancestry.

NEC occurred on 22.4 ± 20.1 days (±1.6 days SEM); 139 (90.2%) presented with abdominal distension, 8 (5.2%) with feeding intolerance, and 7 (4.5%) had bloody stools. All enrolled infants had some radiological evidence of NEC such as *pneumatosis*, mucosal thickening, bowel loop dilatation, ascites, and/or pneumoperitoneum. These patients underwent an exploratory laparotomy if they had evidence of peritonitis with worsening abdominal distention, hypotension, thrombocytopenia, anemia, or metabolic acidosis. The diagnosis of NEC was confirmed by intraoperative inspection and pathological examination of the resected bowel.

After surgery, 51 (33.3%) infants needed hemodynamic support with intravenous fluid boluses and pressor support for >24 hours. Postoperative ileus lasted for 17.3 ± 14.5 days (±6.6 days SEM). Enteral feedings were initiated on 18 ± 14.5 days SD (±1.2 days SEM), and full feeding volumes were achieved on 72.4 ± 44.2 days (±3.6 days SEM). Parenteral nutrition was given for 91.1 ± 53.8 (±1.2 days SEM). Forty-six (30.1%) patients died at a postnatal age of 84.2 ± 84.9 days (±6.8 days SEM). Survivors were discharged after

Table 1: Demographic and clinical summary

		n = 153
Prenatal information		
1	Pregnancy-induced hypertension, *n* (%)	37 (24.1%)
2	Chorioamnionitis, *n* (%)	12 (7.8%)
3	Antenatal steroids, *n* (%)	90 (58.8%)
Infant demographics		
5	Gestational age (weeks; mean ± SD)	27.4 ± 3.4
6	Birth weight (g; mean ± SD)	987 ± 505
7	Male gender, *n* (%)	96 (62.7%)
8	Ethnicity	
	a Caucasian	28 (18.3%)
	b Latino	3 (2%)
	c Mixed	3 (2%)
	d African American	119 (77.8%)
9	Small for gestational age, *n* (%)	40 (26.1%)
10	Mode of delivery	
11	C-section	65 (42.4%)
12	Vaginal	88 (57.5%)
13	Outborn, *n* (%)	87 (56.8%)
Infant medical information prior to NEC		
14	Apgar score <6 at 5 minutes	39 (25.4%)
15	Patent ductus arteriosus, *n* (%)	88 (57.5%)
16	Patent ductus arteriosus, indomethacin treated, *n* (%)	25 (16.3%)
17	Patent ductus arteriosus, surgically ligated, *n* (%)	5 (3.3%)
NEC disease features		
18	Age at NEC onset (days; mean ± SD)	22.1 ± 20.6
19	Corrected gestational age at NEC onset (weeks; mean ± SD)	30.4 ± 4.2
20	Clinical presentation	
	a Abdominal distension, *n* (%)	139 (90%)
	b Bloody stools, *n* (%)	7 (4.5%)
	c Feeding Intolerance, *n* (%)	8 (5.2%)
21	Radiological findings	
	a Pneumatosis, *n* (%)	57 (37.2%)
	b Pneumoperitoneum, *n* (%)	43 (28.1%)
	c Portal venous gas, *n* (%)	10 (6.5%)
22	Length of bowel resected (cm; mean ± SD)	26.6 ± 28.7
Postoperative systemic course		
23	Assisted ventilation (intubated), *n* (%)	138 (90.1%)
24	Pressor support >24 hours, *n* (%)	51 (33.3%)
25	Blood culture positive sepsis, *n* (%)	41 (26.7%)
Postoperative nutrition		
26	Postoperative ileus [days; *n* (%)]	17.3 ± 14.4
27	Surgical wound infection, *n* (%)	21 (13.7%)
28	Surgical wound dehiscence	13 (8.4%)
29	Postoperative day at starting enteral feedings (days; mean ± SD)	18 ± 14.5
30	Feeding at start	
31	Mother's own/donor milk, *n* (%)	41 (26.7%)
32	Infant formula, *n* (%)	63 (41.1%)

33	Mixed	21 (13.1%)
34	Day of attainment of full enteral feedings (120 mL/kg; mean ± SD)	73.5 ± 44.1
35	Duration of parenteral nutrition (days; mean ± SD)	92.8 ± 53.5
36	Intestinal adhesions, *n* (%)	28 (18.3%)
37	Intestinal strictures, *n* (%)	15 (9.8%)
38	Short bowel syndrome, *n* (%)	43 (28.8%)
Discharge		
39	Length of stay (days; mean ± SD)	133.9 ± 81.9
40	All deaths, *n* (%)	46 (30%)

154.8 ± 71.1 days (±6.9 days SEM). The average hospital stay was 133.6 ± 81.9 days (±6.6 days SEM).

Pathological Findings

Intestinal specimens from all 153 patients showed histopathological signs of NEC (Table 2). The average grading for necrosis was 2.3 ± 1.4 (±0.2 SEM), and it correlated with inflammation ($r = 0.38$, $p < 0.001$) and hemorrhages ($r = 0.37$, $p < 0.001$). The length of the resected intestine was 14.9 ± 2.4 cm (±0.3 cm SEM). Inflammation was graded at 3 ± 1 (±0.3 SEM), with 88.3 ± 12 WBCs/hpf (±3.8 WBCs/hpf SEM). Hemorrhages were seen in all infants, with a grade of 3.3 ± 0.9 (±0.2 SEM). Reparative changes were prominent in histopathological sections from 25 (16.3%) patients and were graded 0.6 ± 1 (±0.3 SEM).

NEC lesions were limited to the small intestine in 103 (67.3%) patients and extended into the colon in 50 (32.7%). The two groups had similar gestational ages of 27.2 ± 4 weeks (±0.3 weeks SEM) and 26.9 ± 3.5 weeks (±0.5 weeks SEM), and birth weights of 935 ± 471 g (±46.4 g SEM) and 961 ± 559 g (±77.9 g SEM), respectively. Infants who had NEC in only their small intestine needed a bowel resection of 28 ± 31.9 cm (±2.1 cm SEM), which was significantly shorter than 42.2 ± 40.7 cm (±2.2 cm SEM) in those with colonic involvement ($p = 0.02$). The length of the resected small intestines in the two groups was not different and measured 20 ± 23 cm (±2.3 cm SEM) and 18.7 ± 26.7 cm (±3.8 cm SEM). The second group had colonic involvement of 16.4 ± 11.6 cm (±1.6 cm SEM). Ninety-seven (94.2%) infants with small intestinal NEC had an intact ileocecal valve, whereas only 10 (20%) in the second group had one ($p < 0.001$). Finally, there were 46 (30%) deaths; 32/103 (31.1%) infants who had NEC lesions only in the small intestine died, whereas 14/50 (28%) had lesions extending into the colon. This trend was not statistically significant. Table 3 shows the demographic data, clinical course, and pathological findings in the resected intestine in these two subgroups.

Clinical Outcomes of NEC that was Limited to the Small Intestine or Extended into the Colon

We performed linear regression to identify factors that determined the localization of NEC to the small intestine or its extension into the colon. Birth weight, gender, and indomethacin use were identified to be significant (Supplemental Table 1, section a). However, r^2, the goodness-of-fit measure, was modest at 0.11.

We next investigated for the determinants of mortality in NEC that was limited to the small intestine or extended to the colon (Supplemental Table 1, sections b and c). In small intestinal NEC, gestational age and the length of resected bowel were significant determinants of mortality in the entire groups. In NEC extending into the colon, gestational age remained significant, but a history of PDA and the age at onset of NEC were also important. Finally, we looked for predictors of LOS (Supplemental Table 1, sections d and e). The important clinical determinants in small intestinal disease were gestational age, death, age of onset of NEC, and the length of bowel resection. In disease extending into the colon, gestational age and death were significant predictors of LOS. Considering the importance of gestational age as a key determinant in most comparisons, we focused further analysis on the relationship between gestational maturation and outcomes of NEC.

Gestational Age and NEC Region Involvement

We next plotted Kaplan-Meier curves for mortality against gestational age (Flowchart 1) both for NEC that was limited to the small intestine (blue lines) or extended into the colon (red lines). The 95% confidence intervals (CIs) are shown with dotted, similarly colored lines. Summated data from the entire cohort are depicted with a magenta line and a similarly shaded 95% CIs. In infants with small intestinal NEC, mortality decreased to less than 50% after 37 weeks' gestation. In contrast, NEC extending into the colon showed <50% mortality beyond a gestational age of 27.3 weeks (Fig. 1; $p = 0.008$). The number of deaths prior to and beyond the gestational age thresholds in both groups showed statistically significant differences (Table 4). In all infants studied together, mortality dropped to levels less than 50% beyond 31.3 weeks' gestation (Supplemental Table 2). The clinical information of infants born prior to and beyond these gestational age thresholds is shown in Table 4.

To understand the impact of intestinal maturation on NEC-related mortality, we plotted Kaplan-Meier curves for mortality against the post-menstrual age (PMA). In NEC limited to the small intestine, mortality decreased to less than 50% beyond 38.2 weeks PMA ($p = 0.015$). In contrast, mortality in NEC with colonic involvement dropped below 50% at 33 weeks' PMA. In the entire cohort, mortality decreased to less than 50% at a PMA of 37.3 weeks (Fig. 1; Supplemental Table 3).

In other analyses, we did not find a difference in NEC-related mortality against the birth weights in these two groups ($p = 0.54$; Supplemental Figure 1). The postnatal age at onset of NEC was also not a predictor of its being restricted to the small intestine or extension into the colon ($p = 0.33$; Supplemental Figure 2). The length of the resected small intestine was similar in the two groups ($p = 0.6$). However, infants with NEC involving both the small intestine and the colon had a median survival at a resection of 58 cm, which was significantly longer than those who had to undergo removal of small intestinal disease (median 30 cm, $p = 0.02$; Supplemental Figure 3). Supplemental Figure 4 shows the relationship between survival and the length of colonic resection.

We also developed prediction models for LOS; Supplemental Table 1, sections d and e). In NEC limited to the small intestine ($r^2 = 0.58$), LOS was associated with gestational age, age at onset of NEC, death, and the length of resected bowel. The intercept was significant ($|t| = 3.7$, $p < 0.001$), indicating that the some determinants of small intestinal NEC remain to be discovered. The prediction models for LOS were less robust in infants who had colonic lesions ($r^2 = 0.35$); gestational age and death remained significant. The intercept remained important ($|t| = 4.1$, $p < 0.001$), again emphasizing the importance of hitherto undetermined elements.

Table 2: Pathological changes in resected intestinal tissue

Gross pathological changes in available tissue samples										
		Specimens available from patients with NEC lesions only in the small intestine (n = 40)			*Specimens available from patients with NEC extending into the colon (n = 20)*			*Combined (n = 60)*		
		Total (40)	*Alive (32)*	*Dead (8)*	*Total (20)*	*Alive (17)*	*Dead (3)*	*Total (60)*	*Alive (49)*	*Dead (11)*
Acute changes	Necrosis, *n* (%)	10 (25%)	6 (18.8%)	4 (50%)	7 (35%)	6 (35.3%)	1 (33.3%)	17 (28.3%)	12 (24.5%)	5 (45.5%)
	Dusky, *n* (%)	10 (25%)	8 (25%)	2 (25%)	9 (45%)	7 (41.2%)	2 (66.7%)	19 (31.6%)	15 (30.6%)	4 (36.4%)
	Erythema, *n* (%)	2 (5%)	2 (6.3%)	0	1 (5%)	0	1 (33.3%)	3 (5%)	2 (4.1%)	1 (9.1%)
	Hemorrhage, *n* (%)	9 (22.5%)	6 (18.8%)	3 (37.5%)	6 (30%)	5 (29.4%)	1 (33.3%)	15 (25%)	11 (22.4%)	4 (36.4%)
	Defect/perforation, *n* (%)	20 (50%)	14 (43.8%)	6 (75%)	9 (45%)	8 (47.1%)	1 (33.3%)	29 (48.3%)	22 (44.9%)	7 (63.6%)
Subacute changes	Thinning, *n* (%)	6 (15%)	5 (15.6%)	1 (12.5%)	5 (25%)	4 (23.5%)	1 (33.3%)	11 (18.3%)	9 (18.4%)	2 (18.2%)
	Stricture, *n* (%)	2 (5%)	1 (3.1%)	1 (12.5%)	2 (10%)	2 (11.8%)	0	4 (6.6%)	3 (6.1%)	1 (9.1%)
	Friable, *n* (%)	8 (20%)	7 (21.9%)	1 (12.5%)	7 (35%)	5 (29.4%)	2 (66.7%)	15 (25%)	12 (24.5%)	3 (27.3%)
	Adhesions, *n* (%)	20 (50%)	14 (43.8%)	6 (75%)	9 (45%)	8 (47.1%)	1 (33.3%)	29 (48.3%)	22 (44.9%)	7 (63.6%)
Histopathological changes										
		NEC lesions only in the small intestine (n = 103)			*NEC extending into the colon (n = 50)*			*Combined (n = 153)*		
		Total (103)	*Alive (75; 72.8%)*	*Dead (28; 27.2%)*	*Total (50)*	*Alive (32; 64%)*	*Dead (18; 36%)*	*Total (153)*	*Alive (107; 69.9%)*	*Dead (46; 30.1%)*
Histopathological changes	Necrosis (mean ± SD)	2.3 ± 1.4	2.4 ± 1.5	2 ± 1.1	2.2 ± 1.4	2.4 ± 1.4	2 ± 1.4	2.3 ± 1.4	2.4 ± 1.4	1.9 ± 1.2
	Inflammation (mean ± SD)	3.08 ± 1.05	3.1 ± 1.1	2.9 ± 0.84	2.9 ± 0.99	3.1 ± 0.9	2.5 ± 1.0	3.0 ± 1.0	3.15 ± 1.0	2.7 ± 0.91
	Hemorrhage (mean ± SD)	3.3 ± 0.97	3.3 ± 1.0	3.4 ± 0.88	3.3 ± 1.0	3.5 ± 0.9	3.0 ± 1.1	3.3 ± ± 0.9	3.4 ± 0.98	3.2 ± 1
	Reparative changes (mean ± SD)	0.72 ± 1.4	0.67 ± 1.1	0.8 ± 1.2	0.3 ± 0.6*	0.38 ± 0.76	0.30 ± 0.75	0.6 ± 1.0	0.6 ± 1.03	0.61 ± 1.1

*$p < 0.05$

Table 3: Demographic data and clinical outcomes in infants with small intestinal NEC and those with colonic disease and variable small intestinal involvement

		NEC lesions only in the small intestine			*NEC extending into the colon*			*Combined*		
		n = 103	*75 (72.8%)*	*28 (27.2%)*	*n = 50*	*32 (64%)*	*18 (36%)*	*n = 153*	*107 (69.9%)*	*46 (30%)*
		Total	*Alive*	*Dead*	*Total*	*Alive*	*Dead*	*Total*	*Alive*	*Dead*
Prenatal information										
1	Pregnancy-induced hypertension, *n* (%)	25 (24.2%)	18 (24%)	7 (25%)	12 (24%)	7 (21.8%)	5 (27.7%)	37 (24.1%)	25 (23.3%)	12 (26%)
2	Chorioamnionitis, *n* (%)	9 (8.7%)	7 (9.3%)	2 (7.1%)	3 (6%)	2 (6.2%)	1 (5.5%)	12 (7.8%)	9 (8.4%)	3 (6.5%)
3	Antenatal steroids, *n* (%)	60 (58.2%)	48 (64%)	12 (42.8%)	30 (60%)	19 (59.3%)	11 (61.1%)	90 (58.8%)	67 (62.6%)	23 (50%)
Infant demographics										
5	Gestational age (weeks; mean ± SD)	27.2 ± 4	27.2 ± 3.2	25.8 ± 3	26.9 ± 3.5	27.9 ± 3.8	27.1 ± 3.0	27.4 ± 3.4	27.7 ± 4.1	26.4 ± 3.1
6	Birth weight (g; mean ± SD)	935 ± 471	981 ± 510	812 ± 290	961 ± 559	1134 ± 575	1038 ± 527	987 ± 505	1024 ± 538	900 ± 400
7	Male gender, *n* (%)	69 (66.9%)	60 (80%)	9 (32.1%)	27 (54%)	15 (46.8%)	8 (44.4%)	96 (62.7%)	79 (73.8%)	17 (36.9%)
8	Ethnicity									
	a Caucasian	19 (18.4%)	13 (17.3%)	6 (21.4%)	9 (18%)	7 (21.8%)	2 (11.1%)	28 (18.3%)	16 (15%)	12 (26%)
	b Latino	2 (1.9%)	2 (2.7%)	0	1 (2%)	1 (3.1%)	0	3 (2%)	3 (2.8%)	0
	c Mixed	3 (2.9%)	2 (2.6%)	1 (3.5%)	0	0	0	3 (2%)	3 (2.8%)	0
	d African American	79 (77.8%)	62 (82.7%)	21 (75%)	40 (80%)	29 (90.6%)	11 (61.1%)	119 (77.8%)	91 (85%)	28 (60.9%)
9	Small for gestational age, *n* (%)	28 (27.1%)	21 (28%)	7 (25%)	12 (24%)	7 (21.8%)	5 (27.7%)	40 (26.1%)	28 (26.1%)	12 (26%)
10	Mode of delivery									
11	C-section	40 (38.8%)	28 (37.3%)	12 (42.8%)	25 (50%)	16 (50%)	9 (50%)	65 (42.4%)	44 (41.1%)	21 (45.6%)
12	Vaginal	63 (61.2%)	47 (62.6%)	16 (57.1%)	25 (50%)	16 (50%)	9 (50%)	88 (57.5%)	63 (58.8%)	25 (54.3%)
13	Outborn, *n* (%)	58 (56.3%)	48 (64%)	10 (35.7%)	29 (58%)	20 (62.5%)	9 (50%)	87 (56.8%)	68 (63.5%)	19 (41.3%)
Infant medical information prior to NEC										
14	Apgar score <6 at 5 minutes	30 (29.1%)	21 (28%)	9 (32.1%)	9 (18%)	5 (15.6%)	4 (22.2%)	39 (25.4%)	26 (24.2%)	13 (28.2%)
15	Patent ductus arteriosus, *n* (%)	63 (61.1%)	46 (61.3%)	17 (60.7%)	25 (50%)	19 (59.3%)	6 (33.3%)	88 (57.5%)	65 (60.7%)	23 (50%)
16	Patent ductus arteriosus, indomethacin treated, *n* (%)	21 (20.3%)	13 (17.3%)	8 (28.5%)	4 (8%)*	3 (9.3%)	1 (5.5%)	25 (16.3%)	16 (14.9%)	9 (19.5%)
17	Patent ductus arteriosus, surgically ligated, *n* (%)	4 (3.8%)	4 (5.2%)	0	1 (2%)	1 (3.1%)	0	5 (3.3%)	5 (4.6%)	0
NEC disease features										
18	Age at NEC onset (days; mean ± SD)	21.5 ± 21.3	18.5 ± 16.7	29.6 ± 29.4	23.5 ± 17.04	27.1 ± 19.1	18.7 ± 12.1	22.1 ± 20.6	21.1 ± 17.8	25.2 ± 24.4
19	Corrected gestational age at NEC onset (weeks; mean ± SD)	30 ± 4.2	30.2 ± 4.4	30 ± 4.2	29.6 ± 2.9	31.7 ± 4.2	29.8 ± 2.9	30.4 ± 4.2	30.6 ± 4.4	29.9 ± 3.7
20	Clinical presentation									
	a Abdominal distension, *n* (%)	99 (96.1%)	72 (96%)	27 (96.4%)	40 (80%)	25 (78.1%)	15 (83.3%)	139 (90%)	98 (91.5%)	41 (89.1%)
	b Bloody stools, *n* (%)	2 (1.9%)	2 (2.6%)	0	5 (10%)*	3 (9.3%)	2 (11.1%)	7 (4.5%)	5 (4.6%)	2 (4.3%)
	c Feeding Intolerance, *n* (%)	3 (2.9%)	2 (2.6%)	1 (3.5%)	5 (10%)*	4 (12.5%)	2 (11.1%)	8 (5.2%)	5 (4.6%)	3 (6.5%)
21	Radiological findings									
	a Pneumatosis, *n* (%)	46	41 (54.6%)	5 (17.8%)	24 (48%)	17 (53.1%)	7 (38.8%)	57 (37.2%)	43 (40.1%)	14 (30.4%)
	b Pneumoperitoneum, *n* (%)	26	23 (30.6%)	3 (10.7%)	29 (58%)	18 (56.2%)	11 (61.1%)	43 (28.1%)	31 (28.9%)	12 (26%)
	c Portal venous gas, *n* (%)	14	10 (13%)	4 (14.2%)	3 (6%)	2 (6.2%)	1 (5.5%)	10 (6.5%)	3 (2.8%)	7 (15.2%)

(Contd...)

Table 3: *(Contd...)*

		NEC lesions only in the small intestine			*NEC extending into the colon*			*Combined*		
		n = 103	*75 (72.8%)*	*28 (27.2%)*	*n = 50*	*32 (64%)*	*18 (36%)*	*n = 153*	*107 (69.9%)*	*46 (30%)*
		Total	*Alive*	*Dead*	*Total*	*Alive*	*Dead*	*Total*	*Alive*	*Dead*
22	Length of bowel resected (cm; mean ± SD)	28 ± 31.9	17.1 ± 15.2	28 ± 31.9	42.2 ± 40.7**	37.9 ± 34.4	41.7 ± 39.6	26.6 ± 28.7	23.3 ± 24.5	33.8 ± 35.3
Postoperative systemic course										
23	Assisted ventilation (intubated), *n* (%)	95 (92.2%)	73 (97.3%)	22 (78.5%)	48 (96%)	32 (100%)	16 (88.8%)	138 (90.1%)	100 (93.4%)	38 (82.6%)
24	Pressor support >24 hours, *n* (%)	38 (36.9%)	26 (34.7%)	12 (42.9%)	20 (40%)	7 (21.9%)	3 (16.7%)	51 (33.3%)	36 (33.6%)	15 (32.6%)
25	Blood culture positive sepsis, *n* (%)	26 (25.2%)	22 (29.3%)	4 (14.2%)	15 (30%)	10 (31.2%)	5 (27.7%)	41 (26.7%)	36 (33.6%)	5 (10.8%)
Postoperative nutrition										
26	Postoperative ileus [days; mean ± SD)	14.6 ± 8.9	18.1 ± 17.1	14.6 ± 8.9	18.5 ± 13.6	16.2 ± 9.3	18.5 ± 13.6	17.3 ± 14.4	17.6 ± 15.4	16.1 ± 10.9
27	Surgical wound infection, *n* (%)	14 (13.5%)	10 (13.3%)	4 (14.2%)	5 (10%)	2 (6.2%)	5 (27.7%)	21 (13.7%)	12 (11.2%)	9 (19.5%)
28	Surgical wound dehiscence	9 (8.7%)	9 (12%)	0	8 (16%)	4 (12.4%)	1 (5.5%)	13 (8.4%)	12 (11.2%)	1 (2.1%)
29	Postoperative day at starting enteral feedings (days; mean ± SD)	15.6 ± 8.9	18.8 ± 17.1	15.5 ± 8.9	18.7 ± 13.5	16.8 ± 9.2	18.7 ± 13.4	18 ± 14.5	18.2 ± 15.4	16.8 ± 10.8
30	Feeding after surgery									
31	Mother's own/donor milk, *n* (%)	31 (30.0%)	16 (21.3%)	15 (53.5%)	25 (50%)	19 (59.3%)	6 (33.3%)	41 (26.7%)	41 (38.3%)	0
32	Infant formula, *n* (%)	41 (39.8%)	38 (50.6%)	3 (10.7%)	25 (50%)	22 (68.7%)	3 (16.6%)	63 (41.1%)	54 (50.4%)	9 (19.5%)
33	Mixed	13 (12.6%)	11 (14.6%)	2 (7.1%)	10 (20%)	2 (6.2%)	8 (44.4%)	21 (13.1%)	20 (18.6%)	1 (2.1%)
34	Day of attainment of full enteral feedings (120 mL/kg; mean ± SD)	51.9 ± 33.1	79.4 ± 46.1	51.8 ± 33.1	58 ± 40.7***	63 ± 40.1	58 ± 40.7	73.5 ± 44.1	75.1 ± 44.9	54.4 ± 34.8
35	Duration of parenteral nutrition (days; mean ± SD)	60.5 ± 44.6	103.2 ± 46.2	60.5 ± 44.6	83.6 ± 69.8*	93.4 ± 60.8	78.4 ± 70.5	92.8 ± 53.5		68 ± 56.8
36	Intestinal adhesions, *n* (%)	22 (21.3%)	20 (26.6%)	2 (7.1%)	6 (12%)	4 (12.5%)	3 (16.6%)	28 (18.3%)	23 (21.4%)	5 (10.8%)
37	Intestinal strictures, *n* (%)	9 (8.7%)	8 (10.6%)	1 (3.5%)	5 (10%)	3 (9.3%)	2 (11.1%)	15 (9.8%)	12 (11.2%)	3 (6.5%)
38	Short Bowel syndrome, *n* (%)	27 (26.2)	26 (34.6%)	1 (3.5%)	22 (44%)	13 (40.6%)	9 (50%)	43 (28.8%)	42 (39.2%)	1 (2.1%)
Discharge										
39	Length of stay (days; mean ± SD)	96.04 ± 88.1	152 ± 61.6	96 ± 88.1	69.7 ± 19.1*	161 ± 90	65.8 ± 78.4	133.9 ± 81.9	154 ± 71.0	84.2 ± 84.9
40	All deaths, *n* (%)	32 (31.1%)			14 (28%)			46 (30.1%)		

*p <0.05, **p <0.01, ***p <0.001

Table 4: Demographic data and clinical outcomes of infants with small intestinal NEC, colonic disease, and combined data, above and below the gestational age thresholds for 50% mortality

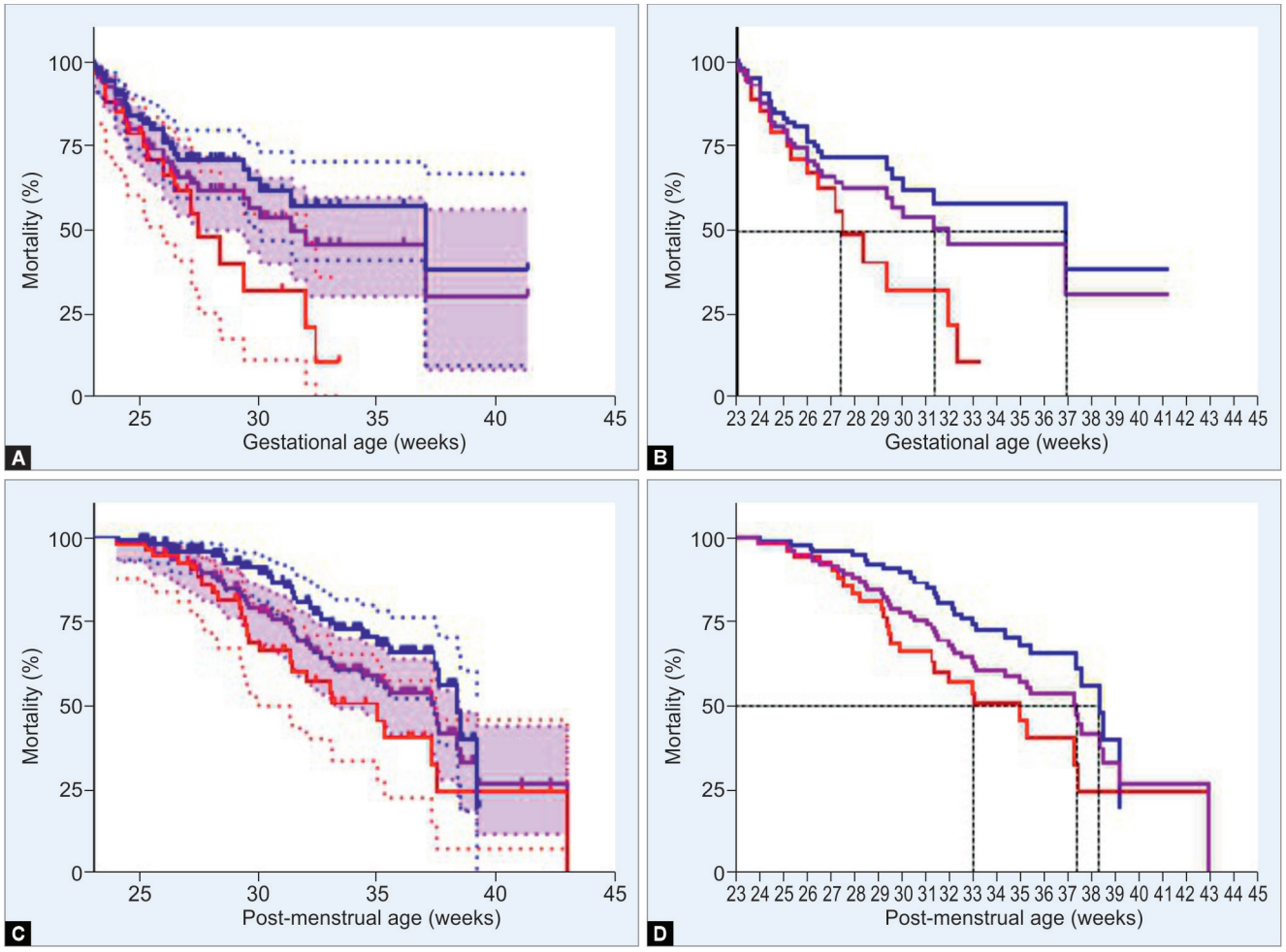
	NEC lesions only in the small intestine <37 weeks, n = 99	*NEC lesions only in the small intestine >37 weeks, n = 4*	*NEC extending into the colon <27.3 weeks, n = 28*	*NEC extending into the colon >27.3 weeks, n = 22*	*Combined <31 weeks, n = 126*	*Combined >31 weeks, n = 27*
	Mortality >50%	*Mortality <50%*	*Mortality >50%*	*Mortality <50%*	*Mortality >50%*	*Mortality <50%*
Prenatal information						
1 Pregnancy-induced hypertension, *n* (%)	26 (26.2)	1 (25%)	3 (10.7%)	9 (40.9%)*	29 (23%)	8 (29.6%)
2 Chorioamnionitis, *n* (%)	9 (9%)	0	3 (10.7%)	0	12 (9.5%)	0*
3 Antenatal steroids, *n* (%)	60 (60.6%)	1 (25%)	18 (64.2%)	12 (54.5%)	78 (61.9%)	12 (44.4%)*
Infant demographics						
5 Gestational age (weeks; mean ± SD)	26.6 ± 2.9	40.4 ± 4.3***	25.1 ± 1.3	29.5 ± 2.8***	25.9 ± 2.0	34 ± 3.4***
6 Birth weight (g; mean ± SD)	922 ± 446	1130 ± 973	758 ± 185	1527 ± 569***	816 ± 265	1809 ± 576***
7 Male gender, *n* (%)	35 (35.4%)	0	16 (57.1%)	7 (31.8%)*	51 (40%)	6 (22.2%)*
8 Ethnicity						
a White	19 (19.2%)	1 (25%)	4 (14.3%)	4 (18.2%)	24 (19%)	4 (14.8%)
b Latino	1 (1%)	0	1 (3.5%)	1 (4.5%)	3 (2.4%)	0
c Mixed	3 (3%)	0	0	0	3 (2.4%)	0
d African American	80 (80.8%)	3 (75%)	19 (67.8%)	17 (77.2%)	96 (76.2%)	23 (85.1%)
9 Small for gestational age, *n* (%)	27 (27.2%)	1 (25%)	4 (14.2%)	8 (36.3%)*	33 (26.1%)	7 (25.9%)
10 Mode of delivery						
11 C-section, *n* (%)	38 (38.3%)	2 (50%)	13 (46.4%)	12 (54.5%)	53 (42%)	12 (44.4%)
12 Vaginal, *n* (%)	61 (61.6%)	2 (50%)	15 (53.5%)	10 (45%)	73 (57.9%)	15 (55.5%)
Infant medical information prior to NEC						
13 Apgar score <6 at 5 mins	29 (29.2%)	1 (25%)	8 (28.5%)	1 (4.5%)*	34 (26.9%)	5 (18.5%)
14 Patent ductus arteriosus, *n* (%)	60 (62.6%)	1 (25%)	17 (60.7%)	8 (36.3%)*	77 (61.1%)	11 (40.7%)*
15 Patent ductus arteriosus, indomethacin treated, *n* (%)	20 (20.2%)	0	4 (14.2%)	0	24 (19%)	1 (3.7%)*
16 Patent ductus arteriosus, surgically ligated, *n* (%)	4 (4.0%)	0	1 (3.5%)	0	5 (3.9%)	0
NEC disease features						
17 Age at NEC onset (days; mean ± SD)	22.2 ± 21.4	4.7 ± 3.2	25.4 ± 18.5	21.6 ± 14.7	24.4 ± 20.8	12.3 ± 11.4**
18 Corrected gestational age at NEC onset (weeks; mean ± SD)	29.7 ± 3.7	29.9 ± 4.2	28.7 ± 2.9	33.8 ± 3.5***	29.3 ± 3.48	35.6 ± 3.5***
19 Clinical presentation						
a Abdominal distension, *n* (%)	96 (96.9%)	4 (100%)	23 (82.1%)	17 (77.2%)	118 (94.4%)	22 (81.4%)*
b Bloody stools, *n* (%)	3 (3.0%)	0	1 (3.5%)	4 (18.1%)	3 (2.3%)	5 (18.5%)***
c Feeding Intolerance, *n* (%)	1 (1%)	0	4 (16%)	1 (4.5%)	6 (4.7%)	0
20 Radiological findings						
a Pneumatosis, *n* (%)	44 (44.4%)	2 (50%)	10 (35%)	14 (63.6%)	54 (42.8%)	16 (59.2%)
b Pneumoperitoneum, *n* (%)	24 (24.2%)	2 (50%)	18 (64.2%)	11 (50%)	40 (31.7%)	15 (55.5%)**
c Portal venous gas, *n* (%)	13 (13.1%)	1 (25%)	2 (7%)	1 (4.5%)	15 (11.9%)	2 (7.4%)

(Contd...)

Table 4: (*Contd...*)

	NEC lesions only in the small intestine <37 weeks, n = 99	*NEC lesions only in the small intestine >37 weeks, n = 4*	*NEC extending into the colon <27.3 weeks, n = 28*	*NEC extending into the colon >27.3 weeks, n = 22*	*Combined <31 weeks, n = 126*	*Combined >31 weeks, n = 27*
	Mortality >50%	*Mortality <50%*	*Mortality >50%*	*Mortality <50%*	*Mortality >50%*	*Mortality <50%*
21 Length of bowel resected (cm; mean ± SD)	19.1 ± 20.0	20.0 ± 21.2	36 ± 38.3	46.6 ± 34.7	24.4 ± 28.6	35.3 ± 26.4*
Postoperative systemic course						
22 Assisted ventilation (intubated), *n* (%)	84	4 (100%)	28 (100%)	20 (90%)	112 (88.8%)	24 (88.8%)
23 Pressor support >24 hours, *n* (%)	39 (39.4%)	0	6 (21.4%)	7 (31.8%)	40 (31.7%)	8 (29.6%)
24 Blood culture positive sepsis, *n* (%)	25 (25.2%)	1 (25%)	11 (39.2%)	4 (18.1%)	34 (26.9%)	7 (25.9%)
Postoperative nutrition						
25 Postoperative ileus [days; *n* (%)]	17.6 ± 16.1	12.5 ± 7.4	14.7 ± 6.0	18.1 ± 12.5	17.7 ± 15.3	15.5 ± 10.1
26 Postoperative day at starting enteral feedings (days; mean ± SEM)	18.4 ± 16.1	13.3 ± 8.5	15.2 ± 6.1	19.3 ± 12.4	18.4 ± 15.4	16.2 ± 10.2
27 Surgical wound infection, *n* (%)	13 (13.1%)	1 (25%)	3 (10.7%)	4 (18.1%)	16 (12.6%)	5 (18.5%)
28 Surgical wound dehiscence	7 (7%)	1 (25%)	4 (14.2%)	1 (4.5%)	11 (8.7%)	2 (7.4%)
29 Intestinal adhesions, *n* (%)	21 (21.2%)	0	3 (10.7%)	4 (18.1%)	25 (19.8%)	3 (11.1%)
30 Intestinal strictures, *n* (%)	10 (10.1%)	0	4 (14.2%)	1 (4.5%)	14 (11.1%)	1 (3.7%)
31 Feeding at start						
32 Mother's own/donor milk, *n* (%)	16 (16.1%)	0	11 (39.2%)	14 (63.6%)	24 (19.0%)	17 (62.9%)
33 Infant formula, *n* (%)	36 (36.3%)	2 (50%)	13 (46.4%)	12 (54.5%)	46 (36.5%)	17 (62.9%)
34 Mixed	9 (9%)	2 (50%)	8 (28.5%)	2 (9.0%)	19 (15.0%)	2 (7.4%)
35 Day of attainment of full enteral feedings (120 mL/kg; mean ± SD)	77.6 ± 45.8	41.5 ± 0.7	67.2 ± 50.9	62.8 ± 29.9	76.5 ± 46.5	55.8 ± 28.5*
36 Duration of parenteral nutrition (days; mean ± SD)	94.1 ± 48.6	64.7 ± 58.6	99 ± 69.9	85 ± 61.9	93.0 ± 53.6	82.5 ± 54.9
37 Short Bowel syndrome, *n* (%)	17 (17.1%)	4 (100%)	15 (53.5%)	7 (31.8%)	33 (26.1%)	10 (37%)
Discharge						
38 Length of stay (days; mean ± SD)	139.3 ± 73.4	74.2 ± 59.2*	144 ± 109	107 ± 82.6	142.3 ± 83.1	93.1 ± 62.9**
39 Death, *n* (%)	29 (29.3%)	3 (25%)*	11 (39.3%)	3 (13.6%)*	40 (31.7%)	6 (22.2%)

*p <0.05, **p <0.01, ***p <0.001



Figs 1A to D: (A) Kaplan-Meier curves for mortality against gestational age (weeks). Blue line shows infants with NEC limited to the small intestine ($n = 103$), red shows patients with NEC extending into the colon ($n = 50$), and magenta shows the entire cohort ($n = 103 + 50 = 153$). The 95% confidence intervals (CIs) are shown with dotted, similarly colored lines. The magenta-shaded zone shows 95% confidence zone for the entire cohort; (B) Kaplan-Meier curves similar to panel A, where dotted black lines show the convergence of 50% mortality with gestational age in NEC limited to the small intestine, disease extending into the colon, and in the entire cohort; (C) Kaplan-Meier curves for mortality against the corrected gestational age (weeks). Color coding of the curves, dotted lines, and the combined zone as in panel A; (D) Kaplan-Meier curves similar to panel C, where dotted black lines show the convergence of 50% mortality with PMA in NEC limited to the small intestine, disease extending into the colon, and in the entire cohort

Discussion

We studied 153 infants with a clinical profile typical for surgical NEC, with an average (±SD) gestational age of 27.3 ± 3.9 weeks and a birth weight of 986.9 ± 505 g. NEC occurred on postnatal day 22.4 ± 20.1 with usual presenting features of abdominal distension, feeding intolerance, and/or bloody stools. Abdominal surgery was done within 24 hours of disease onset. Postoperatively, ileus lasted 17.3 ± 14.5 days; enteral feedings were started on 18 ± 14.5 days, and full volumes were achieved on 72.4 ± 44.2 days. Parenteral nutrition was given for 91.1 ± 53.8 days. Forty-six (30.1%) infants died. The average hospital stay was 133.6 ± 81.9 days.

The findings in this study support our hypothesis that surgical resections may be more likely associated with better outcomes in NEC that extends into the colon than in disease limited to the small intestine. In our cohort, 103 infants had small intestinal NEC, whereas the other 50 had colonic involvement. The two groups had comparable gestational ages and birth weights and, except for some minor differences, had generally similar clinical courses prior to and after surgery for NEC. In the resected tissues, the gross and microscopic pathological changes of NEC, and their severity, were similar except for the relative prominence of reparative changes in some infants with colonic involvement (Table 2). There were significant differences in the LOS of the two groups, and a trend toward significance in mortality (Table 3). The application of Kaplan-Meier statistics, when mortality data were plotted against the gestational age at birth or the corrected gestational age at onset of NEC, showed important differences in the clinical trajectories of the two groups. These results once again emphasize the need for cautious analysis in numerically limited clinical samples.[21] Despite longer bowel resections, infants who had NEC involving the colon were quicker to reach full-volume enteral feedings and had shorter hospital stays. Their mortality rates dropped below 50% at 27.3 weeks' gestation, which was much earlier than the 37 weeks' threshold we noted in NEC limited to the small intestine. The reasons for these differences in the gestational age thresholds for mortality are not clear. Prior to 28 weeks' gestation, the mid-gestation colon is still undergoing histomorphological and functional differentiation and resembles the small intestine in many ways.[22,23] Its mucosa displays villus-like structures that express

digestive enzymes such as sucrose-isomaltase, aminopeptidase, and alkaline peptidase; hormones such as glucagon, somatostatin, and pancreatic polypeptide; absorb glucose, alanine, and methionine; and synthesize apolipoproteins. Interestingly, the two subgroups show major differences in mortality only beyond 28 weeks' gestation. One possibility that needs further investigation is whether the two subgroups could have temporal differences in colonic differentiation of the distal intestine.

We found no difference in the average gestational age of infants who developed NEC exclusively in their small intestine vs others who had disease extending into the colon. These findings contrast with existing information that colonic NEC lesions may occur more frequently in infants born at gestational ages closer to term than in very premature ones.[7,24] One possible explanation for this observed concordance in the gestational age and PMA between the two studied subgroups is that developmentally regulated changes in intestinal structure or function, or predisposing genetics, may have a relatively strong impact on the onset of NEC.[5,25] After surgery, infants with NEC involving the colon had improved survival at a significantly earlier gestational age and PMA. As hypothesized, these differences in survival rates may reflect the greater impact of surgical resections in reducing gut microbial loads, bacterial translocation across the diseased bowel, and consequent local and systemic inflammation. These possibilities merit further investigation. Finally, the two groups developed NEC at a similar postnatal age. This temporal convergence could possibly be rooted in one or more postnatal factors. Increasing evidence emphasizes the role of postnatal exposures such as enteric dysbiosis and overgrowth of gram-negative bacteria, to potentially injurious components of artificial feedings, and the limitations of mucosal antimicrobial defenses such as immature Paneth cells. We also have some information on point triggers of NEC during intensive care, such as hypoxia and hypothermia, ischemia and reperfusion, severe anemia and blood transfusions, and enteral exposure to immunological stimuli.[26–32]

We confirmed the diagnosis, severity, and the anatomic localization of NEC in surgically resected intestines (Table 2). Gross pathological specimens were available from 60 infants, and all showed findings of NEC. Acute changes included gangrenous areas, dusky discoloration suggesting altered perfusion, hemorrhages, and in some samples, perforations. Some samples showed subacute changes such as bowel wall thinning, friability, strictures, and adhesions. Histopathological sections of the resected intestine were available from all 153 patients. All showed findings of NEC, including necrosis, inflammation, and hemorrhages, and some showed regenerative changes. Consistent with our earlier observations,[17] necrosis, inflammatory changes, and hemorrhages correlated with each other. Pneumatosis was seen in about 30% of the sections. Tissue reparative changes were more prominent in mild-moderate NEC than in severe disease.[7] Finally, bowel resections had clean margins in only about 35% of all sections. The rest showed some necrosis in the resected edges, possibly pointing to both the surgeons' efforts to minimize the removal of the intestines and to some difficulty in visually differentiating completely vs partially necrosed bowel. We have recently shown that incomplete resections of necrotic bowel may increase mortality in NEC.[17] There may be some justification for on-site, rapid histopathological screening of the resected tissues to prevent excessive removal of segments that could possibly recover in time, but also to ensure that all clearly necrotic patches have been removed.

The demographic and clinical features of infants who had NEC limited to the small intestines were generally similar to those with colonic involvement (Table 3). Infants in both groups had comparable gestational ages and birth weights. They developed NEC at a similar postnatal age. Infants who developed small intestinal NEC had a more frequent history of symptomatic PDA than those with colonic NEC. These infants also had a higher frequency of feeding intolerance and bloody stools as presentations of NEC. Small intestinal NEC led to shorter bowel resections, but resulted in longer periods to reach full enteral feedings and longer hospital stays. The reasons for these clinical differences are unclear. They could have had a higher incidence of intestinal dysmotility or subclinical strictures, but these data are not available and need further investigation. Their clinical course was likely complicated as their Kaplan-Meier estimates for the gestational age at 50% mortality were much more delayed than in those with NEC extending into the colon (37 vs 27.3 weeks, Table 4).

In our entire cohort, the gestational age threshold for 50% mortality was at 31.3 weeks (Table 4). Infants born prior to this maturational cutoff were more often exposed to chorioamnionitis and had less frequent treatment with antenatal steroids. Their average gestational ages and birth weights were lower, and they were more often male. They had a higher incidence of PDA, developed NEC at an earlier PMA but a later chronological age, developed abdominal distension and bloody stools more frequently at the time of presentation with NEC, and developed pneumoperitoneum more frequently. Many of these differences were also noted across the comparison thresholds in small intestinal NEC or in disease that extended into the colon, although some differences did not reach statistical significance because of the smaller numbers.

African American infants constituted a large proportion of our cohort. Several large studies have now shown the impact of ethnic and genetic influences in the pathogenesis of NEC, but the underlying reasons are still unclear.[33,34] In a recent study, Janevic et al.[35] reported increased risk of NEC in African American [adjusted relative risk (RR) 1.39, 95% CI, 1.00–1.93]. In another study at the Pediatrix medical group, 8,796 (7%) patients were identified to have NEC in a cohort of 126,089 infants.[33] NEC was frequent in African Americans [adjusted odds ratios (AOR) 1.31, 95% CI 1.24–1.39]. The mortality was also higher in these infants than in Caucasians (AORs 1.35, 95% CI 1.15–1.58). These health disparities need further investigation, possibly focusing on environmental exposures and genetic variants associated with NEC.[25]

To conclude, we present novel information suggesting that surgical resections may be more likely associated with better outcomes in NEC involving the colon than in disease limited to the small intestine. There may also be other exciting opportunities; infants who developed NEC extending into their colon had an average time gap of nearly 6 weeks between the gestational age at birth and the PMA at onset of NEC. This interval may provide an opportunity, at least in some infants, for intensive clinical monitoring, risk stratification with analysis of the microbiome or other parameters, or focused interventions with nutritional, pharmacological, or probiotic-based supplementation. Our study had limitations in its single-center design, limited sample size, and the retrospective design, which increase the risk of bias. There is a need to validate these results in a larger, prospectively enrolled, and multicentric cohort, which may also allow the evaluation of additional clinical/laboratory predictors in the statistical models.[36–39]

AM and PMG designed the study, PMG, JLP, MZ, KL, NV, CM, MM and AA collected and analyzed the data. AM and PMG wrote the study. All the authors contributed to and approved the manuscript.

Orcid

Parvesh M Garg https://orcid.org/0000-0001-5949-7304

Supplementary Material

All the supplementary figures and tables are available online on the website of www.newbornjournal.org.

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ORIGINAL RESEARCH

Real-time Echocardiography-guided Weaning of Veno-arterial Extracorporeal Membrane Oxygenation in Neonates

Sharada Hiranya Gowda[1], Alice King[2], Adam M Vogel[3], Ryan D Coleman[4], Corey A Chartan[5], Joseph A Garcia-Prats[6], Caraciolo J Fernandes[7]

Abstract

Objective: The objective of the study is to evaluate the utility of real-time echocardiography (RTE) to provide objective hemodynamic guidance during decannulation of neonates from extracorporeal membrane oxygenation (ECMO).

Design: Retrospective case series.

Patients: Neonates with respiratory and circulatory failure who underwent venoarterial ECMO (VA-ECMO).

Interventions: Use of RTE to assess cardiac function, pulmonary hypertension (PH), and readiness for decannulation from ECMO.

Outcome measures: Data abstracted included clinical parameters, RTE data, and management decisions during weaning from VA-ECMO.

Results: We used RTE during weaning in 12 of 33 patients between 2016 and 2019. Findings prompted inotrope titration in 10 (83%) patients and volume resuscitation in 10 patients. PH was present in 12 (100%) patients and prompted initiation of prostaglandin infusion (in 3 (25%) patients. Ten of 12 patients were successfully weaned off; in 2, RTE was instrumental in halting decannulation.

Conclusions: RTE may serve as a valuable tool in clinical decision-making while weaning neonates from VA-ECMO and providing data to choose appropriate support for successful decannulation.

Keywords: Echocardiography, Weaning from extracorporeal membrane oxygenation, Veno-arterial extracorporeal membrane oxygenation.

Newborn (2022): 10.5005/jp-journals-11002-0006

Introduction

Extracorporeal membrane oxygenation (ECMO) can be life-saving for patients with cardiorespiratory failure.[1] Historically, ECMO was primarily used during respiratory failure in neonates with meconium aspiration syndrome (MAS).[2] Neonatal respiratory failure is the most common indication for extracorporeal life support (ELSO), with over 30,000 neonatal runs listed in the ELSO database.[3] With advancements in medical management and mechanical ventilator support, in conjunction with innovative therapies such as inhaled nitric oxide, neonatal ECMO is now a relatively rare occurrence that is mainly used in patients with congenital diaphragmatic hernia (CDH) with pulmonary hypoplasia and pulmonary hypertension (PH).[4] Initiation of venoarterial (VA)-ECMO improves hypoxemic respiratory failure in these newborns with life-threatening persistent PH and supports end-organ perfusion and allows right ventricle (RV) unloading until pulmonary vascular resistance (PVR) decreases to allow for improvement in ventricular function. Since the successful treatment of Esperanza in 1975, there is remarkable consensus in initiating ECMO in neonates.[5] However, decannulation and assessing readiness to come off ECMO is still very subjective and not consensus or evidence-based. In neonates, accurately assessing time needed to allow for resolution of right heart failure and improvement in PVR can be challenging. Currently, there are limited data to guide clinicians in developing evidence-based protocols to decide when and how to wean neonates off VA-ECMO.[6–9] Successful weaning from ECMO depends on several clinical, hemodynamic, and echocardiographic variables.[10] Given that the most common current indication for VA-ECMO in neonates is severe PH in the setting of CDH, echocardiographic assessment of ventricular interaction, function, ductal patency, shunt directionality, and volume status offers the promise of real-time information to help clinicians decide whether a neonate is ready for VA-ECMO to be discontinued and to direct the support the patient needs for successful decannulation.[11,12] While real-time echocardiography (RTE) is commonly used in the management of pediatric and adult patients on ECMO, it has yet to become a common standard practice in neonatal intensive care units (NICUs).

[1,6,7]Department of Pediatrics, Section of Neonatology, Baylor College of Medicine/Texas Children's Hospital, Houston, Texas, United States of America

[2,3]Department of Surgery, Division of Pediatric Surgery, Baylor College of Medicine/Texas Children's Hospital, Houston, Texas, United States of America

[4,5]Department of Pediatrics, Section of Pediatric Critical Care Medicine, Baylor College of Medicine/Texas Children's Hospital, Houston, Texas, United States of America

Corresponding Author: Sharada Hiranya Gowda, Department of Pediatrics, Section of Neonatology, Baylor College of Medicine/Texas Children's Hospital, Houston, Texas, United States of America, e-mail: Shgowda@bcm.edu

How to cite this article: Gowda SH, King A, Vogel AM, *et al.* Real-time Echocardiography-guided Weaning of Veno-arterial Extracorporeal Membrane Oxygenation in Neonates. Newborn 2022;1(1):27–31.

Ethics committee and approval number: Baylor College of Medicine Institutional Review Board H-27591.

Source of support: Nil

Conflict of interest: None

In this retrospective descriptive study, we share our center's initial experience in using echocardiography to assess cardiac function, intravascular volume status, and titrate vasoactive medications for decannulation from mechanical circulatory support in neonates with hypoxemic respiratory failure undergoing VA-ECMO. We hypothesized that RTE provided useful objective real-time information in determining readiness for decannulation from VA-ECMO.

Materials and Methods

Study Design

We conducted a retrospective observational study of all neonates admitted to our NICU from January 2016 to December 2019 undergoing VA-ECMO and in whom echocardiography was used when weaning from ECMO. The study was performed under a study protocol approved by the Institutional Review Board of Baylor College of Medicine and affiliated hospitals (no H-27591).

Patient Selection

All patients who underwent VA-ECMO in our NICU during the above-mentioned period were identified by our institutional ELSO data abstractor. Determination of use of echocardiography during weaning from ECMO was made by review of notes documented in the medical record during the weaning process.

Measured Outcomes

Maternal and Neonatal Birth Data

Data abstracted included maternal age, type of delivery, infant's gestation and weight at birth, and Apgar scores. All data are presented as mean (+ SD) or (min, max) as appropriate.

ECMO and Vasoactive Support Data

Details abstracted included the diagnosis necessitating ECMO, the day of life of initiation and duration of ECMO. Additionally, we reviewed the types of vasoactive medications used during the ECMO weaning. Specific inotrope or volume resuscitation titration in response to real-time echocardiographic observations was recorded.

Echocardiographic Data

All infants had either a prenatal echo or routine postnatal echocardiographic evaluation prior to placement on ECMO to exclude any complex congenital heart disease. Ventricular volumetric analyses, great artery measurements, and atrio-ventricular valves (A-V) competency were determined using standard echo views. Comprehensive assessment of left ventricle (LV) function was assessed by measuring fractional shortening (FS) and ejection fraction (EF); RV function was assessed in a qualitative manner and interpreted using the American Society of Echocardiography guidelines as normal values for FS and EF in infants and children have been established.[12] In our cohort, a modified pediatric protocol was used at the bedside to subjectively assess RV and LV systolic and diastolic function, tricuspid regurgitation (TR) jet, septal configuration, pulmonary valve regurgitant velocity, and shunt direction across the patent ductus arteriosus (PDA), atrial septal defect, and ventricular septal defect.[11] Because of the volume-unloading that occurs with VA-ECMO and the increased left ventricular afterload induced by the ECMO circuit, there are certain inherent limitations in judging function while on ECMO.[13] In order to address these limitations, we obtained a baseline echo on full flow. We then weaned the flow in a stepwise manner under continuous echo monitoring. Inotropic and pulmonary vasodilator therapy were titrated as indicated in order to allow for more accurate determinations of true cardiac function to be ascertained.

Results

Patient Demographics

During the study period, 39 infants underwent cannulation for ECMO; of the 33 treated with VA-ECMO, 12 had echocardiography utilized during weaning from ECMO at the clinician's discretion. Of the 21 patients who did not get RTE-guided weaning, most were decannulated prior to instituting the RTE protocol. Only 53% of infants were successfully weaned in the non-RTE cohort. Notably, 10 of 21 non-RTE patients underwent withdrawal of life-sustaining therapy due to complications such as bleeding or a life-limiting diagnosis such as alveolar capillary dysplasia or pulmonary hypoplasia secondary to bilateral renal agenesis. Median duration of ECMO for the 11 non-RTE patients compared with the 12 patients who underwent RTE-guided weaning was 327 (82, 554) vs 138 (82, 486) hours (min-max), $p = 0.27$. Duration of ECMO in hours is depicted in Figure 1. Maternal data and infant birth information are shown in Table 1.

Process of Weaning

Candidacy for discontinuing ECMO support was discussed daily in a multidisciplinary bedside meeting, including representatives from neonatology, surgery, PH, ELSO specialists, and transfusion medicine. Fluid balance, arterial blood gas (ABG), and pump mixed venous saturation were monitored. Decreases in requirement for vasoactive medications, pump flow, circuit FiO_2 requirement and sweep gas requirement were evaluated to assess readiness to be decannulated. Once the patient was deemed potentially ready for decannulation, a 30-minute "trial-off" was conducted using the following procedure. A baseline ABG was obtained and ventilator settings adjusted to optimize lung recruitment. Next, ECMO flow was decreased by approximately 10 mL/kg/minute every 10–15 minutes from our usual flow of 100–50 mL/kg/minute. At this flow rate, an ABG was obtained along with baseline evaluation of echo parameters. Ventilator settings

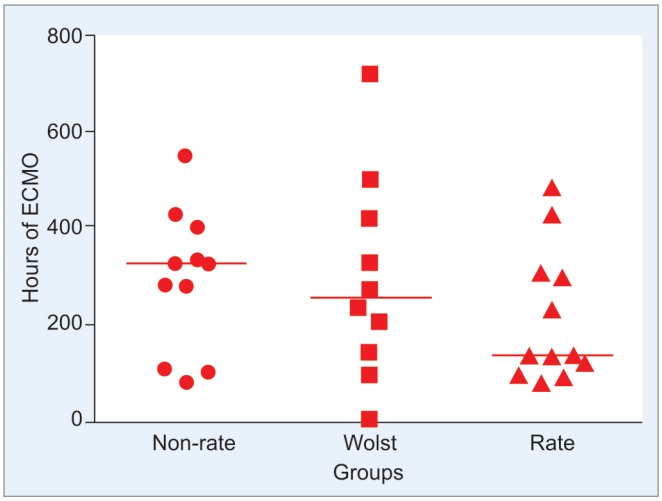

Fig. 1: ECMO duration comparison between the three groups. Median values: Non-RTE (327), RTE (138), $p = 0.27$

Table 1: Patient characteristics

Characteristics	
N	12
Maternal age at delivery (years)	28.3 (7.5, 17–42)
Type of Delivery	
Vaginal	4
CS	8
Elective CS	5
Emergency CS	3
Apgar at 1 minute	5 (1–9)
Apgar at 5 minutes	7 (3–9)
Diagnosis	
CDH*	7
Left CDH	5
Right CDH	2
Other	5
MAS	1
Hydrops	2
HIE/PH/IDDM	1
CPAM	1
Race	
African American	1
Asian	2
Hispanic	1
Caucasian	8
Gestational age in weeks	37.2 (1.6, 33–39)
Birth weight (kg)	3.37 (0.7)
Male	8
Female	4

Data reported as mean (SD, range) and absolute numbers. *All CDH repairs were done on ECMO. CPAM, congenital pulmonary airway malformations; HIE: HIE, hypoxic ischemic encephalopathy; IDDM, infant of a diabetic mother

Table 2.1: Echocardiographic findings

PH*	12 of 12	
Decreased preload	6 of 12	
Decreased LV contractility	2 of 12	
Decreased RV contractility	4 of 12	One patient with weaning halt
Decreased BiV contractility	1 of 12	One patient with weaning halt
Shunt R–L	3 of 12	
Shunt L–R	5 of 12	
Bidirectional Shunt	4 of 12	
PDA Patency	9 of 12	
Echo-guided decannulation completed	10 of 12	
Echo-guided decannulation halted	2 of 12	

*PH based on TR jet, septal position, shunt across PDA/ASD

Table 2.2: RTE-guided interventions

Medication		*Dose Range*
Epinephrine		
Infusing prior	7 of 12	0.02–0.12mcg/kg/minute
Initiated during	2 of 12	0.02–0.04 mcg/kg/minute
Dopamine		
Infusing prior	3 of 12	5–15 mcg/kg/minute
Initiated during	4 of 12	5 mcg/kg/minute
Vasopressin		
Infusing prior	4 of 12	0.01–0.06 u/kg/hour
Initiated during	4 of 12	0.02 µ/kg/hour
Milrinone		
Infusing prior	2 of 12	0.250–0.500 mcg/kg/minute
Initiated during	1 of 12	0.375 mcg/kg/minute
PGE		
Infusing prior	7 of 12	0.0125–0.025 mcg/kg/minute
Initiated during	3 of 12	0.025 mcg/kg/minute
iNO	12 of 12	20 PPM
Sildenafil	12 of 12	1–4 mg/kg/day

and FiO_2 were titrated to target PO_2 and PCO_2. An attending neonatologist, cardiologist or pediatric PH consultant was at bedside to assess the real-time ECHO changes and to assist with management. After approximately 10 minutes of flow at 50 mL/kg/minute flow, another ABG with lactate was obtained. If clinical, echo parameters and ABG were reassuring, the ECMO circuit was clamped. During the 30-minute clamp time, vital signs were monitored continually, echo assessment was performed every 10 minutes or sooner if vital signs changed, and ABGs with lactate were obtained every 10 minutes.

Patient-specific Interventions

Guided by clinical vital sign changes and echo evaluation, vasoactive infusions were adjusted and/or volume boluses were administered (Tables 2.1 and 2.2). During the weaning process, based on biventricular function, we increased epinephrine dosage in 7 of 12 patients and initiated it in an additional 2 of 12 patients. Dopamine was initiated in 4 of 12 patients, but we did not increase it in 3 of 12 patients who were already on it prior to weaning as these patients were already at a maximum therapeutic dosage; instead, we switched over to epinephrine for its better inotropic effect. Evidence of persistent PH was noted in 12 of 12 patients. We initiated vasopressin in 4 of 12 patients and increased the dosage in an additional 4 of 12 patients based on ventricular septal bowing. In one patient with evidence of TR jet acceleration with change in RV volume, we initiated milrinone; 2 of 12 patients were on it at initiation of weaning. In patients with severe PH and depressed RV function, the ductus arteriosus was kept patent using prostaglandin (PGE) infusion as a "pop off" for the RV and to improve preload for the LV.[14] A PDA was present in 9 of 12 patients. PGE was initiated in 3 of 12 patients in addition to 7 of 12 patients who were on it prior to initiation of weaning. Observation of systemic hypotension with adequate ventricular function on RTE prompted volume resuscitation with fresh frozen plasma (FFP), packed red blood cells (PRBCs), cryoprecipitate, normal saline, albumin, and platelets. We used normal saline in 1 of 12 patients, PRBCs in 4 of 12 patients, cryoprecipitate in 1 out of 12 patients, and FFP in 3 of 12 patients. After completion of the weaning trial, the ECMO flow was returned to full flow and the

surgeons, family, and consultants were notified and the decision and timing for decannulation determined.

Patient Outcomes

Using RTE as part of our weaning process allowed us to identify 10 patients who were deemed to be clinically ready for discontinuation of ECMO support and decannulation. All 10 patients were successfully decannulated. In one patient, despite maximizing vasoactive support, severe RV dysfunction persisted and the trial-off was aborted after only 10 minutes of clamping. In another patient, a significant increase in the TR jet and right-to-left shunting across the PDA despite titration of milrinone and epinephrine infusions and prior optimization with sildenafil and iNO prompted us to halt the weaning attempt at 60 mL/kg flow.

Discussion

In this study, we report our center's initial experience using RTE to assess myocardial performance, interventricular interactions, severity of PH, and readiness for decannulation from mechanical circulatory support in neonates with hypoxemic respiratory failure and circulatory failure undergoing VA-ECMO. We found RTE allowed for assessment of biventricular function, septal configuration, ventricular interaction, intracardiac volume, and the presence and direction of intracardiac and ductal level shunts during different loading conditions. These findings in turn allowed us to intervene and tailor our management according to the infant's need. Although the number of patients in which RTE was used in this four-year period was relatively small, our cautious attempts to introduce and test the feasibility and utility of this imaging modality during decannulation yielded some valuable insights.

In our retrospective cohort, RTE was used to help guide changes in vasoactive infusions and/or volume boluses in response to hypotension observed during the trial off mechanical support in a substantial number of infants. Inadequate cardiac function prompted titration of vasoactive infusions, while determination of suboptimal intracardiac volume status prompted a volume bolus. Dopamine and epinephrine were the inotropic medications commonly used for systolic ventricular dysfunction. Milrinone was used for its inotropic, lusitropic functions.[14] If RV dysfunction was noted despite use of pulmonary vasodilators, PGE was used to keep the ductus open as a "pop off" to offload the RV. Vasopressin was used for its alpha-adrenergic property to increase the systemic vascular resistance (SVR) if there was echocardiographic evidence of the ventricular septum flattening or bowing into the LV due to increased right-sided pressures. Inhaled nitric oxide and sildenafil were used to decrease PVR. When volume expansion was deemed necessary, choice of volume expander was based on the hematocrit (Hct) and coagulation status in addition to hypotension. In instances where the Hct was less than our threshold of 40%, PRBCs were chosen as the preferred volume replacement and to increase oxygen-carrying capacity. Infusion of plasma and cryoprecipitate was initiated based on the most recent coagulation profile as appropriate. If fibrinogen levels were low, cryoprecipitate was chosen; if partial thromboplastin time was prolonged, FFP was chosen.

Use of RTE during weaning from VA-ECMO in our retrospective cohort permitted a level of precision in management not available in earlier decades. This precision-based approach to volume resuscitation and inotrope titration not only aided in the successful weaning of 10 of 12 patients but also guided us to abort the weaning trial in 2 of the 12 patients; in one whom cardiac function was suboptimal and one in whom PH worsened despite above interventions. Specifically, in the latter two patients, RTE was a key factor in the decision to abort the weaning process. In the patient whose RV function did not improve despite aggressive titration of vasoactive medications, use of PGE infusion and milrinone, volume resuscitation would have been deleterious and could have precipitated acute cardiovascular collapse in the setting of biventricular failure. In another patient, an infant of a diabetic mother with significant septal hypertrophy, the diastolic dysfunction markedly deteriorated with volume repletion and there was evidence of left atrial hypertension and pulmonary venous congestion. RTE in this case allowed us to identify the futility of initiating inotropes, and recognize the need for additional time for ventricular rest before reattempting a weaning trial.

In our cohort, the main indication for VA-ECMO was PH and RV dysfunction. Assessing cardiac function, especially in the setting of PH, in neonates on VA-ECMO is challenging because of the changes in volume loading of the ventricles, lack of pulmonary blood flow, and alterations in systemic vascular resistance due to VA-ECMO and changes in PVR induced by ventilator management. Using echocardiography to estimate right ventricular performance is technically challenging due to its anatomical and functional distinctiveness.[15] The current adult guidelines for the echocardiographic quantification of RV function recommend using multiple indices to describe the RV in a thorough and comprehensive manner, such as RV index of myocardial performance, tricuspid annular plane systolic excursion, fractional area change, Doppler tissue imaging-derived tricuspid lateral annular systolic velocity (S'-wave), three-dimensional RVEF, RV longitudinal strain /strain rate by speckle-tracking echocardiography.[13] These indices are even more difficult to measure in critically ill neonates at risk for hemodynamic decompensation with prolonged interrogations. RTE during the weaning trial allowed us to evaluate whether the PH had resolved and the infant was hemodynamically ready for decannulation.

The RTE-weaning model provides clinicians real-time data to interpret changes in cardiac-loading conditions. Dynamic changes with stepwise decreases in circuit flow allowing the heart to fill and thus eject more affect the myocardial contractility and resistance to flow. Information regarding the ability of both LV and RV to adapt to these dynamic volume shifts and function optimally after a period of prolonged rest is readily available with RTE. RTE has the unique distinction of providing direct correlation of myocardial function during different loading conditions non-invasively to guide interventions with tailored vasoactive medications and timely decannulation. The goal of this review was to first address the binary question of whether RTE-provided data useful for weaning. Our results suggest that RTE is a valuable and feasible tool that can be easily incorporated into clinical practice to help clinicians decide which patients need intervention and whether they need volume versus inotropic support versus RV support. Additionally, RTE also provides information when the patients are not ready to come off ECMO support.

In summary, our results suggest that RTE serves as an objective diagnostic tool in weaning neonates from VA-ECMO while serving

as a guide toward optimizing medical interventions that may result in successful decannulation. Our retrospective review documents our attempts to bridge an important knowledge gap in understanding the process of physiological readiness for decannulation and utility of RTE to streamline the weaning from ECMO. This is the first article that exclusively studies the use of RTE during decannulation in neonatal ECMO.

What is Known?

- The leading indication for neonatal ECMO currently is hypoxic-respiratory failure due to PH in infants with congenital diaphragmatic hernia.
- Resolution of maladaptive PH, which usually wanes significantly in the first two weeks of life, signals an infant's readiness for discontinuation of ECMO support.
- There are no published guidelines advocating the routine use of echocardiography in determining a neonate's readiness to come off ECMO support.

What this Study Adds?

- Real-time echocardiography is a valuable tool to aid clinical decision-making while weaning neonates from VA-ECMO.
- Use of real-time echocardiography facilitates successful decannulation as it provides information regarding the need for fluid volume versus inotropic support.
- Use of real-time echocardiography during "clamp" trials permits rapid identification of neonates not ready for decannulation.

Competing Interests

None.

Author Contribution

SHG made substantive contributions to the conception, drafting, and design of this paper, reviewed and revised it critically for important intellectual content and approved the final manuscript.

AK, AV, RC, CC, and JGP made substantive contributions to the paper by performing the initial literature review of the subject matter, interpreting the patient data in light of the literature review, conceptualizing, designing and drafting the initial manuscript, and approving the final manuscript.

CJF conceptualized and designed the paper, supervised the work, critically reviewed and revised the manuscript for important intellectual content, and approved the final manuscript.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Orcid

Sharada Hiranya Gowda https://orcid.org/0000-0001-7009-4821

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REVIEW ARTICLE

Intestinal Epithelial Barrier Function and Necrotizing Enterocolitis

Elizabeth Managlia[1], Xiaocai Yan[2], Isabelle G De Plaen[3]

Abstract

Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants. NEC is characterized by intestinal tissue inflammation and necrosis. The intestinal barrier is altered in NEC, which potentially contributes to its pathogenesis by promoting intestinal bacterial translocation and stimulating the inflammatory response. In premature infants, many components of the intestinal barrier are immature. This article reviews the different components of the intestinal barrier and how their immaturity contributes to intestinal barrier dysfunction and NEC.

Keywords: Intestinal barrier, Necrotizing enterocolitis, Preterm neonate.

Newborn (2022): 10.5005/jp-journals-11002-0003

Introduction

Necrotizing enterocolitis (NEC) is a disease affecting the gastrointestinal (GI) tract of premature infants thought to result from an immature immune system, impaired microvasculature development, and an impaired mucosal barrier. In this review, the different components of the intestinal barrier and their functions are discussed (Fig. 1), with emphasis on how these are affected by the NEC disease process and by factors and interventions known to protect against NEC. The GI tract is a highly vascularized organ where the exchange of water and nutrients occurs via a single layer of epithelial cells. Precise regulation of the gut barrier function is essential to maintain the critical balance between its absorptive function and its role at preventing potentially harmful digestive enzymes, bile acids, and bacteria present in the lumen to get into the tissues. Breakdown of the intestinal barrier in neonates is thought to be a critical step in the development and the progression of NEC.

In human neonates, intestinal permeability to sugar decreases during the first week of life.[1] This is more pronounced in breastfed newborns, compared to those that are fed formula,[1] suggesting a beneficial role of breast milk on the intestinal barrier. The intestinal barrier has been shown to be impaired during NEC in humans[2] and animals.[3,4] We showed that the intestinal permeability is increased in response to NEC-inducing stresses prior to the development of intestinal injury in a mouse model of NEC,[5] which suggests that alterations of the intestinal barrier may play a role in NEC pathogenesis. In adults, an increase in intestinal permeability has been shown to precede Crohn's disease[6] and its relapse,[7] and is thought to contribute to the disease. In premature infants, several components of the intestinal barrier are immature and therefore may predispose them to NEC.

The Mucus Layer

The Mucus

The intestinal epithelium is protected against harmful luminal bacteria and toxins by a thick gelatinous mucus layer secreted by specialized epithelial cells called goblet cells. In the colon, while bacteria reside and thrive in the outer mucus layer,[8] the inner mucus layer is physically impenetrable to bacteria.[9] However, in the small intestine, the mucus layer is loose, unattached, and can easily be penetrated by microorganisms.[10] To decrease the risk of infection, the small intestinal mucus layer is regularly flushed by a process consisting of liquid secretion and motor activity. The migrating motor complex generated by the enteric nervous

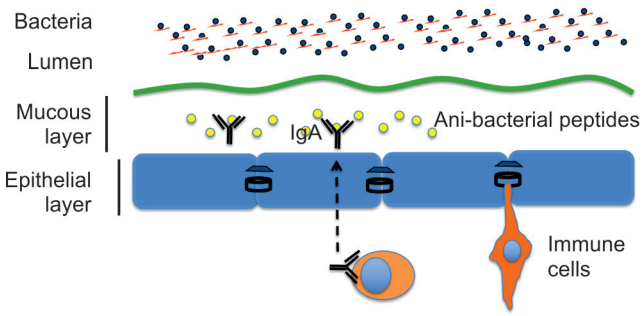

Fig. 1: Components of the intestinal barrier

[1–3]Division of Neonatology, Department of Pediatrics, Ann and Robert H Lurie Children's Hospital of Chicago, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, United States; Center for Intestinal and Liver Inflammation Research, Stanley Manne Children's Research Institute, Ann and Robert H Lurie Children's Hospital of Chicago, Northwestern University, Chicago, Illinois, United States

Corresponding Author: Isabelle G De Plaen, Division of Neonatology, Department of Pediatrics, Ann and Robert H Lurie Children's Hospital of Chicago, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, United States; Center for Intestinal and Liver Inflammation Research, Stanley Manne Children's Research Institute, Ann and Robert H Lurie Children's Hospital of Chicago, Northwestern University, Chicago, Illinois, United States, Phone: +312-503-7179, e-mail: isabelledp@northwestern.edu

How to cite this article: Managlia E, Yan X, De Plaen IG. Intestinal Epithelial Barrier Function and Necrotizing Enterocolitis. Newborn 2022;1(1):32–43.

Source of support: This article was supported by a NIH grant award DK116568 (IGDP).

Conflict of interest: None

system likely allows the loose mucus to migrate to the colon.[10] The mucus is composed of mucins, lipids, and water. Mucins are large glycoproteins made of a central protein backbone rich in proline, threonine, and serine which are *O*-glycosylated with large glycans.[11] These glycans cannot be digested by digestive enzymes thus protecting the central mucins from degradation by endogenous proteases. Mucins are produced, stored, and released by goblet cells. Once secreted, gel-forming mucins lubricate and protect the gastrointestinal tract. While Muc2 is the main secreted mucin in the small intestine,[12] others are MUC5AC, MUC5B, MUC6, and MUC7.[10] As opposed to secreted mucins, membane-bound mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, and MUC17) have a transmembrane domain that enables them to be anchored into the cell membrane. These play a role in protection, apical cell surface sensing, and signaling.[10]

In the fetus, Muc2 mRNA is expressed at 12 weeks of gestation in the jejunum, ileum, and colon.[12] Muc2 was found to be rapidly synthesized in the small intestine of preterm infants who have undergone an enterostomy for necrotizing enterocolitis.[13] While Muc2 developmental regulation is not fully known, a few studies suggest that its deficiency may play a role in NEC. In the intestinal tissue samples of patients with NEC, the number of mucus-containing small intestinal goblet cells is decreased compared to age-matched control samples.[14] In a neonatal rat NEC model, Muc2 mRNA and protein have been found to be decreased as well as the number of Muc2 positive cells when compared to dam fed animals.[15] In immature but not mature mice, TNF injection resulted in the loss of mucus-containing goblet cells but induced Muc2 and Muc3 mRNA upregulation in the mature ileum.[14] Muc2 deficient mice spontaneously develop colitis.[16] Several recent studies correlate increased Muc2 production with decreased NEC severity.[17–20] In addition, in premature neonates, the immaturity of the enteric nervous system and of the migrating motor complexes[21] may delay the normal migration of the bacteria-containing mucous layer from the small intestine to the colon. Both of these mechanisms may increase the risk of bacterial product translocation through the intestinal mucosa and contribute to NEC pathogenesis.

Antibacterial Products, Enzymes, and Soluble Factors

The mucus layer not only limits the diffusion of toxins but allows the generation of a gradient of antibacterial products secreted by Paneth cells and enterocytes.[10] Paneth cells secrete different antibacterial products such as alpha-defensins, cathelicidins, lysosyme, and secreted phospholipase A2 (sPLA2). The production of Paneth cell antimicrobial peptides is affected by the composition of the microbiota[22] and increases with age.[23–26] Paneth cells reside at the base of the crypt in close contact with the epithelial stem cell compartment. The potent cocktail of antimicrobial products produced by paneth cells is therefore thought to play a role in the protection of these vital cells from bacterial invasion. Mice lacking Paneth cells have increased bacterial translocation[27] possibly predisposing them to the development of NEC. Conflicting reports exist on the number of Paneth cells in NEC patients.[28–30] As antibacterial peptide detection is used to identify Paneth cells and immature Paneth cells do not yet produce antibacterial peptides, discrepencies in the number of these cells reported in the literature may ultimately be due to a variable degree of maturation of this cell population or to degranulation of the Paneth cells during the NEC process. NEC typically occurs in the neonatal period when these peptides are not being produced at high levels thus supporting the premise that decreased Paneth cell differentiation and maturation may be a predisposing factor for NEC. Interestingly, Paneth cell metaplasia and increased expression of the paneth cell product alpha-defensin upon recovery from NEC has been reported.[30] A recent study suggests that Paneth cells may play an important role in NEC as immature mice (P14-16) treated with the zinc chelator dithizone to ablate Paneth cells develop NEC-like disease when infected with *Klebsiella pneumoniae*.[31]

The alpha-defensins (called cryptdins in mice) are the most abundant antimicrobial peptides made by Paneth cells. These are produced in an inactive form and converted into an active peptide after cleavage by proteases such as matrilysin (also called MMP-7). Active defensins are able to permeabilize gram-positive and gram-negative bacterial cell membranes. Altered alpha-defensin expression has been shown in NEC.[29]

Cathelicidins and beta-defensin are antimicrobial peptides produced by different cell types including epithelial cells and several populations of immune cells such as neutrophils, NK cells, B cells, and monocytes. They differ in structure from the alpha-defensins but have similar cationic amphipathic properties and are also effective against gram-positive and gram-negative organisms. In addition, they present chemotactic activity for neutrophils, monocytes, and T cells. In mice, their expression is extremely high during the neonatal period and decreases with maturity concomitant to an increase in Paneth cell maturity and antimicrobial peptide production. In a rat model of NEC, treatment with human beta-defensin-3 improved NEC and promoted mucosal integrity by reducing inflammatory mediators and reduced autophagy-activated proteins.[32] Furthermore, rats treated with Bifidobacterium increased beta-defensin-2 which provided protection from NEC.[33] However, whether deficient production of these antimicrobial peptides contributes to NEC remains unknown.

Lysozyme is a highly cationic protein and enzyme which cleaves β-1-4 glycosidic bonds of gram-positive bacteria. This causes the destabilization of the bacterial peptidoglycans leading to cell lysis. Gram-negative bacteria are resistant to this mechanism due to their outer shell that encases their peptidoglycan layer. Lysozyme has been shown to be absent in the Paneth cells of newborn infants with NEC[28] and may play an important role in preventing bacterial invasion in the neonatal intestine.

Secreted phospholipase A2 (sPLA2) is another antimicrobial protein produced by Paneth cells. It acts on gram-positive bacteria through a mechanism that hydrolyzes phospholipids. Group II phospholipase A2 is developmentally regulated after birth and was found to increase from D15 to D21 in the neonatal rat ileum.[34] The expression of sPLA2 has been shown to be increased in a neonatal mouse NEC model.[35] Phospholipase A2 is an enzyme critical for the production of platelet-activating factor (PAF),[36] which has been shown to mediate intestinal injury[37] and experimental NEC.[38]

Intestinal alkaline phosphatase (IAP) is a brush border enzyme produced by intestinal epithelial cells. It is expressed in decreasing concentrations from the duodenum to the ileum. IAP has multiple functions including the regulation of lipid metabolism, the regulation of bicarbonate secretion, as well as the detoxification of bacterial lipopolysaccharide (LPS).[39] By dephosphorylating the Lipid-A moiety of LPS, IAP prevents its interaction with toll-like receptor 4 (TLR-4). The level of IAP expression has been found to be decreased in NEC.[40] Furthermore, IAP supplementation in experimental NEC models decreased the severity of the disease, attenuated the systemic inflammatory response,[41] and increased barrier function with upregulation of claudins-1, and -3, as well as

occludin and ZO-1 following treatment.[42] In the stool of premature infants with NEC, relative IAP content was increased but had biochemical dysfunction.[43]

Trefoil Factor 3 (TFF3), like Muc2, is an important protein secreted by goblet cells. TFF3 is thought to play a role in the maintenance of the mucus layer and surface integrity by facilitating mucin polymerization.[44] Furthermore, TFFs play a significant role in epithelial cell restitution following injury promoting enterocyte migration, proliferation, and survival.[45] Mice lacking TFF and subjected to experimental DSS colitis die of complications due to impaired restitution.[46] Large amounts of TFF3 are present in breastmilk[47] and TFF3 expression has been found to be decreased in NEC tissues.[48] Treatment of mice with recombinant human TFF3 during experimental NEC reduced inflammation which suggests a protective role of TFF3 in NEC.[49]

IgA molecules are transported from the basolateral to apical epithelial surface and secreted. These antibodies act in the mucus layer to inhibit attachment of microorganisms to the epithelial cells. In term neonates, the synthesis of secretory IgA is very low and takes 2 weeks or more after birth for normal production.[50] Colostrum is rich in IgA.[51] Breast milk from mothers of preterm infants was found to have higher IgA compared to those of term infants.[52] Breastmilk-derived IgA has been shown to shape the host-microbiota relationship of preterm neonates[53] and pups reared by IgA-deficient mothers are more susceptible to experimental NEC, suggesting that IgA is critical for preventing NEC in a mouse model.[53]

Lactoferrin is an iron-binding protein present in breast milk and in most exocrine fluids such as tears, saliva, bile, and pancreatic secretions.[54] Lactoferrin provides protection against bacterial translocation via several mechanisms: (1) by binding iron which is necessary for bacterial growth; (2) via the toxic effect of its metabolite, lactoferricin, which disrupts gram-negative bacteria cell membranes; (3) by binding microbe-associated molecular pattern (MAMP) such as endotoxin, CpG, flagellin, and secondary inflammation; (4) by promoting the growth of probiotics;[55] (5) by promoting intestinal epithelial cell proliferation.[56] Lactoferrin concentration is very high in human colostrum.[57] Oral lactoferrin prophylaxis has been found to reduce the incidence of late-onset sepsis in infants weighing less than 1500 g.[58] Recent work describes its effect on neonatal myeloid cells in their conversion to myeloid-derived-suppressor-cells, thus blocking intestinal inflammation and experimental NEC.[59] While large-scale randomized clinical trials are needed, current evidence does not support a protective effect against NEC of exogenous lactoferrin when given alone.[58–60]

The Epithelial Layer

Paracellular Permeability

Paracellular permeability is the passage of molecules across intercellular structures. Indeed, adjacent cells of the intestinal epithelial barrier are secured by several complex structures that are named, from luminal to basolateral side, the tight junction complex, the adherens junction complex, and the desmosome (Fig. 2). Besides a recent study showing that desmoglein-2, a component of desmosome, is increased with increased NEC severity in a pig model,[61] no current data exist on desmosomes in NEC. Detailed studies looking at tight junction and adherens junction complexes have been performed and are discussed below.

The Tight Junction Complexes

Intercellular tight junctions are protein structures formed on the apical surface between adjacent cells of the epithelial barrier which regulates the passage of water, ions, and large solutes across the epithelium via the paracellular pathway (Fig. 3).[62,63] Tight junctions are made of several structural and functional proteins which regulate their function, such as occludin and claudins.[64] Their extracellular domains interact with the proteins on the adjacent cell membrane while their cytoplasmic tails associate with scaffold proteins called zonula occludens (ZO-1, ZO-2, and ZO-3).[65] These protein complexes associate with a variety of kinases and cytoskeletal proteins such as actin and myosin to regulate barrier function. The apical surface of the epithelial cell is circled by a belt of actin and myosin. Upon phosphorylation of the light chain of myosin (MLC) by activated myosin light chain kinase (MLCK), the actin-myosin ring contracts leading to tight junction reorganization and increased paracellular permeability.[66] Several inflammatory mediators such as TNF and IL-6 have been found to increase paracellular permeability.[67,68] TNF for example has been shown to activate MLCK.[69] During human development, TJ complexes have been detected in the fetal intestine as early as 10 weeks of gestation.[70] We have shown that pups exposed to an experimental NEC protocol have TJ restructuring with increased apical-to-basal tight junction length prior to the development of intestinal injury.[5]

The claudin family of proteins has been shown to control charge selectivity, and ion and small molecule permeability[64,71] and mutations in claudins have been shown to disrupt paracellular transport.[72] Several members of the claudin family are expressed in the intestine. While the role of claudins 7, 12, and 15 is unclear, claudins 1, 3, 4, 5, and 8 have been shown to tighten the barrier or decrease permeability. In contrast, claudin-2 is pore-forming and its expression leads to increased permeability. In mice, the gene expression of the intestinal claudins has been shown to be developmentally regulated.[73] In neonatal rat pups, hypoxia/reoxygenation induced the downregulation of claudin 1, 14, and 15 and the upregulation of claudin 8 and of the gap junction protein, beta 3.[74] In a neonatal rat model of NEC, the expression of claudin 3 and occludin has been found to be increased at 96 hours in the intestine of stressed pups compared to controls.[3] This may be a compensatory mechanism of the intestine to re-establish its barrier function. Indeed, in neonatal mice, intestinal claudin 3 expression has been found to increase in the first 2–3 weeks after birth and this increase to be associated with a decreased in intestinal barrier permeability.[75] Claudin 3 increase was dependent upon bacterial colonization.[75] In humans, when measured in the urine, claudin 3 has been shown to be a useful diagnostic marker for the detection of NEC in infants.[76] In a neonatal mouse NEC model, we found that claudin 2 expression is increased at 6 hours while claudin 4 and 7 are decreased at 24 hours.[5] Claudin 2 protein is increased in the intestine of neonatal mice submitted to a NEC protocol for 48 hours and in human NEC tissues compared to controls.[5] Interestingly, claudin 2 is also increased in the intestinal tissues of patients with inflammatory bowel disease[77,78] and to be positively correlated with inflammatory activity.[78] IL-6 has been shown to increase the expression of claudin 2, thus increasing TJ permeability.[79] Whether the increase in claudin 2 is injurious or serves as a compensatory protective mechanism remains to be determined. Indeed, claudin 2 increases paracellular channel-like permeability to monovalent cations such as sodium.[80] The resulting absorption of nutrients such as glucose and amino acids may prevent malnutrition

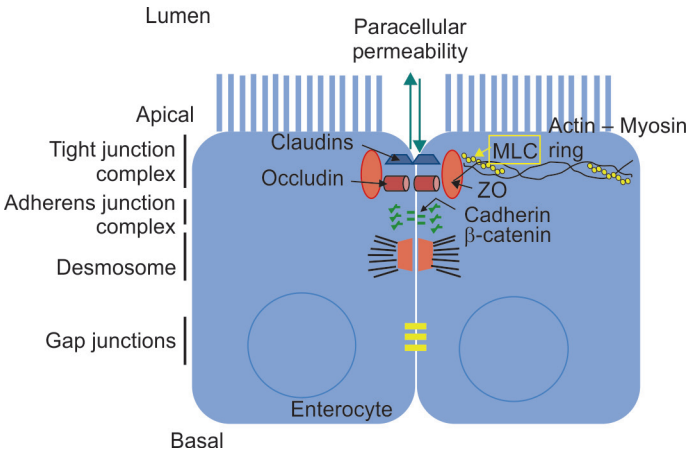

Fig. 2: Intercellular structures playing a role in paracellular permeability

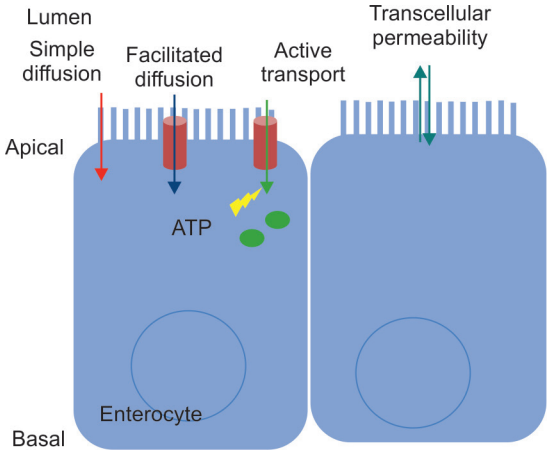

Fig. 3: Transcellular mechanisms responsible for transcellular permeability

during a time of stress, and therefore may be protective. Another potential protective mechanism may be, by increasing intestinal water loss, help flushing pathogens from the intestinal lumen. The distribution of TJ proteins is altered during several disease processes.[77,81] In patients with ulcerative colitis, claudin 4 association with TJs has been noted to be lost in the colonic epithelium.[77] In a neonatal rat model of NEC, the association of ZO-1 with TJ has been found to be lost at day 5.[82] In neonatal mice, we found that claudins 2, 4 and occludin are densely localized to TJ structures and claudin 7 is mainly associated with enterocyte lateral membranes.[5] When neonatal mice were exposed to a NEC protocol for 12 hours, which is a time when injury has typically not yet occured, the association of claudin 4 with TJ structures was markedly decreased, and occludin and claudins 4 were mainly found in the cytoplasm.[5] Furthermore, administration of *Bifidobacter* (*B.) infantis* preserved claudin 4 and occludin distribution at TJ, significantly attenuated stress-induced intestinal permeability and NEC in a mouse model.[5]

The Adherens Junction Complexes

The adherens junction complexes are made of transmembrane epithelial cadherin (E-cadherin) proteins and cytosolic proteins named alpha and beta-catenin. E-cadherin forms homophilic interactions with the neighboring cell thus conferring bonds that are necessary for epithelial strength and support. Additionally, adherens junctions are critical in directing cell polarity along the apical-basolateral axis.[83] While their role in NEC is not known, these proteins have been shown to be differently localized in the intestine of pups submitted to a NEC protocol compared to dam fed controls,[15] and this change was prevented by the inoculation of the probiotic *Bifidobacterium bifidum*.[15] E-cadherin expression has been shown to be decreased in human NEC and experimental NEC, where it was shown to be internalized.[84] Also, the cytoskeletal protein vinculin, known to be associated with adherens junctions, has been found to be decreased in formula-fed neonatal mice compared to dam-fed controls.[85]

Transcellular Permeability

The intestinal epithelium has multiple transport mechanisms for molecules to gain entry into the cellular compartment (Fig. 3). Depending on the specific molecules envolved, it can occur by simple diffusion through the cell membrane, by diffusion through channels and pores, by facilitated diffusion utilizing transport proteins, or by osmosis. Passive transport is driven by concentration gradients. In contrast, active transport is able to move molecules against a concentration gradient and requires the use of energy. Large particles or macromolecules can gain entry into a cell by an active transport process consisting of endocytosis which may or may not be receptor mediated. Transcytosis is the transcellular

transport process that involves endocytosis followed by vesicular transport across the cell to the opposite membrane where exocytosis occurs. Transcytosis thus provides a route of entry from the intestinal lumen to the underlying lamina propria. This process is particulary important in the transport of maternal IgG to the infant, conferring immunologic protection prior to the maturity of their own adaptive immune system.[86] In addition, many pathogens are known to exploit this mechanism leading to dysfunction of the epithelial barrier,[87] and *E. coli* transcytosis has been hypothesized to be an initiating event in necrotizing enterocolitis.[88] Alpha-haemolysin from *E. coli* has been shown to induce focal leaks in colonic epithelial cells which causes increase bacterial translocation.[89] Several strains of commensal bacteria and probiotics have been shown to increase TJ proteins at the cell boundaries and in some cases prevents or reverses the adverse effects of pathogens.[90] In addition, many nutrients have been shown to impact barrier permeability.[90] Luminal antigens that reach the lamina propria have the potential to initiate an immune response. This response includes the production of inflammatory cytokines that may further facilitate transcellular permeability. Specifically, TNF can increase endosomal uptake and enhance transcellular transport.[91] Also, interferron-γ)(IFNγ) which has been shown to play an important role in NEC[92] enhances transcytosis of macromolecules.[93] Increased transcytosis of luminal molecules occurs in conjunction with tight junction reorganization and increased paracellular permeability[91] and barrier function may be affected through alterations of both transcellular and paracellular transport.[94]

Although epithelial cells are able to capture antigens and microbes, transcellular transport is mainly thought to occur at the level of M-cells (M for microfold) which cover isolated lymphoid follicles or Peyer's patches.[87] M-cells are specialized epithelial cells of the follicle-associated epithelium. They take up antigens and microorganisms from the intestinal lumen via transcytosis and present them to dendritic cells and other immune cells such as macrophages and lymphocytes. As their glycocalyx (protective outer cell layer composed of glycoproteins and glycolipids) is thinner than enterocytes, M-cells constitute a functional opening in the intestinal mucosal barrier.[95] During inflammation, there is increased apoptosis of M cells that may contribute to the breakdown of the intestinal barrier.[96] However, not much is known about the developmental maturation of M cells and their potential role in NEC.

Epithelial Cell Layer Integrity

The single layer of epithelial cells is a highly regulated barrier. The cells are replaced approximately every 4–5 days through a process of proliferation, differentiation, migration, and apoptosis. This process is initiated by rapidly cycling epithelial stem cells situated at the base of the small intestinal crypt. Signaling events instruct these newly created immature transit amplifying cells to differentiate into one of four main cell types of the small intestine. Enterocytes are the most numerous and perform the absorptive functions of the barrier. Paneth, goblet, and enteroendocrine cells are of secretory lineage. Paneth cells migrate downward surrounding the stem cells. They are relatively long lived, with a turn-over time of 57 days in mice.[97] The other cell types migrate upward toward the villus tip where they eventually undergo apoptosis and are replaced. During villous tip shedding, MLC gets phosporylated and tight junction reorganization occurs. This process is vital to the normal turnover of enterocytes and does not compromise the barrier.[98] However, when this process is impaired, tight junction function may be affected, impacting barrier permeability. To maintain the integrity of the intestinal epithelial layer, the proliferation and differentiation of intestinal stem cells, and the migration and apoptosis of intestinal epithelial cells are tightly regulated and synchronized.[99] Upregulated apoptotic rate increases permeability and bacterial translocation.[100] Unbalanced epithelial proliferation and apoptosis may be a contributing factor in the loss of the intestinal barrier function and in NEC development. Indeed, NEC has been associated with a decrease in intestinal epithelial cell proliferation and migration and with an increase in intestinal epithelial cell apoptosis.[101] LPS, the ligand of TLR-4, has been shown to play an important role in NEC[102,103] and to inhibit enterocyte migration via increased expression and function of the adhesion molecule alpha 3- and beta-1 integrin[104] and via autophagy.[105] Also, TLR4 expressed on intestinal stem cells regulates enterocyte proliferation and apoptosis and may contribute to the pathogenesis of NEC.[106] Indeed, TLR4 activation has been shown to inhibit beta-catenin signaling via GSK3 β activation thus reducing enterocyte proliferation.[107] An inhibitory interaction between TLR4 and NOD2 signaling in enterocytes leads to the regulation of enterocyte apoptosis and NOD2 may have a protective effect on NEC.[108]

Nonepithelial Influences

Immune Cells and Inflammatory Mediators

Other cell types may affect intestinal barrier permeability indirectly. Indeed, during inflammation, activated macrophages inhibited enterocyte migration and mucosal healing via the release of nitric oxide[109] and the activation of RhoA.[110] IFNγ inhibits enterocyte migration by impairing enterocyte gap junctions, which are intercellular channels composed of connexin43 (Cx43) monomers. Mesenchymal stem cells have been shown to enhance the viability and proliferation of human fetal intestinal epithelial cells following hypoxic injury via paracrine mechanisms.[111]

In the intestine, dendritic cells and CX3CR1^{+} macrophages maintain direct contact with epithelial cells through dendrites that extend from the mucosa to the lumen to sample antigens in the external environment. These cells express tight junction proteins allowing them to span the intercellular space while maintaining an intact barrier.[112,113] The close proximity of epithelial and immune cells facilitates the cytokine and chemokine signaling necessary to initiate an immune response upon a barrier breach.

While the exact mechanism is unknown, the interaction of inflammatory cells with tight junctions may contribute to the TJ restructuring and barrier dysfunction seen in diseases such as NEC. Activated dendritic cells and macrophages secrete a variety of cytokines, which are increased in human and experimental NEC.[114,115] Many of these factors are essential for an appropriate immune response but may also have a negative impact on barrier function. Specifically, TNF at high doses has been shown to induce apoptosis and shedding due to a major redistribution of the tight and adherens junction structures.[116] Low-dose TNF in contrast does not induce cell shedding yet impacts barrier permeability through MLCK activation and endocytosis of occludin.[117] IL-1 β is also known to increase tight junction permeability in an MLCK and NF-κB-dependent pathway without causing apoptosis[118,119] and IL-6 has been shown to increase intestinal permability via the upregulation of claudin 2 mRNA and protein expression both *in vitro* and *in vivo*.[79,120]

During inflammation, effector cells are recruited to the site of injury via the secretion of chemokines such as CCL20 and CCL2 by epithelial and innate immune cells. Recruitment of dendritic cells through the CCL20/CCR6 axis was shown to be responsible for intestinal epithelial damage in a model of NEC.[121] Recruited

T cells secrete Th1 cytokines such as IFNγ and the Th2 cytokine IL-13. Epithelial cells respond to IL-13 by upregulating claudin 2 and thereby increasing permeability.[77] IL-13 also induces epithelial cell apoptosis and decreases proliferation, causing epithelial microerosions and bacterial translocation.[114,122] IFNγ has been shown to increase the intestinal epithelial permeability to macromolecules via the Src kinase pathway. In addition, IFNγ was found to synergize with TNF to induce barrier dysfunction.[123]

Many pro-inflammatory cytokines are under the control of the transcription factor NF-κB, which has been shown to be a major effector molecule in NEC.[124] NF-κB is essential for signaling in both epithelial and immune cells. TNF, IL-1β, and TLR ligands activate the NF-κB signaling pathway and can trigger amplification of the immune response. Also, NF-κB is known to protect the cell against apoptosis and inhibition of NF-κB specifically in epithelial cells leads to a loss of barrier function by inducing apoposis and bacterial translocation.[125] While increased intestinal permeability may be necessary to induce intestinal injury in experimental NEC, it is not sufficient as we found that blocking NF-κB activation in monocytes prevented against NEC without impacting intestinal permeability.[126]

Cytokines produced by immune cells can alter barrier function not only by causing tight junction structure alteration, but also by altering transcytotic mechanisms and by causing apoptotic leaks and mucosal gross lesions.[122]

Commensal Bacteria and Probiotics

Commensal bacteria and probiotics upregulate TJ proteins and may prevent the adverse effects of pathogens on intestinal barrier.[90] The beneficial effect of probiotics such as *Lactobacillus acidophilus* and *B. infantis* in human NEC[127] may be mediated by its effect on intestinal permeability. In human preterm neonates, supplementation of formula with *Bifidobacterium lactis* decreased intestinal permeability at day 30 of life.[128] In a neonatal mouse model of NEC, *B. infantis* has been found to attenuate the increase in intestinal permeability observed at 24 hours and to decrease the incidence of NEC.[5] In this same model, *B. infantis* preserved claudin 2, 4 and occludin integrity at TJ structures and claudin 7 at lateral membranes.[5] *In vitro*, when T84 cells (human colon carcinoma cell line) were treated with *B. infantis* conditioned medium, the expression of claudin 4, ZO-1 and occludin was increased, claudin 2 was decreased and the IFNγ-induced rearrangement of occludin and claudin 1 was prevented.[129] Also, *B. bifidum* reduces intestinal epithelial cell apoptosis in a neonatal rat NEC model.[130] Conditioned medium from *B. infantis* has been found to decrease apoptosis and maintain epithelial cell proliferation in a model of neonatal intestinal inflammation induced by *Cronobacter sakazakii*.[131]

Amniotic Fluid

During development, the intestinal epithelium is exposed to a diversity of bioactive molecules present in the amniotic fluid such as growth factors, which promote mucosal morphogenesis, and cytokines, which have immunomodulatory and anti-inflammatory properties.[132,133] These molecules have protective effects on barrier function, and therefore, postnatal enteral administration of amniotic fluid has been hypothesized to protect against NEC.[134,135] Amniotic fluid has been shown to increase cell migration, proliferation, and cell survival *in vitro* and these effects were dependent on PI3Kinase and were reproduced by HGF treatment.[136] Stem cells isolated from the amniotic fluid have been shown to improve enterocyte cell survival and enhance repair of damaged intestine in NEC via a COX-2 dependent mechanism.[137] Furthermore, isolated amniotic stem cells restored tight junction protein expression in mice.[138]

Breast Milk

Preterm infants who received the majority of feeding as human milk had significantly lower intestinal permeability when compared to infants receiving minimal or no human milk.[139] Breast milk contains many molecules such as trefoil factor[47] and lysozyme[140] that may improve intestinal permeability and protect the neonatal intestine against injury. Several components of breast milk such as hyaluronan 35kD,[141] lactadherin,[61] TIMP-1,[142] and exosomes[143] have been shown to protect TJ proteins. Also, breast milk oligosaccarides protect against NEC by inhibition of TLR4 signaling[144] and were also found to interact with bacterial receptors, inhibiting the binding of pathogenic bacteria with intestinal epithelial cells and preventing bacterial invasion.[145] Breast milk oligosaccharides can also serve as a food source for commensal bacteria promoting their growth and therefore limiting the growth of pathogenic species or preventing intestinal inflammation.[146,147] Heat Shock Protein 70 is induced in enterocytes by exposure to breast milk and has has been shown to preserve barrier function.[148] In addition, breast milk contains many immune cells (such as monocytes and lymphocytes) which downregulate the inflammatory response of the immature intestine.[133]

Growth Factors

Both amniotic fluid and breast milk contain growth hormones which promote the integrity of the intestinal barrier:

Epithelial Growth Factor (EGF)

EGF, which present in breast milk,[149] has been shown to protect against NEC in neonatal rats.[150] There is evidence that some effect of EGF on NEC[150] might be mediated via a protective mechanism on the intestinal barrier. Indeed, in a neonatal rat model of NEC, EGF has been found to abrogate the increase in intestinal occludin and claudin 3 found in the intestine of pups exposed to the NEC model.[3] In caco-2 monolayers, EGF has been found to reverse the increase in epithelial permeability and occludin dephosphorylation and rearrangement induced by bile acids.[151]

Heparin-binding EGF (Hb-EGF)

Hb-EGF is a member of the EGF family produced by macrophages which is present in breast milk.[152] It has been found to increase enterocyte proliferation and migration in a rat model of NEC[153] and has been found to protect against NEC.[154]

Transforming-growth Factor-beta (TGF-beta)

TGF-β is an extracellular peptide which has anti-inflammatory properties and promotes cell differentiation, migration, and cell death in the intestine. TGF-β is present in breastmilk[155] and is secreted by many cell types including immune cells. Monocytes from infants with NEC have reduced TGF-β expression.[156] In rodents, TGF-β administration has been shown to decrease the severity of experimental NEC.[157] TGF-β has been shown to be assosciated with the restoration of intestinal morphology and barrier function in pigs following weaning stress.[158]

Erythropoietin

Erythropoitin is a glycoportein present in breast milk that controls erythropoiesis.[159] It has also been shown to protect epithelial cells

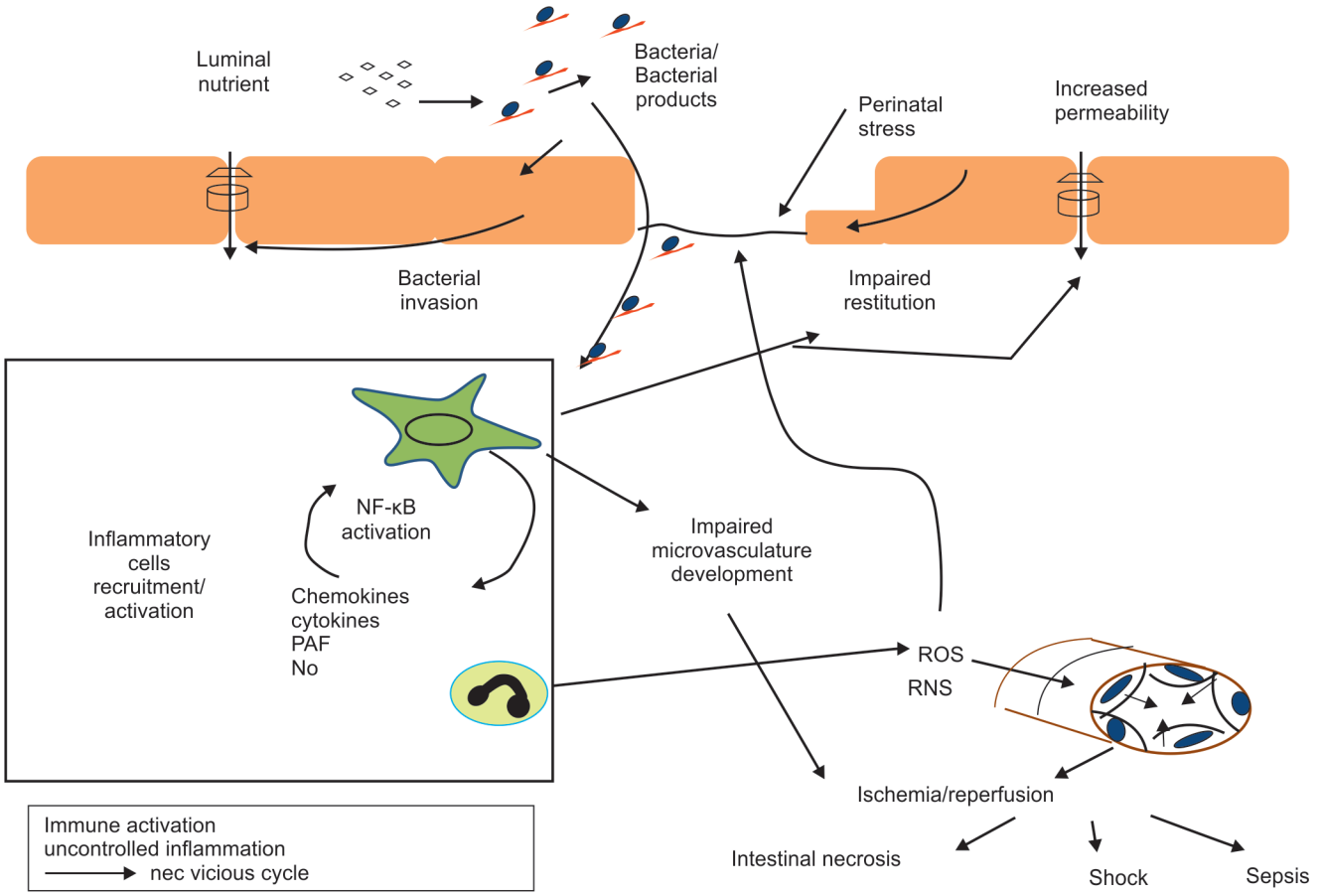

Fig. 4: Illustration of our current understanding of NEC pathogenicity

against autophagy and apoptosis thus preserving intestinal barrier function to protect against NEC.[82,160]

Conclusion

In humans, many mechanisms contribute to tighten the intestinal barrier. In premature infants, several of these mechanisms are immature, which affect the intestinal epithelial barrier function. These include decreased mucus production, decreased amounts of antibacterial peptides, and decreased intestinal motility due to an immature enteric nervous system. This may lead to bacterial translocation, with subsequent activation of NF-κB in lamina propria immune cells, causing them to secrete pro-inflammatory mediators such as chemokines (CXCL2), cytokines (TNF, IL1β), prostanoids, platelet-activating factor, and nitric oxide (Fig. 4). These inflammatory agents further recruit inflammatory cells inducing reactive oxygen species production, and causing further damage to the intestinal barrier resulting in the translocation of bacteria and their products, intestinal epithelial injury, impairment of epithelial cell restitution, apoptosis and mucosal necrosis. In severe NEC, a vicious cycle is thus created where gut barrier failure causes bacterial invasion, immune activation and uncontrolled inflammation, production of reactive oxygen and nitrogen species, vasoconstriction, secondary ischemia-reperfusion injury, intestinal necrosis, sepsis and shock. While the pathogenesis of NEC is multifactorial with involvement of the immune system and the microvasculature, measures aimed at improving barrier function in premature infants may prevent NEC and/or slow down the progression of the disease.

Summary Box

- Intestinal permeability is increased prior to the development of experimental NEC and breakdown of the intestinal barrier is thought to be a critical step in the development and the progression of necrotizing enterocolitis (NEC).
- Immature mucins and decreased antibacterial products such as alpha-defensins, cathelicidins, lysosyme, and secreted PLA2 due to decreased Paneth cell differentiation and maturation may be a predisposing factor for NEC.
- Peri-junctional cytoskeletal condensation occurs at the TJ complex in NEC prior to the development of intestinal injury and tight junction dysfunction may contribute to the increase in permeability preceding NEC.
- Increased apoptosis and decreased epithelial cell restitution may play a role in the altered barrier function seen during NEC.
- Breast milk and amniotic fluid improve intestinal barrier and thus protect the intestinal mucosa against NEC.

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REVIEW ARTICLE

Role of the Endothelium in Neonatal Diseases

Olachi J Mezu-Ndubuisi[1], Akhil Maheshwari[2]

Abstract

In both fetal and neonatal physiologic and pathologic processes in most organs, endothelial cells are known to play critical roles. Although the endothelium is one of the most ubiquitous cell type in the body, the tight adherence to the blood vessel wall has made it difficult to study their diverse function and structure. In this article, we have reviewed endothelial cell origins and explored their heterogeneity in terms of structure, function, developmental changes, and their role in inflammatory and infectious diseases. We have also attempted to evaluate the untapped therapeutic potentials of endothelial cells in neonatal disease. This article comprises various peer-reviewed studies, including ours, and an extensive database literature search from EMBASE, PubMed, and Scopus.

Keywords: Angiogenesis, Bronchopulmonary dysplasia, Endothelium, Necrotizing enterocolitis, Neonate, Retinopathy of prematurity.

Newborn (2022): 10.5005/jp-journals-11002-0025

Impact

- We reviewed the scope of endothelial cell heterogeneity, along with the endothelial cell structure and function as seen in the fetus and neonate.
- Endothelial cells are a diverse subtype of cells and play vital roles in innate immunity, angiogenesis, tissue homeostasis, repair of tissues, tissue inflammation, and cellular apoptosis in numerous inflammatory and infectious diseases.
- Evolutionary mechanisms regulating endothelial cell heterogeneity vary *in vivo* and *ex vivo*.
- Endothelial cells are important therapeutic mediators in the vasculature of numerous neonatal disorders.

Introduction

Endothelial cells are metabolically active cells bordering the blood vessels inner lining, where they have a crucial function in both physiology and pathology. Due to their critical anatomic location, these cells have always been believed to have unlimited therapeutic potential, but the relative inaccessibility of the endothelium in intact organs has curtailed detailed *in vivo* studies. Recent advances in diagnostic microtechnology have provided some solutions to this problem, at least in larger blood vessels, and have renewed the scientific interest in these cells. These cells are important regulators of trans-vascular blood-to-tissue barrier to macromolecules and nutrients, trafficking of leukocytes between blood and inflamed tissues, and of tissue respiration via both hemodynamic homeostasis and neoangiogenesis. With the dispersive, arboreal vascular arrangements, endothelial cells are distributed throughout our body.

Abnormalities in the function of endothelial cells are depicted in several neonatal conditions, such as intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), hypoxic-ischemic encephalopathy (HIE), bronchopulmonary dysplasia (BPD), acute kidney injury (AKI), and necrotizing enterocolitis (NEC). Endothelial markers may be helpful in the diagnosis, monitoring, prognosis, and clinical management of many neonatal conditions. Therapeutic targeting of microvascular structure and function may also be useful in neonatal conditions. The current article merges peer-reviewed evidence arising from our research as well as extensive literature review from notable databases, such as Scopus, EMBASE, and PubMed.

[1]Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States of America

[2]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Olachi J Mezu-Ndubuisi, Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States of America, Phone: +6082638558, e-mail: olachimezu@pediatrics.wisc.edu

How to cite this article: Mezu-Ndubuisi OJ, Maheshwari A. Role of the Endothelium in Neonatal Diseases. Newborn 2022;1(1):44–57.

Source of support: The study was made possible by the McPherson Eye Research Institute's Retina Research Foundation Edwin and Dorothy Gamewell Professorship Award (to OJM), and NIH awards 1K08EY032203-01 (to OJM) and HL133022 and HL124078 (to AM).

Conflict of interest: None

Origin of Endothelial Cells

The vascular system plays a vital homeostatic role in all vertebrates by promoting nutrient transport, oxygen, waste products and metabolites, immune surveillance, and the autoregulation of perfusion via chemical stimuli and hormones that help in the communication between the blood vessels and underlying tissues. Endothelial progenitor cells (EPCs) were discovered in the late 1990s resulting in a paradigm shift in our understanding of angiogenesis. The endothelium consists of a single layer of cells lining blood vessels in the body and is formed very early in gestation. Angiogenesis refers to the formation of new capillaries from existing vessels, while vasculogenesis refers to *de novo* formation of blood vessels during embryonic development.[1–3] There are several sources of endothelial cells, including the neural crest cells, embryonic mesoderm, and hemangioblasts. These have been summarized in Figure 1.

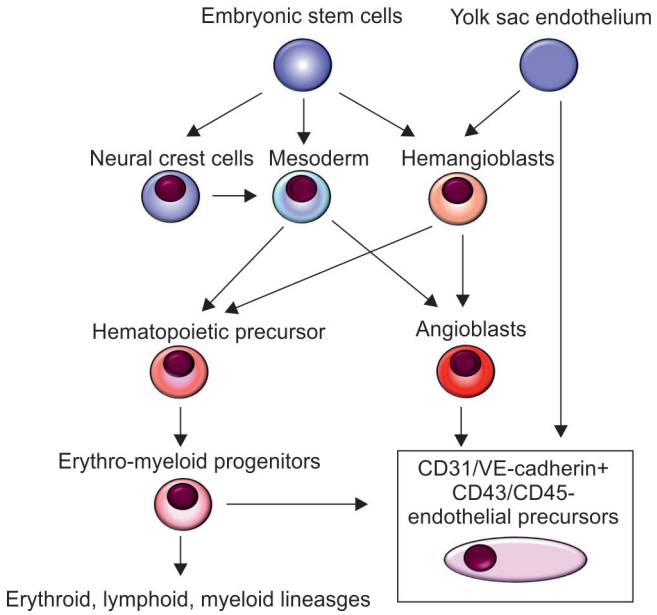

Fig. 1: Origin of endothelial cells. Overall schematic of the common origin of endothelial progenitor cells and the erythroid, lymphoid, myeloid precursors. Hematopoietic and endothelial progenitor cells are derived from a common precursor, the hemangioblast. Embryonic stem cells give rise to neural crest cells, mesoderm, and hemangioblasts. Hemangioblasts are derived from the yolk sac endothelium. Neural crest cells differentiate into mesenchymal stem cells, which tissue-resident precursors through chondro-, osteo- and adipogenesis. Endothelial precursors can arise from the yolk sac, myeloid precursors, and hemangioblasts

- *Differentiation from embryonic mesoderm:* These primitive mesodermal cells differentiate into hematopoietic precursors or angioblasts, and these cellular subsets can both develop into endothelial cells.[4,5] The intraembryonic endothelium forms a primitive vascular labyrinth[2,6] shortly after gastrulation in the extraembryonic yolk sac. The endothelial/vascular maturation of mesodermal cells is induced by signals emanating from visceral endoderm,[7] such as increased production of growth factors such as the basic fibroblast growth factor (bFGF) or FGF2, bone morphogenetic protein 4 (BMP4), and the vascular endothelial growth factor (VEGF).[3,8]

The transition of mesenchymal into endothelial cells may be a reversible, bidirectional process. The activation of transforming growth factor-β, bone morphogenetic protein, wingless/integrated (Wnt), and the Notch signaling pathways may be important in a mechanistic sense.[9,10] The "dedifferentiation" and activation of endothelial cells involve a change in appearance from a characteristic cobblestone to a more elongated, "mesenchymal" shape with increased migratory and proliferative capacity. These transformed cells lose some of the intercellular junctional proteins and related barrier function[7,10] but become pro-inflammatory with higher levels of leukocyte adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion molecule 1), cytokines, and various growth factors.[11] However, with alterations in function, these changes may also shorten the lifespan of these cells. Such endothelial-to-mesenchymal transitions have been noted in various pathological conditions marked by vascular injury, chronic inflammation, and shear stress.[10,11]

- *Differentiation from hemangioblasts:* Plein et al.[12] showed that nearly a third of all endothelial cells in the brain and up to 60% of those in the liver may originate from hemangioblasts differentiating into erythro-myeloid progenitors (EMPs). These EMP-derived endothelial cells express high levels of the gene *Hoxa*. In another study, Feng et al.[13] showed that these intraembryonic endothelial cells likely do not originate from circulating EMPs that express the cluster differentiation (CD) 45 (protein tyrosine phosphatase receptor type C)+. Csf1r-expressing EMPs may also not consistently differentiate into endothelial cells in the brain, liver, heart, and lungs.

The term "hemangioblast" was coined by Murray[14] early in the 20th century to describe a subset of cells that can differentiate into either endothelial or hematopoietic cells during embryogenesis. This hypothesis found favor in the physical proximity of hematopoietic and endothelial lineages within blood islands,[14,15] but the conclusions were not definitive due to the structural complexities in the developing blood islands and also because of the limited number of cells available to study during these early stages of development. Previous imaging and tissue engineering indicating spatiotemporal associations between these embryological hematopoietic and endothelial lineages[16,17] and studies indicating that human embryonic stem cells are differentiated *in vitro* into both hematopoietic and endothelial cell lineages[18] have largely been refuted.

Recent literature suggests that some of the hemogenic endothelium may be a source of hematopoietic stem cells (HSCs). Lineage-tracing studies, *ex vivo* culture, and time-lapse confocal imaging show that hematopoietic cells, including HSCs herald from a hemogenic endothelium, and form an intermediate endothelial state.[4,19,20] Hemogenic endothelium is a specialized subset of the endothelium with only a transient capacity to produce hematopoietic cells through endothelial-to-hematopoietic transition.[21] In murine models, endothelial cells that lose endothelial characteristics to assume a more hematopoietic phenotype begin to co-express surface markers CD144, CD31, KDR, CD117, and CD34, but not the hematopoietic markers, such as CD41, CD45, CD73, and Ter-119.[21–24] Human hematogenic endothelial cells express the surface markers CD43, CD34, CD144, CD117, CD90, CD45, and CD105, but low CD38, and almost not CD45RA.[22,25]

The HSCs are self-renewing cells with multilineage reconstitution potential following transplantation into a recipient. After birth, HSCs are seen predominantly in the bone marrow and form a self-renewing pool at the apex of the hierarchical network of hematopoiesis. Some HSCs are also known to differentiate into hematopoietic progenitor cells (HPCs).[22,26] The HPCs differ from HSCs with relatively limited self-renewal and engraftment potential.

Bone marrow-derived EPC: EPCs in the bone marrow have been redefined in numerous recent consensus statements to possibly originate from the following:

- Endothelial Progenitor Cells: EPCs express surface markers, such as factor VIII, CD31, CD34, E-selectin (CD62E), intercellular adhesion molecule (ICAM)-1 (CD54), von Willebrand factor (vWF), and VCAM-1 (CD106).[1,27–29] EPCs can migrate to the peripheral blood and express surface adhesion molecules that regulate the movement of these cells to and away from the blood.
- Mesenchymal Stem Cells: Data on mesenchymal stem cells (MSCs) being a source of endothelial cells are controversial. Colony-forming units of fibroblasts (CFU-Fs) in the bone marrow, also known as the MSCs, express CD29, CD71, CD73, CD90, CD144,

CD120a, CD105, CD106, and CD 124,[30–33] but no CD34, CD31, vWF, vascular endothelium cadherin (VE-cadherin), VEGFR2, CD62E, VCAM-1, and ICAM-1.[30–33] CD44 was detected in some studies,[31,33] but not in others.[30] MSCs expressing VEGFR2, vWF, and VE-cadherin are most likely endothelial progenitors.[30,32,33] The discovery that mesenchymal cells can rescue damaged endothelial cells was demonstrated using laser scanning confocal microscopy to show that mitochondrial transfer was facilitated by a tunneling nanotube-like structure between human umbilical vein endothelial cells and MSCs.[34]

Endothelial Cell Phenotypes

The phenotypic markers on endothelial cells can vary between various vascular structures in a particular organ and also between different organs. There may also be important structural variations notable within capillaries, veins, and arteries. The endothelium found in veins and arteries may seem to be comprised of an uninterrupted, continuous layer of cells; the capillary endothelium in various tissues can show more obvious differences and may appear continuous, discontinuous, or fenestrated.[35] These spatial and temporal variations have been correlated with differential expression of various messenger RNA (mRNA) and proteins.

The first reported arterial EC marker was a transmembrane ligand ephrinB2 arterial.[36] Notch signaling is vital to arterial EC differentiation. Loss of Notch signaling leads to a loss of the expression of ephrinB2 in the arteries in zebrafish.[37] The first reported marker for venous EC was the ephrin B2 receptor tyrosine kinase EphB4.[36] Venous EC differentiation is recognized as a default EC differentiation pathway, resulting from inadequate activation of Notch signaling during the differentiation of angioblasts to ECs.[38,39] Lymphatic ECs are formed as a result of differentiation from venous ECs. Prox-1 is the most functional and specific EC of lymphatic origin. Disruption of mouse Prox-1 disrupts lymphatic vessel development and budding of lymphatic ECs.[40] Insufficient activation of VEGFR3 signaling leads to hypoplastic lymphatic vessels.[41]

Endothelial cells have diverse microenvironments across vascular beds and display unique structural and morphologic heterogeneity across organs. The EC-translating ribosome affinity purification (TRAP) has emerged as a powerful tool to analyze the *in vivo* EC translatome across several diverse vascular beds to provide greater accuracy, sensitivity, and cellular resolution instead of whole-tissue RNASeq. TRAP identified 82 gene markers shared by five vascular beds (lung, heart, kidney, liver, and brain), such as *Tek* and pan-EC markers such as *Eng*, *Nos3*, *Cdh5*, and *Robo4*.[42]

Table 1 summarizes various endothelial cell markers; some are expressed after activation of growth factors and inflammatory cytokines, and others refer to specific endothelial cells in different organs or tissues. Endothelial cell phenotypes include the following:

- Endothelial cells precursors: Embryonic ECs as a cell lineage expand without contribution circulating precursors or new angioblasts are the current consensus.[12] The relationship of circulating endothelial progenitors to myeloid cells remains subject to controversy.[43] Cells of myeloid origin are $CD14^+$, while EPCs are $CD14^-$. However, monocytes or macrophages ($CD14^+$ cells) can adopt an endothelial phenotype during angiogenesis.[44]
- Brain Endothelium: In the central nervous system, endothelial cells regulate plasma filtration and the movement of circulating cells through the blood-brain barrier, most likely via the assembly of tight junctions.[45,46] Cerebral microvasculature likely originates from the meningeal vessels, but the subsequent angiogenesis involves the whole brain.[47]

Table 1: Specific human and murine endothelial cell markers

Type of marker	*Name of marker*	*Species expressed*	*Cells expressed*
Constitutive markers expressed in different endothelium	CD31/PECAM-1[154]	Human, murine	Endothelial cells, B and T lymphocytes, platelets, monocytes, neutrophils
	Bandeirea simplicifolia lectin binding[155]	Murine	Endothelial cells
	Vascular endothelial cadherin[39,156]	Human, murine	Endothelial cells, trophoblasts, macrophages
	CD34[20]	Human, murine	Endothelial cells, hemopoietic precursors
	Thrombomodulin[157]	Human, murine	Endothelial cells, smooth muscle cells
Monoclonal antibodies used to identify specific endothelial cells	BMA-120[158]	Human	ECs, mesothelium, glomerular epithelium
	EN4[158]	Human	Endothelial cells, leukocytes, platelets
	EN 7/44[159]	Human	Endothelial cells in tumors and inflammatory tissues
Endothelial cell markers induced by inflammatory cytokines	CD54/ICAM-1[22,160]	Human, murine	ECs, leukocytes, epithelium, fibroblasts
	CD62E/E-selectin[21]	Human, murine	Endothelial cells, postcapillary venules
Endothelial cell markers induced by angiogenesis	KDR/Flk-1 (VEGFR-2)[120,132,161]	Human, murine	Endothelial cells
	Flt-1 (VEGFR-1)[120,126,162]	Human, murine	Endothelial cells
	Tie-1[57,163]	Human, murine	Endothelial cells
	Tie-2/Tek[57,163]	Human, murine	Endothelial cells

CD, cluster differentiation; PECAM-1, platelet endothelial intercellular adhesion molecule; BMA, biotinylated monoclonal antibody; EN, endothelium antibody; E-selectin, endothelial cells selectin; KDR/Flk-1, kinase insert domain receptor/fetal liver kinase 1; Flt-1, Fms-related receptor tyrosine kinase 1; VEGFR, vascular endothelial growth factor receptor; Tie and Tek, receptor tyrosine kinase genes

Heterogeneity in EPCs

EPCs are a heterogeneous population of mononuclear cells originating in the bone marrow and can be mobilized to the fetal/postnatal circulation.[48–50] EPCs make up 1–5% of all bone marrow cells and about 0.0001–0.01% of monocytes circulating in the peripheral blood.[51] These cells express endothelial antigens, like CD31, vWF, VE-cadherin, endothelial nitric oxide synthase (eNOS), and VEGFR2.[52–55] The differentiation of hemangioblasts into endothelial cells has been studied in greater detail (Fig. 2). Based on phenotypical and biological properties, the EPCs are believed to be comprised of early and late EPC subgroups. Early EPCs give rise to the conventional colony-forming unit-endothelial cells (CFU-Es) and augment angiogenesis in a concentration-dependent or paracrine manner, whereas the outgrowth and differentiation of late EPCs promote the development of vascular networks.[56] Early EPCs are spindle-shaped, CD133 + CD45 +, and have limited proliferative capacity, a relatively short lifespan of about 3–4 weeks, and secrete angiogenic factors, such as VEGF, interleukin-8, and the CXC-ligand 8/CXCL8. Late EPCs are cobblestone-shaped, CD31 + KDR +, appear at 2–3 weeks, may live up to 12 weeks, proliferate rapidly, and express VE-cadherin, Flt-1, and CD45.[56,57] Both early and late EPCs seem to have comparable vasculogenic capacities.

Based on gene expression profiles, endothelial cells increasingly seem to be a heterogeneous population. Endothelial subpopulations have been identified that show differences in the expression of bone morphogenic protein-2, -4; ephrin-4, and neuropilin-1. In the skin, distinct endothelial cells express platelet and endothelial cell adhesion molecule 1 (PECAM-1), notch-1, and leukocyte markers (ICAM-1, L-selectin, notch 2, CD36, and CD163).[55] The aorta shows at least 3 distinct subpopulations, one comprised of lymphatic endothelial cells, whereas the other two seem to be specifically involved in angiogenesis, lipoprotein processing, and extracellular matrix production.[58] The adult mouse lung contains a distinct subpopulation of endothelial cells that expresses high levels of carbonic anhydrase 4 (Car4) and is distinct from arterial and venous macrovascular, and microvascular endothelial cells.[59] Car4-high endothelium is located throughout the lung periphery, expresses high levels of CD34 and VEGF receptors, and responds to VEGF-A. High numbers of Car4-high ECs can be seen in lung regions regenerating after influenza- or bleomycin-induced injury. The discovery of endothelial subsets with differing capacities for angiogenesis has opened exciting therapeutic possibilities.

Endothelial Cell Function

Endothelial cells show a vast heterogeneity in function. The vascular endothelium is exposed to and responds to numerous tissue microenvironments, resulting in a substantial phenotypic heterogeneity in the vascular system. Epigenetic and non-epigenetic factors are responsible for determining this heterogeneity in the endothelium. Marcu et al.[60] studied endothelial cells isolated from

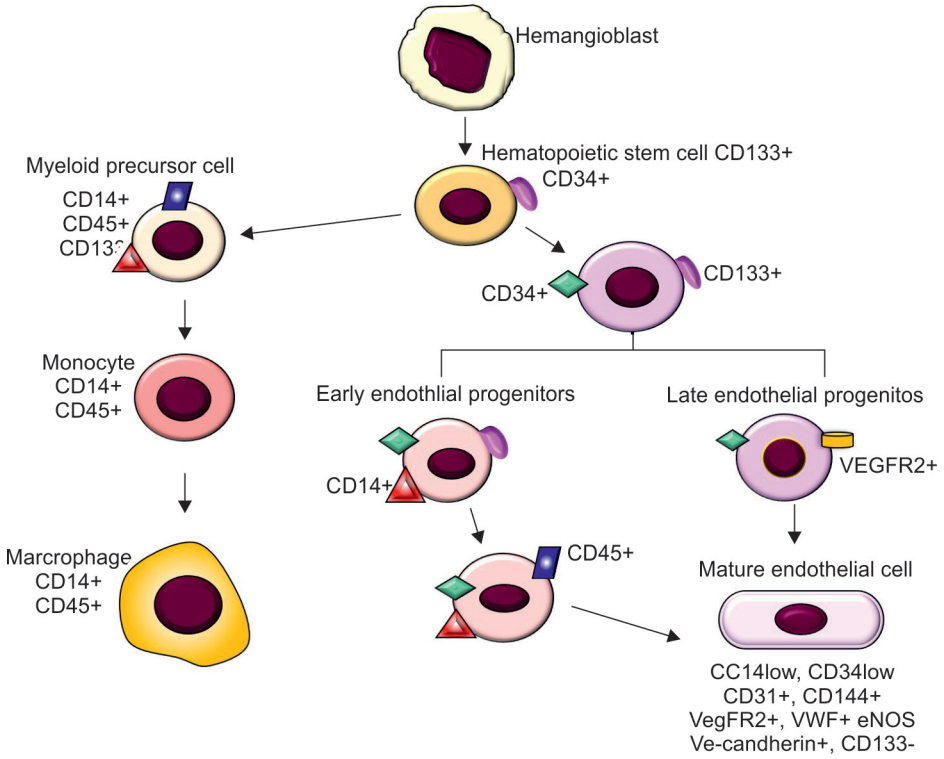

Fig. 2: Differentiation of endothelial progenitor cells. Hemangioblasts differentiate into hematopoietic stem cells and endothelial progenitor cells. Hematopoietic stem cells and endothelial progenitor cells express three markers cluster of differentiation (CD) 34, CD 45, CD133, and vascular endothelial growth factor receptor-2 (VEGFR2). CD133 is a marker for immature hematopoietic stem cell, while CD34 is a classic hematopoietic stem cell marker. Hematopoietic stem cells give rise to myeloid cell lineage, which express CD14 and CD45, and are CD133 negative, which ultimately give rise to monocytes and macrophages. As endothelial progenitor cells differentiate, they lose CD133 and begin to express CD31, CD144, vascular endothelial cadherin, VEGFR2, endothelial nitric oxide synthase (eNOS), and von Willebrand factor (vWF). Endothelial progenitor cells are positive for both hematopoietic stem cell marker CD34 or CD133 and an endothelial marker, such as VEGFR2. Endothelial progenitor cells do not have exclusive surface markers, rather share similar markers with mature endothelial cells

the lungs, heart, liver, and kidneys, and showed organ-specific ECs to have a unique expression of gene clusters, potential for angiogenesis, barrier properties, and metabolic rates, each of which enables their organ-specific functional and development properties. Endothelial cells are known to be highly ubiquitous and one of the most functionally diverse cell systems. Vascular endothelial lining regulates blood flow, nutrient delivery and waste removal; blood coagulation; inflammation; angiogenesis; and vascular remodeling through autonomous and intercellular signaling mediated via neurotransmitters, hormones, and cytokines; and interaction with several cells, such as smooth muscle cells, pericytes, cytokines, and blood cells.[61] Prostacyclins and endothelium-derived nitric oxide (NO) cause vasodilation, while superoxide, endothelin, and thromboxane induce vasoconstriction; both sets of mediators regulate tissue perfusion.[62]

Endothelial Cell Barrier Function

The endothelial lining surface area is large and facilitates the substance exchange between blood and tissues. In humans, the endothelial surface area is estimated to be about 350 m^2.[63,64] Cells in the endothelial cell monolayer are linked to one another via tight, adherent, and gap junctions, which then connect to cytoplasmic proteins and the cytoskeleton.[65] Interestingly, endothelial cells maintain a tight barrier function throughout the process of vascular remodeling; vasculogenesis stimulants, such as VEGF-A, do not change microvascular permeability in the inner blood-retina barrier *in vivo* or *in vitro*, even when specific changes may be seen in transcellular transport or in tight or adherens junctions.[66–68]

The plasma membranes of closely aligned endothelial cells form an important barrier with tight junctions. The main transmembrane constituent of these junctions is the occludins.[69] Below the tight junctions, the adherens junctions are comprised of several proteins, including the surface adhesion glycoproteins, VE-cadherins, which form a zipper-like component at the base of endothelial cells. These proteins connect with their cytoplasmic tail to the underlying actin-based microfilament cytoskeleton.[70,71]

Endothelial Cell Response to Shear Stress

Endothelial cells react actively to blood flow, predominantly to mechanical cues with polarizing changes in conformation, electrical charge, or to the release of biochemical stimuli, such as nitric oxide or prostacyclin.[30,31,72] At rest, endothelial cells typically are shaped like a polygon, but under conditions of stress, they elongate in the direction of flow, thereby reducing the resistance to moving fluids.[30] In response to shear stress, cultured endothelial cells elongate and become oriented along the direction of blood flow[32] by reorganizing the cytoskeleton.[33] Shear stress is known to directly activate the endothelial NO synthetase (eNOS) promoter and increase its expression, and also promote the release of endothelial cell factors that promote endothelial cell survival while inhibiting leukocyte migration, coagulation, and smooth muscle proliferation.[30,72]

Endothelial Cell as Regulator of Vascular Tone

Endothelial lining of vessels regulates vascular tone and function in response to numerous neurotransmitters, hormones, and vasoactive factors.[62] The endothelium releases various vasoactive factors that can be vasodilatory, such as NO, prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factors (EDHF) or vasoconstrictive, such as thromboxane (TXA_2) and endothelin-1(ET-1).[62] Any imbalance of these vasoactive factors leads to dysfunction of the endothelium.

Endothelial Cells in Angiogenesis

The onset of neovascularization or angiogenic switch[73] has several triggers, such as metabolic stress, hypoxia, inflammatory stimuli, and immune response, and may also be related to genetic mutations.[74] During hypoxic conditions, hypoxia-responsive transcription factors regulate the expression of genes that allow tissues and cells to acclimatize to low oxygen conditions.[74] VEGF as an endothelial cell-specific mitogen is unique for its roles in promoting endothelial cell proliferation and vascular permeability.[49,75] VEGF can stimulate blood vessel development through the process of vasculogenesis or angiogenic sprouting, whereas ephrinB2 and Ang1 promote vascular remodeling and maturation of the vasculature.[49,76] VEGF has three major isoforms that originate from alternative splicing, namely VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188};[77] these isoforms also exhibit anti- and pro-angiogenic splice variants. VEGF is known to have two transmembrane receptors, VEGFR1, otherwise known as the feline McDonough sarcoma (fms)-related receptor tyrosine kinase 1 (Flt1), and VEGFR2, otherwise known as the kinase insert domain receptor (Flk-1). VEGFR1 is known to be expressed either as a soluble Flt1 receptor (sFlt1) formed through alternative splicing of the Flt1 mRNA[75] or as the membrane-bound Flt1. The two isoforms of VEGFR1 have a binding affinity that is tenfold higher for VEGF-A than VEGFR2.[78] VEGF can prevent apoptosis in umbilical vein endothelial cells and human dermal microvascular endothelial by inhibiting the activity of stress-activated protein kinase/c-junNH2-kinase (SAPK/JNK) and activating the mitogen-activated protein kinase (MAPK) pathway.[79]

VEGF and Notch show synergistic effects to promote the formation of blood vessel branches. VEGFR2, not VEGFR1, stimulates the induction of tip cells and promotes vascular sprouting (Fig. 3).[80] Notch is activated by the delta-like ligand 4 (DLL4) in neighboring endothelial cells; conversely, DLL4 inhibits tip cell behavior through the upregulation of VEGFR1 and the downregulation of VEGFR2 and VEGFR3 receptors.[80,81] For effective angiogenesis, VEGF acts cooperatively with several factors, such as the angiopoietins (Ang).[82] VEGF and Ang both have receptors on endothelial cells. Ang-1 and -2 bind to tyrosine kinase receptors, Tie 1 and Tie 2[83] (Fig. 3), while Ang-1, -2, and -4 all bind to the Tie 2 receptor.[84] Ang-1 promotes vascular integrity by promoting endothelial cell migration, inhibiting endothelial cell apoptosis, promoting the generation of capillary-like structures, and recruiting pericytes to vascular tissues.[84,85] Ang-1–Tie 2 signaling is shown to assist the maintenance of quiescent endothelial cell phenotype. Tie 2 interacts with the p85 subunit of phosphatidylinositol-3-kinase (PI3K) to activate the PI3K-AKT pathway, leading to increased survival and chemotaxis of endothelial cells.[86,87] AKT activation inhibits the forkhead transcription factor FKHR (FOXO1), which may protect endothelial cells from apoptosis.[88] Ang-1 and its binding to Tie 1 can promote vascular remodeling and are generally considered pro-angiogenic, whereas Ang-2 counteracts these effects and may be anti-angiogenic.[89,90] Ang-2 is regarded as an agonist of Tie 2 and has been shown to stimulate Tie 2/Akt signaling, as well as inhibit the expression of FOXO1-target gene to enable the regulation of transcription and apoptosis. Ang-2 may also inhibit vascular permeability and acts as an autocrine agonist of Tie 2 and protect stressed endothelial cells.[91]

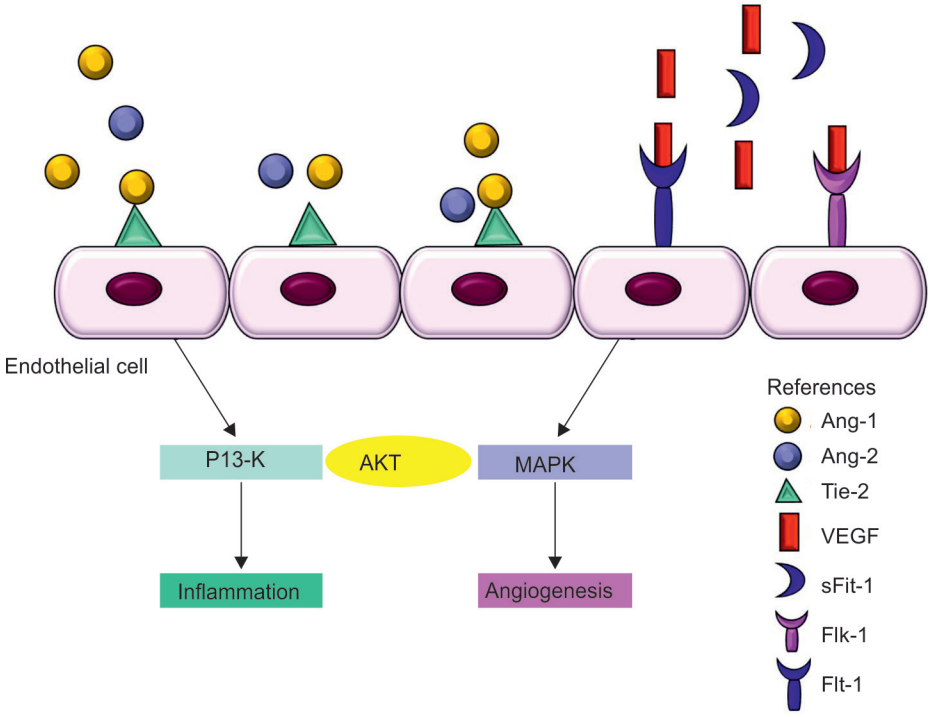

Fig. 3: Endothelial markers in inflammation and angiogenesis. VEGF works together with angiopoietins during inflammation and angiogenesis, and both have receptors on endothelial cells. Ang-1 and -2 bind to their receptors Tie 2. Ang-1–Tie 2 signaling contributes to maintaining a quiescent endothelial cell phenotype. Ang-1 is pro-angiogenic and required for vascular remodeling, while Ang-2 counteracts their effects as anti-angiogenic. VEGF has two transmembrane receptors, Flt1 or VEGFR1 and Flk-1 or VEGFR2. VEGFR1 has two forms generated by alternative splicing, a membrane-bound Flt1 and a soluble Flt1 receptor, VEGF signals through VEGFR2 to promote angiogenesis. VEGFR1 (Flt-1) serves to limit the actions of (VEGFR2) Flk-1. Ang-2 binds to Tie 2 to activate P13-K/Akt signaling. VEGF-VEGFR2 activates MAPK/AKT signaling pathways

In the brain, Ang-1 can be neuroprotective and inhibit apoptosis in brain neurons by activating phosphatidyl-inositol 3-kinase and also promoting the phosphorylation of Akt and restoring caspase-3 cleavage.[92] Coadministration of VEGF-A and Ang-1 synergistically increased DNA synthesis, cell proliferation, endothelial cell migration, and sprouting more than either agent alone.[93]

Endothelial Cells and Inflammation

Endothelial cells can modulate the recruitment of inflammatory cells to locations of injury and produce cytokines, growth factors, colony-stimulating factors, and chemokines in response to mechanical or chemical stimuli.[52,94,95] These cytokines can then induce a feed-forward cycle by promoting cell–cell interactions and the proliferation and survival of endothelial cells and also by inducing an endothelial cell pro-inflammatory phenotype that produces cytokines [interleukin (IL)-1], chemokines [IL-8, monocyte chemoattractant protein (MCP)-1], tumor necrosis factor (TNF), and adhesion molecules [vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and endothelial (E)-selectin], all of which recruit leukocytes to sites of injury.[53,54] Activated endothelial cells recruit leukocytes to sites of infection, which is critical to host defense. Upregulation of related adhesion and ligands on these leukocytes by bacterial or host pro-inflammatory mediators promotes adherence to endothelial cells and focused migration to the sites of infection, where these cells may phagocytize and kill the pathogens.[96]

During inflammation, leukocytes migrate across the vascular endothelium into the tissues in a series of steps. The first steps involve relatively weak, adhesive interactions with the rapidly flowing leukocytes to slow these cells down, followed by a few halting, rolling tumbles on the endothelial surface. These interactions are gradually strengthened with the leukocyte activation and their subsequent adherence in the endothelium. These stationary leukocytes then migrate into the interstitium through spaces between adjacent endothelial cells. As one can imagine, this is an area of intense study. During transmigration across the vascular endothelium, leukocytes can take either the paracellular path to squeeze their way through between adjacent endothelial cells, or less frequently, show transcellular migration across individual endothelial cells.[97] The principal endothelial adhesion molecules engaged in the attachment and transmigration of leukocytes include CD34, intercellular adhesion molecule 1 (ICAM1, CD54), endomucin (a membrane-bound glycoprotein expressed luminally by endothelial cells), ICAM2, the glycosylation-dependent cell adhesion molecule-1 (GLYCAM1), podocalyxin (a member of the sialomucin protein family), mucosal vascular addressin cell adhesion molecule 1 (MADCAM1), P-selectin, junctional adhesion molecule A (JAM-A), JAM-B, CD 99, vascular cell adhesion protein 1 (VAM1), CD106PECAM1, E-cadherin, and single-chain type-I glycoprotein.[98–101]

Endothelial Cells and Coagulation

Endothelial cells are important modulators of coagulation both in the physiological conditions and also during inflammation and infection. Endothelial cells express anticoagulant factors on their outer membrane surface. Loss of surface thrombin-binding proteins, such as thrombomodulin, and downstream protein C-mediated signaling play a vital role in minimizing thrombin activation and

clotting in physiology. The loss of these factors leads to decreased ability of endothelial cells to modulate coagulation and inhibits the release of endothelium-derived factors, such as PGI2 and NO.[102]

Endothelial Cells in Neonatal Disorders

Fetal organs, especially the eye, lungs, and kidneys, show important vascular development in the third trimester of gestation. Therefore, impaired vascular development has been implicated in numerous conditions of prematurity, such as retinopathy of prematurity (ROP), bronchopulmonary dysplasia (BPD), and acute kidney injury (AKI). In neonates, endothelial cell function is well regulated in physiology and known to be altered in pathological states. Table 2 lists biomarkers of endothelial cell in various neonatal diseases.

Endothelial Cells in Neonatal Sepsis

The incidence of early-onset neonatal sepsis with positive cultures in newborns is about 0.98/1,000 live births and most likely higher in very-low-birth-weight (VLBW) infants.[103] The incidence of late-onset sepsis is more variable and could be as high as 30% in extremely low-birth-weight (ELBW) infants.[104] A dysregulated immune host response is associated with the pathogenesis of neonatal sepsis. Gram-negative sepsis has high mortality rates, with most mortality occurring in the acute phase, within the first three days of onset of sepsis.[105]

During inflammation, the vascular endothelium expresses a plethora of cytokines with a local chemotactic gradient that recruits the leukocytes into peripheral tissues. Such recruitment responses in neonates may be weaker in most organs when compared to adults and in preterm in comparison to term neonates.[106,107] In other organs such as the intestine, particularly during necrotizing enterocolitis, the recruitment may be enhanced. Inflammation of vascular endothelium during sepsis leads to altered chemotaxis and leukocyte transmigration because of the impaired endothelial expression of adhesion molecules, such as E-selectin, ICAM-1, and P-selectin.[108] Some of these changes may be related to altered expression of pro-inflammatory ligands such as TNF, which can affect the expression of adhesion molecules VCAM-1 and ICAM-1.[29]

Biomarkers that regulate endothelial cells and reflect their microenvironment may be useful in monitoring sepsis. Angiopoietins stimulate endothelial cells to increase or suppress inflammation. Ang-1 expressed in peri-endothelial cells can suppress inflammatory responses and stabilize the microvasculature by inhibiting nuclear factor κB (NFκB) activation. In contrast, Ang-2, which is expressed preferentially in endothelial cells, is pro-inflammatory and can increase the permeability of vessels and destabilize them. Ang-1 binds to Tie 2, the tyrosine kinase receptor to maintain the endothelial resting state, thereby suppressing vascular permeability during inflammation (Fig. 3).[109]

Endothelial Cells in the Neonatal Brain

Endothelial cells in the brain microvasculature have an intricate relationship with neuronal development and function, suggestive of a neurovascular crosstalk. Endothelial cells stimulate the

Table 2: Endothelial biomarkers in neonatal diseases

Neonatal disease	*Biomarker*	*Functional properties*	*Functional use*
IVH	IL-6[86]	Pro-inflammatory	Increased serum levels in IVH
	IL-8[87]	Pro-inflammatory	Increased serum levels in IVH and white matter injury
ROP	VEGF-A	Pro-angiogenic	Increased in ROP
	sVEGFR-2[120]	Pro-angiogenic	Elevated in premature infants with ROP
	sTie2[120]	Pro-angiogenic	Elevated in premature infants with ROP
	IL-6[118,119]	Pro-inflammatory	Increased amniotic fluid levels
	IL-8[119]	Pro-inflammatory	Increased amniotic fluid levels
NEC	PAF[70,154,165]	Pro-inflammatory	Elevated in blood early in NEC
	TGF-β[153,166]	Pro-inflammatory	Increased blood levels in NEC
Sepsis	Ang-1[81]	Anti-angiogenic	Decreased in sepsis
	Ang-2[81]	Pro-angiogenic	Elevated in sepsis
BPD	Ang-1[128]	Anti-inflammatory	Reduced serum levels in BPD
	Ang-2[128]	Pro-inflammatory	Increased levels in BPD
	ICAM-1[128]	Pro-inflammatory	Increased serum levels correlate with BPD severity
	IL-1β[128]	Pro-inflammatory	Increased serum levels in infants with both BPD and pulmonary hypertension.
	MCP-1[128]	Pro-inflammatory	Lower serum levels of MCP-1
	VEGF[126]	Pro-angiogenic	Decreased levels in BPD
	Tie 2[126]	Pro-angiogenic	Decreased levels in BPD

IL, interleukin; VEGF, vascular endothelial factor; VEGFR, vascular endothelial factor receptor; Tie, receptor tyrosine kinase gene; TGF-β, transforming growth factor-beta; PAF, platelet-activating factor; Ang, angiopoietin; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein

proliferation and differentiation of neuronal precursors toward neuronal lineage.[50] During postnatal development, endothelial cells promote excitatory synaptogenesis through upregulation of VEGF expression in cortical neurons by increased signaling through the P38/MAPK pathway.[110] The premature infant is sensitive to neurologic injury partly due to the exposure of their immature vascular network to extrauterine physiologic abnormalities in oxygen tension, biochemical, and environmental factors.

Intraventricular hemorrhage occurs in about 20% of infants born before 32 weeks' gestation and is a major cause of neurodevelopmental morbidity and mortality in premature infants.[111,112] IL-6 may be an important early biomarker for IVH. In one study, serum IL-6 levels were elevated in infants with IVH and were associated with increased risk of neonatal morbidity at less than 28 days after birth.[113] If high levels of IL-8, an important neutrophil and monocyte chemokine that is produced by macrophages, smooth muscle cells, and the endothelium, persisted for >1 day, the risk of IVH and white matter injury was higher.[114]

Hypoxic-ischemic encephalopathy (HIE) is a encephalopathy resulting from perinatal asphyxia that leads to neuronal death from activation of inflammatory cells and overexpression of apoptosis-related proteins.[115] In HIE, elevated inflammatory cytokine levels such as IL-6, TNF, and IL-8 recruit leukocytes to the site of injury and damage endothelial cell integrity.[116,117] In infants with HIE, early microvascular injury may have a critical impact on neuronal damage.[118]

Endothelial Cells in Retinopathy of Prematurity

Premature infants continue to develop the retinal vasculature after birth and are susceptible to altered vascular development such as in retinopathy of prematurity (ROP). These abnormalities in angiogenesis can be recapitulated in murine models such as those of oxygen-induced retinopathy (OIR).[119–121] ROP involves altered endothelial cell proliferation and survival,[122–124] and consequent abnormal retinal vascularization. Increasing data suggest that ROP involves dysregulation of VEGF expression.[125,126] The vascular development in ROP shows two distinct phases: an initial phase of vaso-obliteration that is triggered by hypoxia and a subsequent period of abnormal neovascularization triggered by retinal hypoxia to meet the demands of the metabolically hyperactive retinal cells and neurons.[127] These abnormalities can be seen in the mouse model of OIR, where mouse pups exposed first to hyperoxia develop vaso-obliteration of retinal vessels and then show abnormal neovascularization.[128,129]

In vivo assessments in a mouse oxygen-induced retinopathy model have revealed several physiologic and functional phenotypes in the developing retinal as a result of aberrant angiogenesis. Alterations in arterial and venous oxygen tension (PO_2) result in increased arterio-venous PO_2 gradients, which indicate increased oxygen extraction and possible underlying ischemia.[130] Whole-mount staining of retinas shows central vaso-obliteration in neonatal OIR mice with recovery to full vascularization by P21.[120,128,129] However, longitudinal live retinal imaging using fluorescein angiography revealed capillary avascularity, arterial tortuosity, and venous dilation in neonatal OIR mice compared to fully vascularized, normal caliber arteries and veins in room-air-raised mice, and consequent prolonged loss of capillary density with the paucity of neovascular buds on capillaries of adult OIR mice in spite of full peripheral vascularization. Spectral-domain optical coherence tomography revealed thinner retinas in neonatal mice with OIR,[131–134] more pronounced in the hypovascular retinal areas,[132] and restricted to the inner retina.[133] Electroretinograms correlate retinal vascular abnormalities to inner retinal dysfunction in OIR mice.[134,135] Comparative retinal histology following *in vivo* imaging showed prolonged over-expression of VEGF, microglial activation, abnormal malaligned neuronal synapses, and apoptosis in OIR mice.[136] A subpopulation of resident macrophages (M2) has been shown to be an important phenotype during angiogenesis.[137,138] Exogenous administration of pro-angiogenic isoform of $VEGFA_{165a}$ in a mouse model of OIR promoted earlier revascularization,[126] likely by targeting endothelial cell proliferation via increased angiogenic signaling through VEGF receptors.

Several proteins and support cells are intricately linked to endothelial cell function. Endothelial cells have surface protein receptors for integrins that play a role in angiogenesis and inflammation.[139] VEGF induces expression of the collagen receptors, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins.[140] The recruitment of pericytes has been demonstrated to be important in vascular maturation, for stabilization of the vasculature and remodeling of the early endothelial plexus into a more mature vascular network.[141,142] Disruption of endothelial-pericyte connections leads to exaggerated regression of vasculature and abnormal remodeling.[141] Angiopoietins play a role in the pathogenesis of ROP. Ang-2 was inhibited by hyperoxia and increased during relative hypoxia in a rat model of OIR.[143] Biomarkers have been investigated for ROP monitoring and disease severity. IL-6 levels in the umbilical cord were noted to be elevated in preterm infants with severe ROP, while high cord levels plasma C5a were associated with ROP that required laser therapy.[144]

Inflammatory cytokines have been associated with ROP in both the peri- and postnatal periods. Studies of amniotic fluid samples from 175 premature infants born between 23–32 weeks showed that higher IL-6 and IL-8 levels were associated with a higher risk of advanced ROP.[145] Similarly, Pieh et al. showed that premature infants with high plasma levels of the soluble VEGF receptor 2 (sVEGFR-2) and its soluble membrane-bound tyrosine kinase receptor (sTie) are associated with an increased risk of ROP.[146]

Endothelial Cells in Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD), also known as chronic lung disease of prematurity, is related to increased supplemental oxygen use during the early neonatal period[147,148] and occurs in about 40% of infants born below 29 weeks gestation.[149,150] A coordinated development of the pulmonary vasculature is required for normal lung development growth. Preterm birth may disrupt the lung vascular growth during the saccular and alveolar stages of pulmonary development, and aberrant development of the pulmonary vascular bed may lead to impaired alveolar development.[148,150,151] Postmortem lung examination of infants with BPD showed low levels of VEGF mRNA and reduced VEGF immunostaining, as well as a reduction in angiogenic receptors Flt-1 and Tie 2 in the infants with BPD compared to those without BPD.[152] Inhibiting VEGF during development decreases alveolarization and pulmonary arterial density.[153,154] Higher levels of ICAM-1, Ang-2, and IL-1β, and reduced levels of Ang-1 and MCP-1 are correlated with BPD severity.[155]

Endothelial Cells in Pulmonary Hypertension

Endothelial dysfunction is centrally implicated in pulmonary hypertension. Pulmonary hypertension is a multifactorial and complex condition, associated with the aberrant endothelial cell

proliferation with concurrent neoangiogenesis and the alteration in the secretion of vasoactive mediators, such as prostacyclin, NO, serotonin, ET-1, and thromboxane. The lung endothelium is heterogenous and different from systemic endothelium in both function and structure. The pulmonary endothelium's function includes maintaining barrier integrity, homeostasis, vascular tone, leukocyte trafficking, and production of necessary growth factors.[156] The normal endothelium is typically in a stable, "quiescent" state. When the endothelium is disturbed and "activated" by stress, infection, disease, or injury, endothelial cells tend to express specific proteins and markers, such as ICAM-1, VEGF, and E-selectin, which causes exaggerated proliferation, coagulability, and vasoconstriction.[156,157] In pulmonary hypertension, some of the triggers of endothelial activation are inflammation, shear stress, reactive oxygen species, genetic mutations, and defect in angiogenesis.[156]

Endothelial Cells in Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease seen in premature infants that is associated with high morbidity and mortality. Maldevelopment of the microvasculature of the intestinal mucosal and abnormally altered intestinal blood flow are implicated in the pathogenesis of NEC. There is low resistance of the intestinal vasculature across the intestines of the newborn infant, mediated by increase in the production of nitric oxide by the endothelium. Neonatal swine models showed abnormal vasoconstriction responses to severe hypoxemia, resulting in intestinal ischemia.[158] Hypoxia in the preterm neonate can inhibit NO production and result in intestinal injury and NEC.[159] There is also evidence of VEGF dysregulation; premature infants exposed to hyperoxia may show decreased VEGF expression and VEGF/VEGFR-regulated pro-angiogenic signaling pathways and diminished development of the intestinal microvasculature. These limitations in the splanchnic vasculature may not be insufficient for the relatively limited metabolic needs in the first few days after birth but may become inadequate with increasing feeding volumes in the later neonatal period. In experimental NEC, VEGFR2 protein and VEGFR2 activity have been shown to be low preceding the onset of intestinal injury.[160,161] Similarly, inhibition of VEGFR2 led to decreased endothelial cell proliferation and intestinal microvascular network development.[161] Administration of dimethyloxalylglycine (DMOG), a propyl hydroxylase enzyme inhibitor, increased the expression of VEGF-A in the intestines of neonatal pups, but the splanchnic effects of DMOG were abolished by inhibiting VEGFR2 signaling.[162] Further investigations are needed to investigate the strategies to modulate angiogenic signaling through the VEGF-VEGFR2 pathway, which may possibly protect against NEC.

Endothelial Cells in Neonatal Acute Kidney Injury

Early changes in capillary blood flow and endothelial cell injury leading to inflammation, ischemia, and pro-coagulation may play a crucial role in the pathogenesis of early and chronic ischemic AKI. In rat models, ischemic kidneys were unable to autoregulate blood flow and exhibited vasoconstriction when renal perfusion pressure decreased.[163] The organization of the cytoskeletal network of endothelial cells and small arterioles is altered during renal ischemia-reperfusion injury, which disrupts endothelial cell tight junctions as indicated by the disintegration of VE-cadherin in renal microvasculature.[164,165] The loss of the integrity of barrier function could have been the result of matrix metalloproteinase-2 or -9 activation.[166] There is also some evidence to show impaired endothelial-dependent vasodilator activity in AKI. L-arginine and eNOS cofactor tetrahydrobiopterin may attenuate acute ischemia-reperfusion renal injury by preserving medullary perfusion.[167–169]

Endothelial Cells as Therapeutic Targets

Therapeutic advances to regulate angiogenesis have been challenging and limited in success employing pro- and anti-angiogenic factors. This could be due to the complex biology of angiogenic factors, their multiple receptors, and versatile functions. Preclinical studies of pro-angiogenic cell therapies or microRNAs targeting show promise of alternate therapeutic strategies.[170]

Bevacizumab (Avastin) is a promising non-selective anti-VEGF drug that was first used to treat metastatic cancers[171] but was subsequently approved for the treatment of ROP and other ocular conditions.[171–173] Selective pro-angiogenic VEGF isoforms are being explored preclinically, such as administration of $VEGFA_{165a}$ microparticles for the treatment of ROP.[126] Ranibizumab, a humanized Fab fragment that can block all VEGF isoforms, reverses VEGF-stimulated delocalized tight junctions, proliferation and migration of cells, and delocalization of tight junction proteins in retinal endothelial cells, may also be useful in some stages of ROP.[68] Targeting endothelial-to mesenchyme transitions may also be useful in specific stages of vascular disease. Relaxin, a calcimimetic agent, Cinacalcet, and Losartan are shown to inhibit endothelial-mesenchymal transitions.[174–176]

There may be some utility in monitoring biomarkers indicative of damage to the endothelium during neonatal sepsis, such as endothelial growth factors or components of tight junctions (TJs) that shed into circulation upon endothelial damage and quantifying plasma and urine levels of soluble components of endothelial wall and glycocalyx and degraded glycocalyx.[177] Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is able to differentiate sepsis from non-sepsis cases, with an area under curve (AUC) of 0.97 to diagnose proven or suspected neonatal sepsis, compared to 0.96 of IL-6, and 0.8 of Endocan.[178] The ratio of Ang-1 is shown to correlate with bacteremia.[179] Higher Ang-2 levels correlate with clinical sepsis.[180] Endothelial cell dysfunction has also been implicated in NEC, and several therapies are being explored to modulate the ensuing inflammatory necrosis. Enteral administration of TGF-β_2 was protective in mice with experimental NEC-like injury.[181] PAF has been implicated in NEC pathogenesis and shows promise as a biomarker.[182] Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a naturally occurring polyphenol found in red wine, berries, and peanuts and has been shown to improve endothelial NO production and endothelial redox balance, as well as inhibit the activation of the endothelium following pro-inflammatory and metabolic stress.[183] Protocols have been developed that enable the differentiation of h-iPSCs very efficiently into competent h-iECs, thereby enabling the development of perfused vascular networks *in vivo*.[184] Despite the early promises of tissue engineering involving endothelial cells, applications to clinical practice are limited. Understanding the cellular and molecular mechanisms related to physiologic and pathologic angiogenesis, both in pediatric and adult tissues, will enhance advances in tissue engineering.[185]

Conclusion

Endothelial cells are critical regulators of vascular homeostasis through intricate interactions with vascular smooth muscle

cells, circulating cells, and surrounding support cells, and their connections to blood and tissue components make them vulnerable to minute alterations in the composition of blood, mechanical stress of blood flow, injury, or inflammation. Endothelium based on the microenvironment can transform from pro-inflammatory to anti-inflammatory properties, as well as vasodilation or vasoconstriction, and pro- and anti-thrombotic properties. Future investigations focused on understanding endothelial cell heterogeneity may provide insights into vascular-bed-specific therapies in neonates.

Author Contribution

The two authors together developed the study concept, acquired, and interpreted the data, wrote, and approved the final version of the manuscript.

Statement on Patient Consent: No patient consent was needed for this study.

Category of study: Clinical Science.

Orcid

Akhil Maheshwari https://orcid.org/0000-0003-3613-4054

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REVIEW ARTICLE

Patent Ductus Arteriosus: A Diagnostic and Treatment Dilemma

Rachana Singh[1], Ruben Vaidya[2], Ravi Ashwath[3], Akhil Maheshwari[4]

Abstract

Ductus arteriosus is a critically important vascular structure that functions as an extracardiac shunt in fetal life between the pulmonary and systemic circulations for optimal utilization of the placenta as a gas exchange organ and fetal well-being. While morbidities and mortality are well known to be associated with persistence of patent ductus arteriosus (PDA) in postnatal life, the treatment options have concerns for adverse outcomes. Additionally, high spontaneous closure rates, lack of clear definition of hemodynamically significant PDA (hs-PDA), ideal diagnostic tools, conflicting evidence regarding timing of treatment, and lack of clear benefits of PDA treatment from randomized trial in reducing adverse outcomes continue to pose challenges for clinicians managing preterm infants with PDA. This review focuses on the pathophysiology, current diagnostic and management practices, as well as the potential of utilizing unique diagnostic tools to support precision medicine for preterm infants with hs-PDA.

Keywords: Ductus arteriosus, Neonate, Patent ductus arteriosus, Prematurity.

Newborn (2022): 10.5005/jp-journals-11002-0023

Introduction

The patency of the *ductus arteriosus*, which is a critically important vascular structure, results in an extra-cardiac shunt between the pulmonary (pulmonary artery) and systemic (aorta) circulations. Galen was the first to describe a patent ductus arteriosus (PDA) and Gross to report its successful closure.[1] Although rare, premature, *in utero* closure of the PDA due to maternal medications and/or due to unknown etiology can lead to right ventricular overload, congestive heart failure, fetal hydrops, and/or intrauterine fetal demise.[2,3] In most infants, the PDA closes within 24–72 hours after birth. In term infants, a persistent PDA can account for up to 5–10% of all congenital heart disease of newborns.[4] On the contrary, the PDA is a common occurrence in preterm, very low birth weight (VLBW) infants where continuous pulmonary over circulation continues until the duct remains open. Ductal closure leads to the improvement of lung compliance and stops the "stealing" from the systemic circulation. These hemodynamic alterations have been historically known to be associated with neonatal morbidities, such as accentuated respiratory distress syndrome (RDS), pulmonary hemorrhage, bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), renal insufficiency, intraventricular hemorrhage (IVH), and/or cardiac dysfunction.[5,6] About 20–60% of preterm infants have a PDA beyond the first 72 hours of life.[7] The prevalence of PDA in preterm infants is inversely related to gestational age and birth weight with an incidence of 70% in infants <28 weeks gestation.[8] Over the last decade with advances in diagnostic modalities, awareness of natural history with spontaneous closure,[9,10] and limited benefits of treatment,[11–13] the management of PDA has posed a significant dilemma to clinicians. The objective of this review is to discuss the utilization of potential innovative diagnostic tools for identification and thereby targeted treatment of hemodynamically significant PDA (hs-PDA).

Pathophysiology

The three *in utero* shunts, *ductus venosus*, *ductus arteriosus,* and foramen ovale, play a critical role in fetal hemodynamics and are essential for efficient utilization of the placenta for gas exchange, normal cardiovascular growth, and function. The relatively low fetal oxygen tension with partial pressure of oxygen (pO_2) ranges of 25–28 mm Hg (65% O_2 saturation),[14] and circulating prostaglandins, specifically prostaglandin E2 (PGE2), formed by the action of cyclooxygenase (COX) enzymes maintains the PDA through their vasodilatory effects.[15] The *ductus arteriosus* smooth muscle relaxation occurs due to activation of the G protein–coupled receptor EP4 by PGE2.[15,16] At birth, increasing oxygen tension and decreasing PGE2 production lead to spontaneous PDA closure by 24–48 hours of life. Fan et al. described the PDA maturation pathway in their rabbit model and demonstrated that patency of the preterm ductus is maintained by high levels of PGE2, which binds the EP4 receptors under conditions of hypoxia as opposed

[1]Division of Newborn Medicine, Tufts Children's Hospital, Tufts University School of Medicine, Boston, Massachusetts, United States of America

[2]Division of Newborn Medicine, Department of Pediatrics, University of Massachusetts Medical School-Baystate, Springfield, Massachusetts, United States of America

[3]Division of Pediatric Cardiology, University of Iowa, Iowa City, Iowa, United States of America

[4]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Rachana Singh, Division of Newborn Medicine, Tufts Children's Hospital, Tufts University School of Medicine, Boston, Massachusetts, United States of America, Phone: +617 6365322, e-mail: rsingh2@tuftsmedicalcenter.org

How to cite this article: Singh R, Vaidya R, Ashwath R, *et al*. Patent Ductus Arteriosus: A Diagnostic and Treatment Dilemma. Newborn 2022;1(1):58–66.

Source of support: Nil

Conflict of interest: None

to the term ductus where the EP3 receptor levels are higher and exposure to PGE2 caused vasoconstriction under normoxic conditions.[17] Once functional closure of the ductus occurs, then anatomic closure follows with intraluminal remodeling in response to hypoxemic conditions.[18] Hence, delayed ductal closure in term infants is primarily related to structural alterations, whereas the lack of spontaneous closure in preterm infants is due to immaturity.[18]

Spontaneous PDA closure rates increase with both advancing gestational age and postnatal age. In the meta-analysis by de Klerk et al., they noted that 34% of premature infants (gestational age ≤28 weeks and/or BW ≤1000 g) had spontaneous ductus closure by the 3rd day after birth (72–96 hours) and up to 41% by the 7th day.[19] Although most ductus will close spontaneously, without intervention,[20] infants with GA ≤28 weeks have the lowest chance of spontaneous PDA closure in the first week of life.[9,19] Liu et al. noted that maternal chorioamnionitis, lower gestational age and birth weight, BPD, IVH, NEC, RDS, sepsis, surfactant treatment, ventilation, and lower platelet count were all positively related to persistence of the PDA, whereas a small for gestational age (SGA) status had a converse effect among preterm infants. Additionally, premature rupture of membrane (PROM), preeclampsia, antenatal steroids, male gender, and platelet indices were not associated with PDA.[21] Our current limited knowledge about predictive factors[22] as well as processes for identification of hemodynamically significant PDA further adds a layer of complexity to successful management of PDA in these critically ill preterm infants.

Diagnosis

In making a diagnosis and determining the need for treatment, clinicians usually grapple with two issues: (a) the presence/absence of a PDA and (b) its hemodynamic significance. Clinically, PDA can be suspected based on clinical signs and confirmed with a two-dimensional (2D) echocardiography. The clinical signs include the presence of a continuous murmur, although "silent" large PDAs may exist; bounding pulses and widened pulse pressures; unexplained metabolic acidosis, due to systemic under perfusion; evidence of pulmonary over circulation manifesting as tachypnea; increased work of breathing, increased need for respiratory support; hypotension and low diastolic blood pressure; oliguria; and biochemical alterations as persistent metabolic acidosis and elevated creatinine. Once suspected, PDA is confirmed by a 2D echo wherein structural and functional measurements are made (Fig. 1). Echocardiography reports provide information about the PDA size, left atrium (LA)-to-aorta (LA/Ao) ratio, LA and left ventricular (LV) size, and LV output. However, the presence of a PDA alone does not provide guidance for further management. Determination of hemodynamic significance if the PDA with a potential for negative impact on multiple organ systems is also considered as an indication for treatment (Fig. 2).

Defining Hemodynamically Significant Pda

The definition of an hs-PDA is controversial.[23] As reported by Zonnenberg et al., most clinical trials use clinical and ultrasound-based criteria to define an hs-PDA, but there is considerable variability in the inclusion criteria and cutoffs without a clear consensus. Of the clinical criteria, a murmur or hyperdynamic circulation is most commonly used. The LA/Ao ratio is most commonly utilized ultrasound criteria.[24] In addition, the likelihood of spontaneous closure and vulnerability to the severity of illness related to gestational or chronological age may also be considered.[25] Kluckow suggested even though echocardiography may be the mainstay of diagnosis, assessment of clinical manifestations,

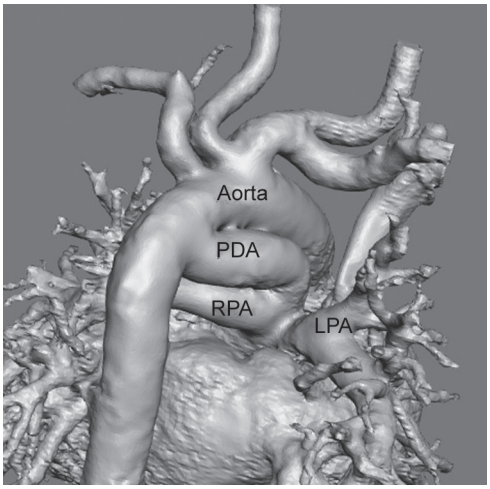
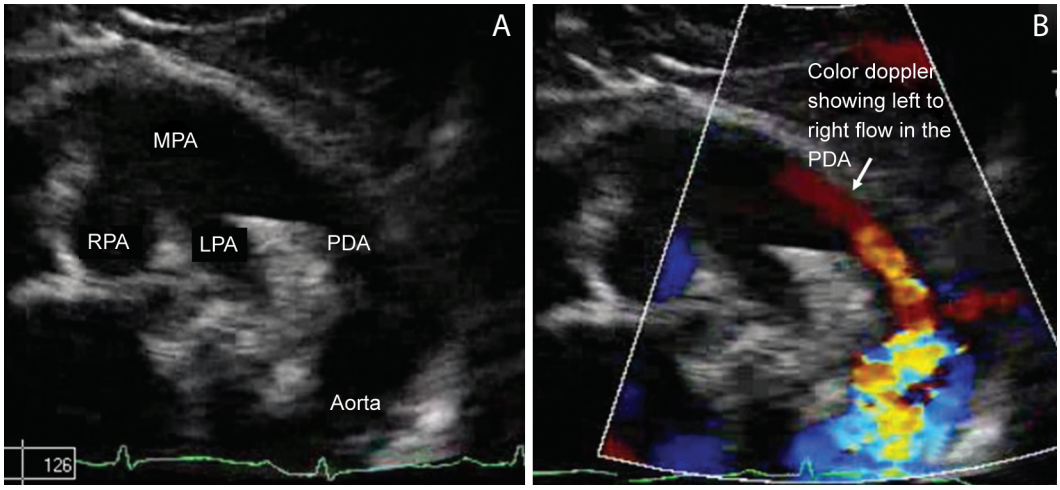


Fig. 2: 3D Volume rendered image depicting the PDA anatomy, posterior view. RPA, right pulmonary artery; LPA, left pulmonary artery; PDA, patent ductus arteriosus



Figs 1A and B: 2D Echocardiographic images of PDA shown in the "ductal view" without (A) and with color Doppler; (B) Showing left to right flow across the duct. MPA, main pulmonary artery; RPA, right pulmonary artery; LPA, left pulmonary artery; PDA, patent ductus arteriosus

biomarkers and physiological markers of the end-organ effects such as with cerebral Doppler or near infra-red spectroscopy should be considered.[26] A combination of these modalities may provide a clearer picture of hs-PDA for judicious, individualized treatment, as opposed to generalized application of therapy for all and/or none. Multiple modalities have been used to assess the hemodynamic significance of a PDA, with each having their own pros and cons:

- *Clinical scores for hs-PDA:* Initial diagnosis of an hs-PDA still relies largely on clinical parameters. Accurate clinical scoring tools can allow for better noninvasive monitoring over time, but also limit the repeated need for echocardiography and other stress-inducing procedures. One such scoring system was proposed by Kindler et al., wherein they measured eight clinical variables with each variable receiving a score of 1. A composite score ≥2 prompted further evaluation with an echocardiogram. These initial variables were further improvised to create the final scoring system composed of four significant symptoms—precordial pulsations, bounding femoral pulses, apnea/need for mechanical ventilation, and metabolic acidosis. This score demonstrated a calculated sensitivity of 84% and specificity of 80%. They further adapted the need for apnea/mechanical ventilation variable to "pulmonary deterioration," as indicated by increased oxygen supplementation, increased noninvasive or invasive respiratory support, increased apnea frequency, and mounting hypercapnia.[27] Another scoring tool utilizing 14 clinical items, the Scoring preterm Infants for PDA clinically without Echocardiographic evaluation (SIMPLE) score, warrants further evaluation in future studies. The authors identified these items based on a literature review and then weighed them by severity on an arbitrary 1–4 scale, the sum of which represented the final SIMPLE score. This objective, bedside administrable score was found to be consistently high in infants with hs-PDA as compared to infants without hs-PDA.[28] A PDA severity score (PDAsc) was developed to predict the diagnosis of chronic lung disease and/or death prior to discharge through an observational study. Using echocardiographic data from 141 infants in a prospective observational study, the PDAsc on postnatal day 2 was noted to be consistently higher in infants who either developed chronic lung disease or died prior to discharge. A PDAsc cutoff of 5 in this study had a sensitivity and specificity of 92 and 87%, respectively, and positive and negative predictive values of 92 and 82%, respectively.[29] However, these clinical scores need further validation.
- *Biomarkers for hs-PDA:* The degree of metabolic acidosis, urinary output, and the blood urea nitrogen (BUN) and creatinine (Cr) levels are the most common biochemical indicators of an hs-PDA and probably most widely used markers of end-organ insult secondary to the ductal "steal" in perfusion. Serum brain natriuretic peptide (BNP) and serum/urinary N-terminal pro-brain natriuretic peptide (NTproBNP) have also generated interest as predictors for chronic lung disease with or without associated pulmonary hypertension in preterm infants.[30,31] Given the association between hs-PDA and chronic lung disease, exploration of NTproBNP as a potential biomarker for assessment of hs-PDA is being explored, given its increased secretion by ventricular myocardium in response to volume overload.[32,33] Additionally, Olsson et al. demonstrated that high levels of BNP, interleukin (IL)-6, -8, -10, and -12, growth differentiation factor-15, and monocyte chemotactic protein-1 were associated with persistent PDA, as were low levels of platelet-derived growth factor. High levels of both inflammatory markers and erythropoietin were associated with persistent PDA and failure to respond to pharmacological treatment.[34] However, prior to translation into clinical decision-making, further work is needed to determine the most optimal timing for testing, cutoff thresholds for treatment, and/or the role of trending these biomarkers over time to assess for severity and/or improvement.
- *Echocardiographic assessment of hs-PDA:* To date, echocardiography remains to be the gold standard for diagnosis of hs-PDA, and the determination of the anatomical characteristics of the ductus, evidence of systemic hypoperfusion and pulmonary over-circulation have been useful.[35] In the order of relevance, the most useful characteristics of hs-PDA are the presence of descending aortic diastolic flow reversal, increased LV output, isovolumic relaxation time (IVRT), PDA diameter, pulmonary vein D wave, LA:Ao ratio, and the mitral E wave (Table 1).[36,37] An E/A ratio is utilized to assess left ventricular function and is defined as the ratio of peak velocity blood flow from left ventricular relaxation during early diastole (the E wave) to the peak velocity flow during late diastole caused by atrial contraction (the A wave). In preterm infants with hs-PDA and signs of volume overloading, the E/A ratio may approach ≥1 or be reversed and can be used in conjunction with other markers, such as the isovolumic relaxation time (IVRT), which may be <40 with pulmonary over-circulation.[38] As decisions are made for medical and/or surgical treatment, defining the anatomy of the PDA becomes critical to rule out ductal-dependent cardiac lesions; the assessment for ductal length and diameter to plan type of closure; determining the directionality of the shunt not only through the PDA but also other intracardiac shunts that may exist as foramen ovale, atrial, and/or ventricular septal defects; and any other structural aberrations as sidedness of the arch and/or presence of vascular rings. While 2D echo is performed most often in clinical setting, emerging evidence supports the role of real-time 3D dimensional echocardiography (RT3DE) in clinical practice They were submitted with the original article and are beign resubmitted (Videos 1A and B). Roushdy et al. imaged 42 older postneonatal patients (mean age of 3.6 years; ranging from 2 months to 14 years) who were referred for elective percutaneous PDA closure after having been assessed with a full 2D echocardiogram, and RT3DE, and off-line analysis using Q lab software within 6 hours from their angiograms. They concluded

Table 1: 2D Echocardiographic markers of hemodynamically significant PDA (Adapted from Boradhouse KM, Price AN, Durighel G, et al. Assessment of PDA shunt and systemic blood flow in newborns using cardiac MRI. NMR Biomed 2013;26(9):1135–1141. DOI: 10.1002/nbm.2927)

Measurement	*Hemodynamically significance*
PDA size	>1.5 mm
LA–Ao ratio	>1.5 mm
IVRT	<50 ms
LA size	Increased ≥2 SD
LV size	Increased ≥2 SD
LV output	>1.5 × RV output
Mitral E wave	>45 cm/sec
PV D-wave	>30 cm/sec
Aortic diastolic flow	Reversed

PDA, patent ductus arteriosus; LA, left atrium; Ao, aorta; IVRT, isovolumic relaxation time; LV, Left ventricle; PV, pulmonary vein; mm, millimeter; SD, standard deviation

that RT3DE was more accurate than 2D echocardiogram in determining the length and the ampulla of the PDA, determining type A and type E PDA and correlated well with angiography.[39]

- *Magnetic resonance imaging (MRI) for hs-PDA:* The role of cardiac magnetic resonance imaging (CMR) is increasingly being explored as a complement for patients during pre- and/or postintervention for structural cardiac disease. As a noninvasive imaging technique, CMR can allow detailed visualization of cardiac anatomy and functional assessment, including wall motion analysis, quantification of chambers size and volume, systolic function, and myocardial tissue characterization, without exposure to ionizing radiation.[40] It can also provide spatial resolution and 3-dimensional (3D) multiplanar reconstruction allowing for assessment of the PDA anatomy (Fig. 2), evaluation of associated abnormalities in the aortic arch, and quantification of ductal shunt volume. While larger PDAs can be seen on spin-echo images, breath-hold magnetic resonance angiogram (MRA), the flow disturbances produced by even small PDA in the pulmonary artery can be visible as signal loss on cine MRA. Additionally, sagittal reconstructions using 3D noncontrast MRA can demonstrate the small PDA.[40] Since direct quantification of the PDA shunting can be difficult with CMR, Broadhouse et al. attempted to indirectly assess for hemodynamic shunting through the PDA with phase-contrast MRI sequences. They assessed 75 infants with median (range)-corrected gestation 33^{+6} (26^{+4}–38^{+6}) weeks, of whom 15 had PDA. In 60 infants without PDA, left ventricular outflow (LVO) matched total systemic flow; while in infants with PDA, ductal shunt volume was 7.9–74.2% of the LVO. Multiple linear regression analysis correcting for gestational age showed a significant association between ductal shunt volume and decreased upper and lower body flow ($p = 0.01$ and $p < 0.001$).[36] PDA is uniquely the only shunt type that gives a Qp/Qs <1, when pulmonary blood flow is increased. Since PDA causes a systemic-to-pulmonary shunt from the descending aorta to the left pulmonary artery, quantification of Qp/Qs cannot be interpreted the same way it is interpreted in an intracardiac shunt. The aorta usually has higher flow than the pulmonary artery so that one way to calculate the shunt volume is to subtract the pulmonary flow from the systemic one (Qshunt = Qs − Qp). Another possible method is MRI measurement of the relation between the flow in the superior cava, the left cardiac output, and the flow of the descending aorta in order to obtain information about the amount of flow subtracted from the inferior systemic circulation (distal to the ductus). This quantitative data about the steal to the descending aorta circulation can be relevant for the indication of PDA closure in premature infants in order to avoid complications.[41] The severity of PDA can potentially be quantified in terms of Qp/Qs and surgery is generally reserved for patients with Qp/Qs >1.5. CMR although has great potential, it also comes with logistic challenges of transporting an infant to MRI suite, obtaining detailed MRI images, need for breath-holding and spatial resolution in a small preterm infant.
- *Near-infrared spectroscopy for hs-PDA:* Persistent shunting from systemic-to-pulmonary circulation results in hypoperfusion of tissues with resultant hypoxemia, as the PDA becomes hemodynamically significant. Ability to measure tissue perfusion with near-infrared spectroscopy (NIRS) can provide further information in the assessment of hs-PDA and has been evaluated. Chock et al., in their study of 47 infants, noted that after adjusting for gestational age found that lower renal saturation (Rsat) was associated with an hs-PDA by echo (OR 0.9, 95% CI 0.83–0.98, $p = 0.01$), while there was no significant change for cerebral saturations (Csat). Using receiver-operating characteristic (ROC) curves, Rsat <66% identified an hs-PDA with a sensitivity of 81% and specificity of 77%.[42] Since the cerebral blood flow has a preductal origin, it has been assumed that if the hemodynamics are stable, then the presence of an hs-PDA does not impact cerebral tissue perfusion. However, in a prospective case–control study, mean arterial blood pressure and regional cerebral oxygen saturation were significantly lower and fractional tissue oxygen extraction significantly higher for infants with PDA when compared with the control infants during PDA (mean arterial blood pressure: 33 ± 5 vs 38 ± 6 mm Hg; regional cerebral oxygen saturation: 62 ± 9% vs 72 ± 10%; and fractional tissue oxygen extraction: 0.34 ± 0.1 vs 0.25 ± 0.1, respectively). These improved after treatment with indomethacin and became similar to controls after successful closure.[43] However, in a recent prospective observational study of 49 preterm infants with a closed PDA, non-hs-PDA, and hs-PDA within 2 weeks after birth, no differences were noted for Csat and/or Rsat and fraction tissue oxygenation extraction (FTOE) within the three groups as well as with the presence of by retrograde diastolic blood flow in the descending aorta.[44] Given these conflicting reports, further evaluation of NIRS monitoring for hs-PDA is needed.

Video 1A: Real-time 3D view of the PDA
Video 1B: 3D view of PDA aneurysm

Management

Management of PDA continues to be controversial with no consensus regarding the need for treatment, optimal timing, and choice of treatment. As most PDAs close spontaneously, treatment of PDA has generally failed to show a decrease in adverse outcomes in preterm infants[45] and the medications may be associated with significant adverse events, like intestinal perforations; routine treatment to induce early closure of a persistent PDA in preterm infants has fallen out of favor.[46]

Timing of PDA Treatment

Historically, treatment considerations have focused on prophylactic vs therapeutic as well as conservative vs medical/surgical management plans.[22] Prophylactic indomethacin administration for prevention of intraventricular hemorrhage for infants born <1000 g and/or <28 weeks has been associated with decreased rates of hypotension, symptomatic PDA, and rates of any PDA.[47,48] Due to the reports showing high rates of spontaneous closure with increasing chronological age,[9,10] and those of complications reported with treatment and limited benefits on long-term outcomes,[11–13] there has been a shift in practice for delaying treatment until the physiological compromise becomes evident due to hs-PDA.[49,50] Based on current literature, the appropriate timing for initiation of treatment still remains an unanswered question with wide variations in practice.

Medical Management

Medical management of hs-PDA, with fluid restriction and administration of nonselective cyclooxygenase (COX) inhibitors, like indomethacin and ibuprofen, as well as prostaglandin-H_2 synthase inhibitors, like acetaminophen, are the first line of treatment before considering surgical options.[51–53]

COX inhibitors promote the constriction and eventual closure of the ductus[54] by inhibiting the synthesis and release of prostaglandins, which play a major role in maintaining ductal

patency during fetal life.[55] Although indomethacin has been the traditional "drug of choice" for treatment of PDA, the US Food and Drug Administration approved the use of ibuprofen lysine in April 2006 for closure of clinically significant PDA in premature infants <32 weeks and weighing between 500 and 1500 g. However, several adverse effects have been reported with these medications, including gastrointestinal bleeding and perforation, decreased platelet aggregation, hyperbilirubinemia, renal failure, thrombocytopenia, and hyponatremia.[52,56,57] In comparison, while acetaminophen appears as a safer alternative to indomethacin and ibuprofen with potentially fewer adverse effects,[58] it has lower efficacy, probably influenced by the gestational age and the size of the PDA.[59,60] The commonly utilized dosing protocols for all prophylactic and treatment protocols for these three medications are presented in Table 2.[52,61–66] The comparative efficacy of these medications is presented in Table 3. However, the protocols and subject selection in these studies were highly variable, making it difficult to generalize the results. Mitra et al., in their meta-analysis evaluated 68 randomized clinical trials of 4,802 infants, where 14 different variations of indomethacin, ibuprofen, or acetaminophen were used as treatment modalities. The overall PDA closure rate was 67.4% (2,867 of 4,256 infants). A high dose of oral ibuprofen was associated with a significantly higher odds of PDA closure vs a standard dose of intravenous ibuprofen [odds ratio (OR), 3.59; 95% credible interval (CrI), 1.64–8.17; absolute risk difference, 199 (95% CrI 95–258) more per 1,000 infants] and a standard dose of intravenous indomethacin [OR, 2.35 (95% CrI, 1.08–5.31); absolute risk difference, 124 (95% CrI, 14–188) more per 1,000 infants]. They concluded that a high dose of oral ibuprofen was associated with a higher likelihood of hemodynamically significant PDA closure vs standard doses of intravenous ibuprofen or intravenous indomethacin; placebo or no treatment did not significantly change the likelihood of mortality, necrotizing enterocolitis, or intraventricular hemorrhage.[67] In their Cochrane meta-analysis,

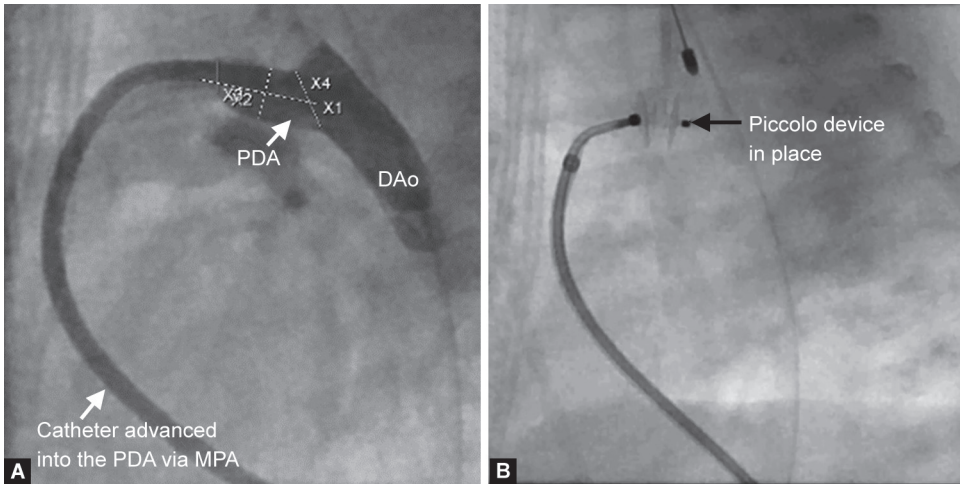
Table 2: Dosing regimen of pharmacological agents for PDA closure (Adapted from Neofax, 2021 [Accessed May 20, 2021])

Drug	*Dosing regimen*
Indomethacin prophylaxis[61–63]	1. 0.1–0.2 mg/kg/dose IV every 12–24 hours beginning within the first 6–24 hours of birth for a total of three doses **OR** 2. 0.1 mg/kg/dose IV every 24 hours for three doses
Indomethacin treatment[#]	• Age-based dosing: – <48 hours: first dose: 0.2 mg/kg IV; second and third doses 0.1 mg/kg IV Q 12–24 hours after first dose – 2–7 days: first dose: 0.2 mg/kg IV; second and third doses 0.2 mg/kg IV Q 12–24 hours after first dose – >7 days: first dose: 0.2 mg/kg IV; second and third doses 0.25 mg/kg IV Q 12–24 hours after first dose **OR** • Longer course 0.2 mg/kg/dose IV every 24 hours for a total of 5–7 days
Ibuprofen treatment[64,65,67]	• Loading dose of 10 mg/kg IV/PO on day 1, followed by 5 mg/kg/dose at 24 and 48 hours subsequently • A second course may be required **OR** • High dose of ibuprofen as 15–20 mg/kg followed by 7.5–10 mg/kg administered every 12–24 hours for a total of three doses
Acetaminophen treatment[52,66]	1. 15 mg/kg/dose IV/PO every 6 hours × 3 days 2. A second course may be required

[#]Product Information: Indomethacin IV injection. APP Pharmaceuticals, LLC (per Manufacturer), Schaumburg, Illinois, 2010

Table 3: Comparative efficacy of pharmacological agents for medical treatment of PDA

				Success rate (%)/OR [CrI, CI]				
Study	*Infants studied (N)*	*Study type*	*Diagnostic criteria for hs-PDA*	*Acetaminophen*	*Indomethacin*	*Ibuprofen standard dose*	*Ibuprofen high dose*	*Placebo*
Davidson et al., 2020	37	RCT	Echo only	5.9%	55%	n/a	n/a	
Mitra et al., 2018	4,802	Meta-analysis (68 RCTs)	Clinical + Echo	2.93 [1.52–5.62]	2.35 [1.08–5.31]	2.22 [1.44–3.40]	3.59 [1.64–8.17]	n/a
Overmeire et al., 2000	148	RCT	Clinical + Echo	n/a	66%	70%	n/a	n/a
Luecke et al., 2017	41	Observational	Clinical + Echo	66%	n/a	n/a	n/a	n/a
Meena et al., 2020	105	RCT	Clinical + Echo	71.43%	68%	77.14%	n/a	n/a
Kumar et al., 2020	161	RCT	Clinical + Echo	64% 1 course 89% 2 courses	n/a	78% 1 course 89% 2 courses	n/a	n/a



Figs 3A and B: Transcatheter closure of PDA in a preterm infant. (A) Measurements of the PDA prior to device deployment; (B) Image showing the successful deployment of the Piccolo device in the duct. PDA, patent ductus arteriosus; Dao, descending aorta; MPA, main pulmonary artery

Ohlsson et al. included 8 studies that reported on 916 infants. One study compared paracetamol to both ibuprofen and indomethacin, the other 5 compared the treatment of PDA with paracetamol (acetaminophen) vs ibuprofen and enrolled 559 infants. There was no significant difference between paracetamol and ibuprofen for failure of ductal closure after the first course of drug administration [typical risk ratio (RR) 0.95, 95% confidence interval (CI) 0.75–1.21; typical risk difference (RD) −0.02, 95% CI −0.09 to 0.09]; $I^2 = 0\%$ for RR and RD; moderate quality of evidence. They concluded that moderate-quality evidence according to GRADE suggests that paracetamol is as effective as ibuprofen; low-quality evidence suggests paracetamol to be more effective than placebo or no intervention; and low-quality evidence suggests paracetamol as effective as indomethacin in closing a PDA, but suggest need for neurodevelopmental follow-up data in future RCTs.[51]

Surgical Management

Surgical ligation of a symptomatic PDA in preterm neonates is successful in closing the ductal shunt in 98–100% of cases.[68] While surgical ligation of a hemodynamically significant PDA can improve hemodynamics, lung compliance, and reduce the duration of mechanical ventilation, complications associated with the procedure are well known, including but not limited to pneumothorax, hypothermia, intraoperative bleeding, phrenic nerve palsy, wound infection, vocal cord palsy, and thoracic scoliosis.[69–71] With increasing conservative management of hs-PDA and higher rates of morbidity and mortality with surgical treatment, the overall rates of ligation for PDA have decreased.[72,73] Ngo et al. examined the trends in a retrospective cohort study of very low birth weight infants (<1500 g) between 2008 and 2014 across 134 California hospitals. They described a trend toward lower annual rate of infants who received pharmacologic intervention (30.5 vs 15.7%) or both pharmacologic intervention and surgical ligation (6.9 vs 2.9%) as well as higher rate of infants who were not treated (60.5 vs 78.3%) or received primary ligation (2.2 vs 3.0%).[74]

In a retrospective cohort study, the cumulative mortality rates at 7 days, 30 days, and at hospital discharge were 2, 8, and 20%, respectively.[75] These concerns have led to exploration of transcatheter closure (TC) of PDA in preterm infants, based on successful and positive experiences from adults and children.[4] Initial assessments of TC of PDA in infancy (<1 year of age) were presented by Backes et al. in their meta-analysis of 38 observational studies and noted technical success rate of 92.2% [95% confidence interval (CI) 88.8–95.0] with overall adverse event and clinically significant adverse event incidence of 23.3% (95% CI 16.5–30.8) and 10.1% (95% CI 7.8–12.5), respectively.[76] These initial reports were followed by a RCT followed by subsequent FDA approval of the Amplatzer Piccolo Occluder to treat PDA in patients ≥700 g (Fig. 3). The trial reported an implant success rate of 95.5% (191/200) overall and 99% in patients ≤2 kg (99/100). Four patients experienced a primary safety endpoint event (two transfusions, one hemolysis, and one aortic obstruction), no branch pulmonary artery obstructions were noted and five patients, all ≤2 kg, were noted to have worsening of tricuspid regurgitation (TR) after the procedure.[77] Technical feasibility in extremely low birth weight infants, potential respiratory and length of hospitalization benefits make TC of PDA an attractive option.[78–80] Additionally, there seems to be a benefit of TC vs surgical ligation of PDA in relation to the occurrence of "postligation syndrome" seen in preterm infants.[81] It is defined as cardiac dysfunction observed due to a sudden increase in afterload and decrease in left ventricular preload with resultant decreased cardiac output and hypotension.[82,83] Careful patient selection, timing, and choice of surgical intervention as well as postprocedural management practices are areas requiring further investigation.

Conclusion

In conclusion, while great strides have been made in understanding the natural history of PDA closure as well as the identification of many promising diagnostic and therapeutic choices there remains the need for consensus guidelines for the management of PDA. Future clinical trials need to focus not only on universal, practical diagnosis of hs-PDA but also identify optimal timing and therapeutic choices.

Orcid

Rachana Singh https://orcid.org/0000-0001-7783-1214
Ruben Vaidya https://orcid.org/0000-0001-6674-4692
Akhil Maheshwari https://orcid.org/0000-0003-3613-4054

Videos

Both the videos are available online on the website of www.newbornjournal.org

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REVIEW ARTICLE

Extra-uterine Growth Restriction in Preterm Infants

Nitasha Bagga[1], Nalinikant Panigrahay[2], Akhil Maheshwari[3]

Abstract

Extra-uterine growth restriction (EUGR) is frequently seen in premature and critically ill infants. Even though advancements in neonatal intensive care have improved the survival of these high-risk infants, many new questions have emerged about the relationship between postnatal growth and neurodevelopmental outcome of these infants. EUGR has traditionally been ascribed to caloric restriction during postnatal periods of critical illness. Nutritional compromise, particularly during the first few weeks of life, may affect the overall growth and could also cause long-term neurodevelopmental impairment. The accidental and premature interruptions of pregnancy could also alter the normal mobilization and utilization of major nutrients from the ways that would have otherwise occurred during the last trimester of pregnancy, which is normally a period of maximal *in utero* growth. In this article, we review our current understanding of defining EUGR, various risk factors for EUGR, its pathophysiology, and possible ways with which our current healthcare protocols could prevent EUGR.

Keywords: Development, Growth restriction, IUGR, Premature, Skeletal.

Newborn (2022): 10.5005/jp-journals-11002-0019

Key Points

- Premature and critically ill infants often develop extra-uterine growth restriction (EUGR). In this article, we reviewed the risk factors, definitions, assessment of severity, and management of EUGR, and the likelihood of its association with altered neurodevelopmental outcome.
- We have briefly reviewed the association between changes in weight and skeletal parameters (skull growth, length).
- Chronic illnesses such as bronchopulmonary dysplasia, necrotizing enterocolitis, chronic liver disease, and cardiac conditions such as patent ductus arteriosus can alter postnatal growth.
- EUGR is usually secondary to chronic neonatal illnesses, but it may be a primary condition needing nutritional, medical, and genetic evaluation in some infants.

Introduction

Extra-uterine growth restriction (EUGR) is a "Nutritional Emergency" in preterm and critically ill term infants, which can arise from multiple clinical pathways and remains a challenge (Fig. 1). This term was first used in literature in 1982 by Hack et al. where weight <–2 *Z* scores at term gestation was used to define EUGR.[1] Subsequently, a few more groups were defined to have EUGR if their postnatal weight was <2 SD or <10th centile at 36 weeks or at discharge.[2,3]

Poor weight gain and EUGR have been associated with adverse medium- and long-term clinical outcomes. For instance, many studies have identified an association of poor in-hospital growth, whether it be in terms of weight gain,[4–6] length,[7,8] or head circumference,[4–6,9,10] with developmental delay. Even though causality remains unclear, these associations need study. The EUGR in some of the sicker infants could be rooted in feeding intolerance or in iatrogenic ultra-cautious provision of calories, but an alternative, equally valid explanation could also be in increased metabolic rates due to high severity of illness related to multiple comorbidities.[4,11–13] There are questions on whether education and course correction in nutrition could fully restore growth and prevent EUGR in the first scenario. We also do not know whether hyperalimentation in critically ill infants can, at least partially, mitigate the effects of increased metabolic rates and enable both weight gain and skeletal growth. Further study is needed before we can confidently tailor the nutritional strategies without adverse metabolic changes. We also need information to identify infants who are likely to respond to hyperalimentation.[14–16]

We need better definitions of EUGR. A recent review questioned the concept of defining EUGR based on only weight <10th centile at 36–40 weeks or at discharge.[17] In this article, we have extended this discussion and have included weight, skeletal, and cranial growth; modifiable and non-modifiable clinical associations; and the determinants of intrauterine growth. We also describe potential remedies to improve EUGR and the growth potential of these infants.

Defining EUGR

An accurate definition of EUGR is needed. As evident in literature, most of the studies defined EUGR based on weight at one point of time. It is important to appreciate that there is always biological variation in size of preterm infants; genetic determinants; social determinants; ante-, peri-, and postnatal morbidities; and inadequate nutritional support affecting the growth.[17] With the

[1,2]Rainbow Children's Hospital and Birthright, Hyderabad, Telangana, India

[3]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Nitasha Bagga, Rainbow Children's Hospital and Birthright, Hyderabad, Telangana, India, e-mail: nitashabagga@gmail.com

How to cite this article: Bagga N, Panigrahay N, Maheshwari A. Extra-uterine Growth Restriction in Preterm Infants. Newborn 2022;1(1): 67–73.

Source of support: Nil

Conflict of interest: None

evident literature, EUGR can be classified on the basis of consensus statement[18] and also described by Fenton et al. in their recent review[17] as "Identifying malnutrition in preterm and neonatal population-recommended indicators " of defining malnutrition in preterm infants. These classifications are shown in Table 1.

Extra-uterine Head Growth Restriction (EUHGR)

EUHGR is another important parameter to follow up in infants with faltering postnatal growth. It is defined as decreased head circumference-for age-Z scores to <2 SD, and has been associated with suboptimal neurodevelopmental outcomes.[4–6,10] The growth of the head circumference may be spared in some preterm infants with relatively recent onset of EUGR,[13,19] but other chronically undernourished infants may show restricted growth of all parameters, including head circumference, weight, and length. If there is a restriction in only the growth of head circumference but not in weight and length, there may be a need to evaluate for antenatal or postnatal neurological morbidities.[18] In preterm infants, post-discharge head growth may be more important as an indicator of cognitive outcome than in-hospital head growth.

Pathophysiology of EUGR

EUGR has been associated with multiple factors,[20] where one of the most critical ones is inadequate nutrition.[15] Despite consistent advancements to improvise preterm nutrition over decades, 28–97% of preterm infants develop EUGR as reported in various neonatal units.[21–23] Most of the nutritional determinants of postnatal growth are modifiable if followed rigorously.

- Modifiable risk factors of EUGR (Fig. 1):
  - Failing to meet required energy and protein needs during immediate postnatal period: Premature birth is the most critical period (third trimester) for nutritional accretion and rapid growth of fetus.[24] There is a considerable discrepancy in recommended daily intake (RDI) (to match the intrauterine growth) to the actual intake that builds up a cumulative mean energy and protein deficits within few weeks after birth.[15,25] Embelton et al. explained the cumulative energy and protein loss at the end of 5th week is around 813 ± 542 kcal/kg/day and 23 ± 12 g/kg respectively.[25] The nutritional goals during the initial days should seek to prevent the catabolism typically seen during the postnatal transition following preterm birth.
  - Variable nutritional practices: There is lack of consensus among individual neonatologists working in the same neonatal intensive care unit (NICU) and adhering to same nutrition protocol.[26] Variability in the time of initiation and amount of parenteral and enteral nutrition leads to poor accretion in these infants, cumulative energy and protein loss, and is the iatrogenic cause of EUGR.[27,28]
  - Overcautious increase of feeding volumes and frequent disruption of feeds: Neonatologists across the globe consider that delayed introduction, slow and cautious increase in feeding volumes may reduce the risk of necrotizing enterocolitis (NEC) in neonates.[29] This overcautiousness often leads to energy deficit and hence growth faltering. Likewise, the feedings are often interrupted in many infants with non-specific abdominal signs that are perceived as feeding intolerance. In many instances, the feeds are not re-initiated in a timely fashion.
  - Unintentional administration of low calories: In many premature infants born with lower-than-average birth weight, the nutritional goals may need to be carefully adjusted and if possible, aimed for the 50th percentile for that gestation.[30] Apart from this, many times the daily feedings may be below the RDI, as no changes in the amount of nutrients administered are made with respect to the increasing birth weight, which if continued for long periods causes cumulative energy and protein deficits.[24] Significant cumulative nutiritional deficit lies "in wait" in NICU as the clinicians hesitate about resumption of feedings.
  - Insufficient standard fortification: Breast milk is known to show considerable variation in nutritional content. Donor human milk, the next best option of milk for preterm infants if mother's own milk (MOM) is unavailable, is usually term milk or donated by mothers who are into many months of lactation. Thus, standard fortification may not be sufficient to meet the energy and protein needs of preterm babies because of the variability in mother's milk contents itself.[24,31,32]

Table 1: Classification of EUGR

Criteria	*Mild EUGR*	*Moderate EUGR*	*Severe EUGR*	*When to apply*
1. Weight-for-age *Z* scores[a]	Decline of 0.8–1.2 SD	Decline of >1.2–2 SD	Decline >2 SD	Not appropriate for first 2 weeks of life
2. Weight gain velocity[b]	<75% of expected weight gain for that particular age	<50% of expected weight gain for that particular age	<25% of expected weight gain for that particular age	Not appropriate for first 2 weeks of life
3. ≥2 of the following: • Length-for-age *Z* scores[a] • Length gain velocity[b] • Days to regain birth weight (in conjugation with nutrient intake)	Decline of 0.8–1.2 SD <75% of expected weight gain for that particular age 15–18 days (>3–5 consecutive days of <75% intakes of estimated protein/calorie)	Decline of >1.2–2 SD <50% of expected weight gain for that particular age 19–21 days (>5–7 consecutive days of < 75% intakes of estimated protein/calorie)	Decline >2 SD <25% of expected weight gain for that particular age >21 days (>7 consecutive days of <75% intakes of estimated protein/calorie)	Not appropriate for first 2 weeks, after that can be used in conjunction with other parameters if accurate length measurement is available Preferred for first 2 weeks of life

[a]Expected *Z* score for weight for age, length for age; [b]Weight gain velocity and linear growth velocity were estimated using online calculator (www.peditools.org). In this calculator, weight gain velocity is estimated by using the World Health Organization methods; Weight increments are classified by birth-weight category presented in 1- and 2-week intervals from birth to 60 days

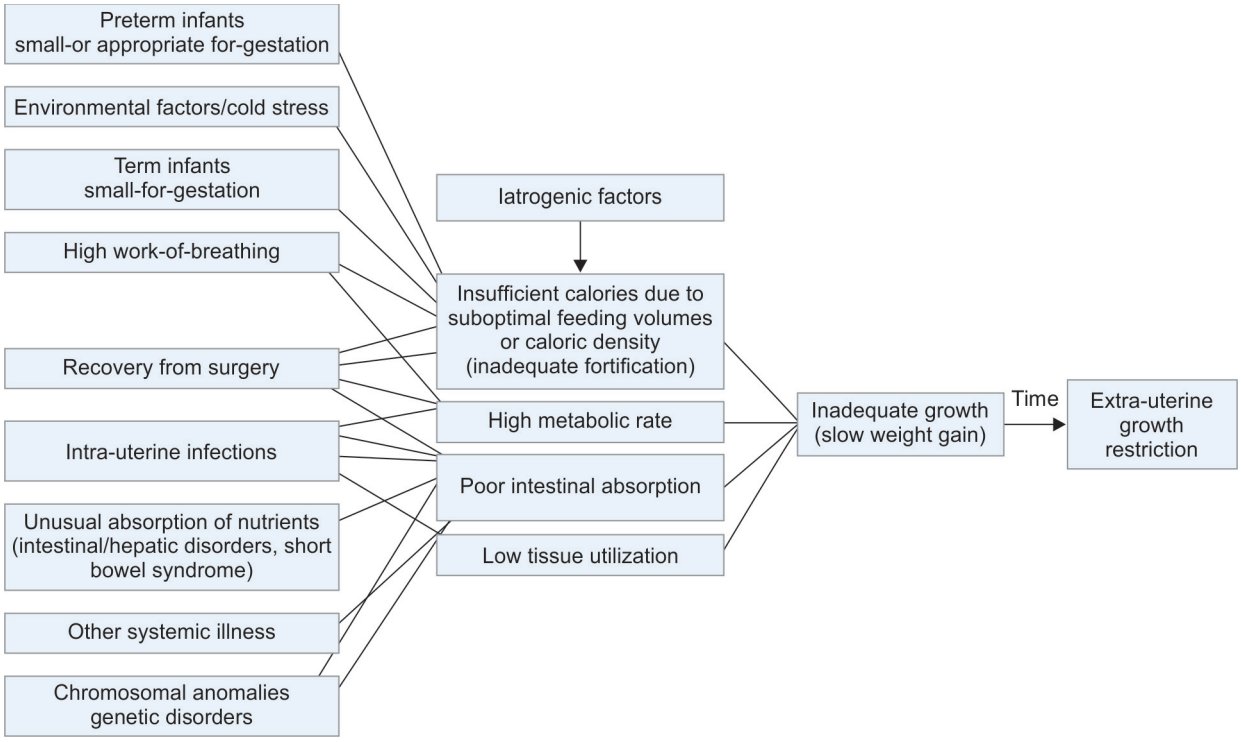

Fig. 1: Possible risk factors of EUGR (Extra-uterine Growth Restriction)

- Small for gestation (SGA): In preterm infants, particularly those who are also small-for-gestation, associated comorbidities may mandate frequent adjustments in feeding volumes. These babies, when cannot be given larger volumes of feeds, struggle with the similar concerns of inadequate protein and energy supply through routine fortification.[33]
- Multiple prematurity-related morbidities affecting growth during postnatal period: Prematurity is an important risk factor for EUGR, growth faltering, high morbidities, and poor neurodevelopmental outcomes.[14] Duration of hospital stay and ventilator support, bronchopulmonary dysplasia, patent ductus arteriosus, and NEC are independent risk factors for EUGR.[23]
- Postnatal growth failure in preterm and intrauterine growth retardation (IUGR) infants: Healthy fetuses who deliver at term do not encounter interruptions in nutritional supply, depletion of nutritional stores, deprivation of growth factors provided by mother and placenta, or increased energy consumption at the gestational ages that preterm infants have to experience *ex utero*.[34] Premature infants typically show considerable weight loss during the early neonatal period as feedings are still being established and due to multisystem illnesses mentioned above. However, even after achieving clinical stability, many do not achieve growth statistics similar to those of fetuses *in utero*, at least in terms of weight.[29,35] Similarly, many infants who were born Small for gestation (SGA) in terms of weight, length, and head circumference do not respond to nutritional interventions and continue to show EUGR. Some may even show worsening Z-scores from birth to discharge. More comprehensive measurements of total body composition are needed, at least in infants who are no longer on multiorgan system support. We are currently engaged in these measurements and should be able to report some data soon.

- Non-modifiable risk factors for EUGR
  - Epigenetic pathways and EUGR: EUGR is presumed to activate several reprogramming mechanisms. Various factors, both nutritional and environmental, regulate gene expression through epigenetic modifications[36–38] that might be responsible for intrauterine and extra-uterine growth. Tozzi et al.[39] reported that EUGR for weight and head circumference is associated with reduced intake of lipids and proteins in early days of life with hypermethylation of the IC1 (imprinting center 1) gene. Some authors emphasized that poor nutrition during the early part of life could be associated with epigenetic mechanism and underlined the relationship of decreased protein intake with DNA methylation.[40,41] In another study, Gong et al. highlighted the association of low maternal protein intake in animal models with IC1 methylation.[42] Once established, these epigenetic changes are difficult to reverse. Further research is needed to understand the value of these markers in EUGR for prognostication and as markers of response to potential therapeutic measures.
  - Plasma metabolome alterations and EUGR: Dudzik et al.[43] showed lower plasma levels of both essential and non-essential amino acids (especially branched chain amino acid) and several phospholipids (glycerophospholipids and sphingolipids) in EUGR preterm infants and also found further decline as per severity of EUGR (moderate >severe), which was irrespective of total parenteral and enteral nutrition in first week of life. Various bile acid metabolites were also found to be increased in severe EUGR infants, which could be hypothesized with the association of liver injury and growth failure. Further larger studies are required to understand the

pathways of growth failure in preterm infants and their long-term effects on developmental outcomes. These biomarkers may facilitate early identification of growth failure and help evaluate clinical/nutritional interventions.

- EUGR and genomic imprinting: Molecular alterations in parentally imprinted genes lead to various human imprinting disorders, which are associated with effects on intrauterine and postnatal growth. This knowledge of human imprinting disorders can be extrapolated to understand the complex regulation and interaction of genomic next-generation sequencing, transcriptomics, as well as methylomics in postnatal growth of preterm infants.[44]

Best Ways to Prevent EUGR (Fig. 2)

Prevention of EUGR in preterm neonates is one of the biggest challenges to neonatologists. Lack of standardized and evidence-based nutritional practices in a neonatal unit are the most common and modifiable risk factors responsible for EUGR.[15,20,21] The prerequisite in achieving optimal extra-uterine growth is the early identification of growth failure, timely intervention, and prevention. Although there are no evidence-based guidelines available, practice standardization and its consistent application can be done.[45,46] Various ways that can improve nutritional status of preterm infant and hence decrease the frequency of postnatal growth failure are as follows:

- Multidisciplinary nutrition support team: A team comprising neonatologists, nutritionists, lactation consultants, and dedicated nursing staff can strive to provide a consistent, individualized nutritional support to all neonates admitted in the nursery and provide a higher growth rate during NICU admission.[26,46] The support team can take the responsibility of regular nutrition specific rounds, growth chart plotting, and early identification of growth failure in preterm infants, which is an important milestone to prevent EUGR. Hence, it is advisable to have this support team observe and follow-up these babies during the hospital stay and after discharge for a sufficiently long period.
- Standardized feeding guidelines: Variation in the feeding practices guidelines in preterm infants is considered as one of the major determinants of postnatal growth failure.[47] It is recommended that every unit must have and strictly adhere to the standardized feeding guidelines to avoid discrepancies between the neonatologist and patient-to-patient variability. This approach helps in[48–50]
  - Immediate parenteral nutrition after preterm birth
  - Initiating feeding and guide to advance them
  - Rapid (or faster) achievement of full enteral nutrition
  - Manage feeding intolerance
  - Procedure and timing to introduce fortified human milk feedings
  - Reduction in the duration of parenteral nutrition

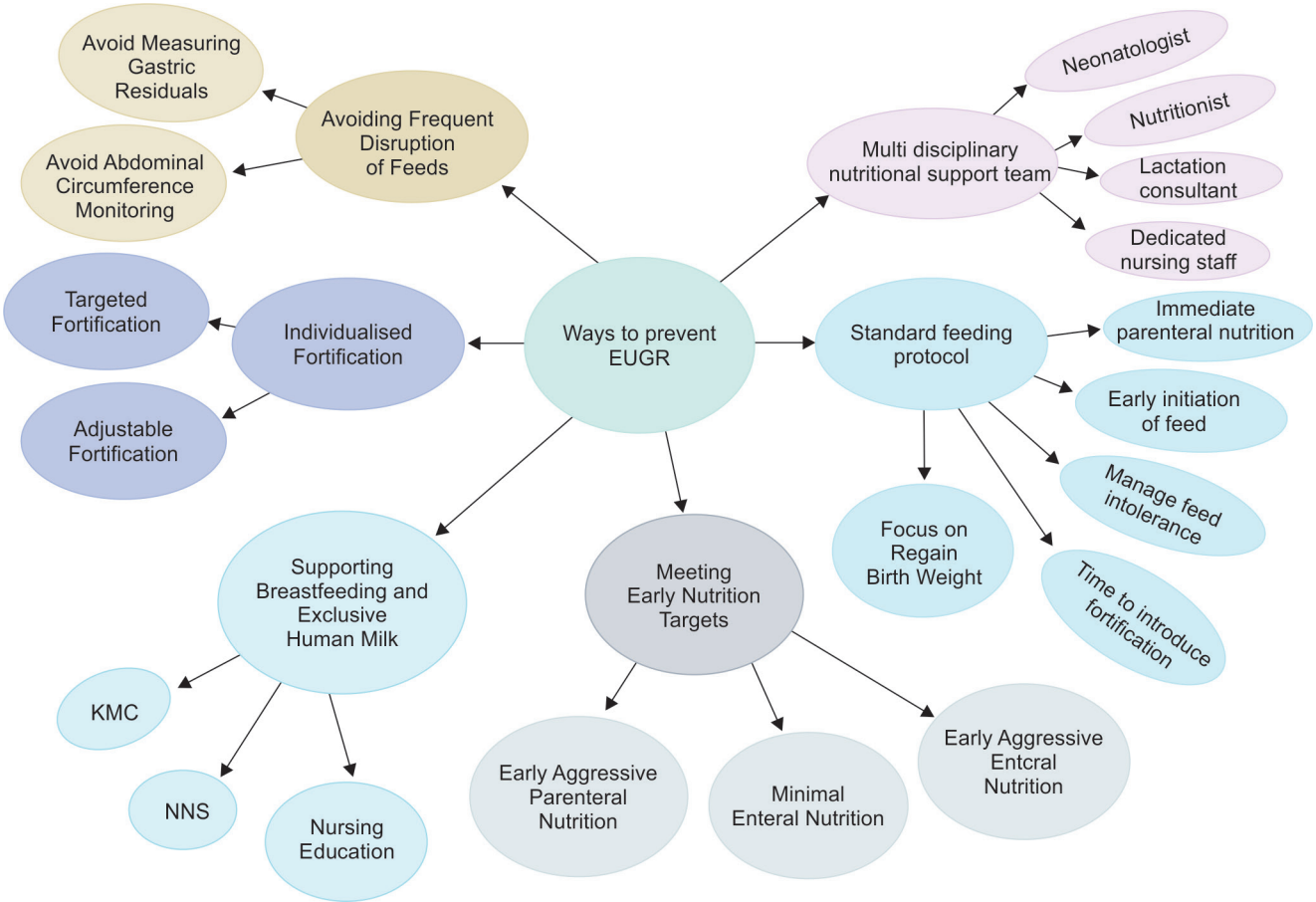

Fig. 2: Ways to prevent EUGR (Extra-uterine Growth Restriction) related to inadequate caloric intake

- Rapid regaining of birth weight
- Improved anthropometrics at 36 weeks' postmenstrual age (PMA).

Standardized feeding guidelines have been associated with lesser rates of NEC and late-onset sepsis, which are the two most important factors for growth failure in preterm infants.[51,52]

- Meeting early nutrition targets
  - Early aggressive parenteral nutrition: Immaturity of the gut in very preterm babies impedes enteral nutrition in adequate amounts during initial days. Early, aggressive parenteral nutrition not only minimizes the initial weight loss, cumulative protein, and calorie deficit during the acute, sensitive phase but also helps in improving the long-term growth and neurodevelopmental outcomes.[53,54] It is suggested to start amino acid in higher doses of 2–3 g/kg/day immediately after preterm birth.[55,56] along with lipids in dose of 1–2 g/kg/day in early hours of life[57] and subsequently increase the doses as recommended.
  - Minimal enteral nutrition: Minimal amounts of human milk (minimal enteral nutrition/trophic feeds) ranging from 10 to 20 mL/kg, starting as early as possible, must be a part of the standard feeding guideline. It is proven that early vs late (<48 hour vs >72 hour) initiation of enteral feeds significantly decreases the time required to reach full feeds, lesser time to regain birth weight, and shorter duration of total parenteral nutrition (TPN).[58]
  - Early aggressive enteral nutrition: In first 2 weeks of life, the EUGR group was given lesser enteral nutrition than non-EUGR group, which was correlated with healthy metabolomics profile (both amino acid and lipid profile) at the time of discharge in non-EUGR group.[43]
  - Supporting breastfeeding and ensuring exclusive human milk: Evidence-based, locally acceptable and relevant strategies to focus on exclusive MOM feeds should be made in every unit like antenatal and postnatal counseling, providing lactation support and educating mother for milk expression,[59,60] early and frequent pumping of milk,[61] role of Kangaroo mother care (KMC) and non-nutritive sucking (NNS),[62] and nursing education should be reemphasized.[63] Donor human milk (DHM) should be considered as the second best choice in the absence of MOM, as there is sufficient evidence in literature that DHM decreases the risk of NEC, chronic lung disease, retinopathy of prematurity and other prematurity-related morbidities, either used alone or along with MOM, when compared with formula feeds, which indirectly affects the postnatal growth outcomes.[64–66]
  - Targeted and adjustable fortification: Mother's milk alone is considered insufficient to meet the higher energy demands of preterm infants and hence, needs multicomponent fortification. It significantly helps in better weight gain, length, and head circumference,[67] safe in terms of feed intolerance and gastric emptying.[68] However, at the same time, standard multicomponent fortification may not be sufficient for adequate growth of these small premature babies.
  - Individualized fortification, which is a customized way of fortification guided by the growth and metabolic response of the baby, must be the focus of therapy.[69] This includes target fortification, adjustable fortification, and super-fortification.[70] However, none of these are considered ideal fortification, requiring further studies to formulize and draw optimal fortification strategies.
  - Avoiding frequent disruption of feeds: Altered gastric aspirates and increase in the abdominal girth are the most common causes for frequent disruption of feeds. It has been proven on various occasions that evaluations of gastric residuals delay the feeding process and can even damage the gastric mucosa.[71,72] Increase in abdominal circumference during prematurity is also variable and normal.[73] Therefore, frequent abdominal girth monitoring and checking gastric aspirates before every feed is not recommended.

Conclusions

Extra-uterine growth retardation should not be defined only on the basis of one-time weight assessment at 36–40 weeks or at discharge but all the three anthropometric parameters—weight, length, and head circumference—should be used together as an assessment tool for overall postnatal growth of preterm infants. Refinement in defining EUGR will not only help in appropriate growth assessments but also aid timely assessment of true growth faltering and interventions to deal with it. Lack of uniformity and inconsistency in nutritional practices are the most common causes. Nutritional assessment should be done on at least weekly basis during the NICU stay so that EUGR can be diagnosed and addressed timely. A lot of research is required to understand the deviation in body compositions of these preterm infants, which affects the postnatal growth than just to optimize and establish the recommended nutrition intakes.

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REVIEW ARTICLE

Advanced Cardiac Imaging in Neonatology

Bijoy Thattaliyath[1], Prashob Porayette[2], Ravi Ashwath[3]

Abstract

Imaging of congenital heart disease (CHD) starts in the intrauterine period by fetal echocardiography. The anatomy and physiology are confirmed postnatally by transthoracic echocardiogram. However, complex CHDs require further imaging to delineate anatomy for further management and surgical intervention. Cardiac magnetic resonance imaging (MRI) and cardiac chest tomography (CT) complement the role of transthoracic echocardiogram in delineating further details of anatomy and physiology in the neonatal period. This review covers the basic sequences and terminologies used in cardiac MRI and cardiac CT. A brief description of the indications and the ideal modality of imaging is described, including the limitations of each modality of imaging.

Keywords: Cardiac, Contrast, CT, Imaging, Indications, MRI, Neonate, Radiation, TnEcho.

Newborn (2022): 10.5005/jp-journals-11002-0020

Introduction

We are living in an exciting era of cardiac imaging as technological advancements now allow quick and accurate non-invasive imaging of a neonatal heart. Non-invasive cardiac imaging has come a long way since 1954 when Edler and Hertz first described the use of reflected ultrasound for imaging of the heart.[1] It was not until the late 1970s before M-mode echocardiography (echo) became available for clinical use in patients.[2] Invasive diagnostic cardiac catheterization was still frequently used for the diagnosis of congenital heart disease (CHD). By the 1980s, two-dimensional echo and color-flow Doppler[3] greatly improved the non-invasive diagnostic imaging capabilities in pediatric patients with CHD. Advancements in fetal ultrasonography made it possible to recognize CHD *in utero*.[4] Nowadays, echo is the first-line of non-invasive imaging tool in pediatric and adult cardiology with multiple advanced features such as tissue Doppler imaging, strain imaging, speckle tracking imaging, and three-dimensional imaging.[5] There are fascinating emerging techniques such as intracardiac[6] and portable ultrasonography.[7] In addition, the growth of neonatal hemodynamics program has allowed performance of targeted neonatal echocardiography (TnECHO) in premature infants with hemodynamic instability[8] with high diagnostic concordance between trained neonatal hemodynamics specialists and pediatric cardiology.[9]

Computed tomography (CT) technology was developed in the early 1970s.[10] Multiple software and hardware advancements, including helical imaging, multi-detector CT, and reconstruction methods, allow ultrafast imaging of the cardiac structures. In addition, low radiation makes CT an appealing imaging modality for CHD in newborns, children, and adults.[11,12]

Nuclear magnetic resonance (MR) was discovered in the 1940s.[13,14] MR did not enter the field of clinical cardiac imaging, however, until the early 1980s.[15] The initial ungated acquisition techniques were slowly replaced by electrocardiographic gating techniques resulting in improved image quality.[16–18] The first injection of gadolinium in human was in 1984[19] soon followed by first patient series.[20] Cardiac MR (CMR) is now frequently used for anatomical and functional evaluation of CHD.[21] Fetal cardiovascular magnetic resonance imaging (MRI) is an upcoming and intriguing technique showing promise as a clinical diagnostic tool in the setting of CHD when the cardiac anatomy is unresolved by ultrasound or when complementary quantitative data on blood flow, oxygen saturation, and hematocrit are required to aid in management.[22–25]

In most developed countries, severe CHD is diagnosed during gestation allowing appropriate medical management of these critically ill newborns immediately after birth. A postnatal chest radiography and echo can often provide the necessary details for diagnosis and management planning of neonate with CHD. When echo cannot provide the comprehensive details of relevant cardiovascular anatomy, advanced non-invasive cardiac imaging techniques such as CT and CMR play an important complementary role. Invasive diagnostic catheterization is reserved exclusively for neonates with complex cardiac anatomy needing further clarification of cardiac structures or direct measurement of pressure or oxygen saturation. This review focuses on the indications, techniques, and safety of non-invasive advanced cardiac imaging in neonates.

Goals of Advanced Neonatal Cardiac Imaging

The cardiac morphology and physiology play a critical role in the clinical decisions regarding surgical or interventional palliation in neonates with CHD. A segmental approach[26,27] is used to delineate cardiac anatomy from images obtained from echo and advanced imaging modality. The physiologic status is assessed using physical examination, chest radiography, echo, advanced imaging, and if needed cardiac catheterization.

[1–3]Department of Pediatrics, University of Iowa, Iowa City, Iowa, United States of America

Corresponding Author: Ravi Ashwath, Department of Pediatrics, University of Iowa, Iowa City, Iowa, United States of America, Phone: +3193563537, e-mail: ravichandra_ashwath@yahoo.com

How to cite this article: Thattaliyath B, Porayette P, Ashwath R. Advanced Cardiac Imaging in Neonatology. Newborn 2022;1(1):74–80.

Source of support: Nil

Conflict of interest: None

Indications for Neonatal Cardiac MRI and CT

The indication for advanced neonatal cardiac CT or CMR could be the assessment of intra-cardiac anatomy or extra-cardiac vasculature. Intra-cardiac assessment can be performed in patients with unusual complex CHD,[11,12,28] cardiac tumors,[29] cardiomyopathy[30] or to help decide single vs biventricular repair in patients with borderline ventricular hypoplasia.[31] Extracardiac indications include the assessment of vascular structures,[32] such as anomalous pulmonary veins, vascular ring, pulmonary sling, aorto-pulmonary collaterals, and aortic arch anomalies. In addition, patients with multiple visceral anomalies such as in heterotaxy syndrome[33] (also known as isomerism)[34] frequently need extracardiac assessment.

Echocardiography remains the main diagnostic tool in the neonates for cardiovascular imaging. However, there are certain instances where additional information needs to be obtained to confirm the diagnosis or add information when echocardiography has not been able to provide complete information. Historically, cardiac catheterization, which is an invasive procedure and needs general anesthesia, was performed to sort out some of the complex anatomy when echocardiography could not provide all the answers. With advancement in CT and MRI technologies, the need for cardiac catheterization as a diagnostic modality has largely become obsolete. Advancements in non-invasive complementary tools such as cardiac MRI and CT are extremely helpful as additional imaging modalities for obtaining high-quality imaging.

The tenets of full basic understanding of the cardiac anatomy should include imaging that sorts out abdominal situs, atrial situs, ventricular looping, great artery relationship, systemic and pulmonary veins, atrial and ventricular septal morphology, atrioventricular valves, semilunar valves, coronary arteries, great arteries, intracardiac and extracardiac vascular anomalies. The diagnostic utility of echocardiogram can be limited by poor acoustic windows.[35]

Cardiac MRI in the Neonates

Multimodality assessment of CHD is essential for anatomical and functional evaluation for diagnosis and planning.[36] Indications for cardiac MRI in the neonate can be broadly classified into two categories:

- Based on cardiac MRI sequences
  - Anatomy delineation
  - Flow quantification
  - Magnetic resonance angiography (MRA)
  - Tissue characterization
- Based on clinical indications for a cardiac MRI study
  - Complementary modality to avoid multiple echocardiographic scans when there are poor acoustic windows
  - Understanding complex spatial orientation of the cardiac chambers
  - Obtaining accurate volumes and function, including end-diastolic volumes and ejection fraction quantification, calculating Qp:Qs, regurgitation fractions of valves

    Example: Volumetric data to determine single ventricle vs biventricular repairs
  - Sorting out coronary artery anatomy in select cases
  - Tissue characterization to sort out cardiac masses and tumors
- To delineate extracardiac vascular anatomy accurately where echocardiogram is unable to sort out intricate anatomy of the aortic arch, pulmonary arteries, systemic veins, and pulmonary veins. Examples include:
  - Branching pattern of the aorta, which may constitute a vascular ring
  - Delineation of collateral vessels supplying pulmonary arteries from the aorta
  - Pulmonary artery slings
  - Aortic arch morphology for surgical planning, including cases of complicated coarctation of aorta and hypoplastic aortic arches
  - Interrupted aortic arches with complicated branching patterns
  - In cases like pulmonary atresia where one needs to sort out all the sources of pulmonary blood flow
- To assess airway issues secondary to vascular malformation or chamber enlargement causing compression. Examples include:
  - Understanding tracheal anatomy in case of heterotaxy syndrome, or major lung abnormalities
  - Tracheal anatomy to quantify the degree of stenosis secondary to vascular ring
- Postoperative period with patient instability to assess patency of aortopulmonary shunt, RV to PA conduit, any sources of external compression to the bronchus or the vessels, ventricular aneurysms, right ventricular outflow tract aneurysms after Norwood type I procedure with a Sano shunt, etc.
- Venous anatomy and abnormalities:
  - Assess venous anatomy to make sure there is no superior vena cava (SVC) obstruction in patients with Glenn and after arterial switch operation
  - Pulmonary vein abnormalities, which can include various forms of total anomalous pulmonary venous return, especially the mixed type, partial anomalous pulmonary venous return, and scimitar syndrome
- Obtaining 3D data sets for 3D printing and virtual reality

Cardiac MRI Techniques

Performing a cardiac MRI is slightly tedious due to the fact that during the imaging period, there is dynamic motion of the heart coupled with respiratory motion, both of which interferes in image acquisition. Methods have to be taken to mitigate this with specialized sequences and breath-holding techniques, which could be voluntary or via sedation/general anesthesia. Sedation in neonates and young infants (<6 months old) can be achieved by scanning during their natural sleep after a feeding.[37] It is beyond the scope of this article to go over the physics of different sequences utilized in cardiac MRI. We will briefly go over some of the common sequences used in routine clinical practice.

- **Spin Echo Black Blood Imaging:** These images are generally T1-weighted images with short echo times. The most common variants of this technique are fast (turbo) spin echo and single-shot fast (turbo) spin echo. They have high tissue to blood contrast with blood pool appearing black and the tissue appearing gray to white. This sequence is particularly useful to evaluate anatomy and relationships of blood vessels and

airways. These are static images (Fig. 1A). This sequence is also forgiving when there are metallic artifacts when compared to steady-state free precision (SSFP).[38]

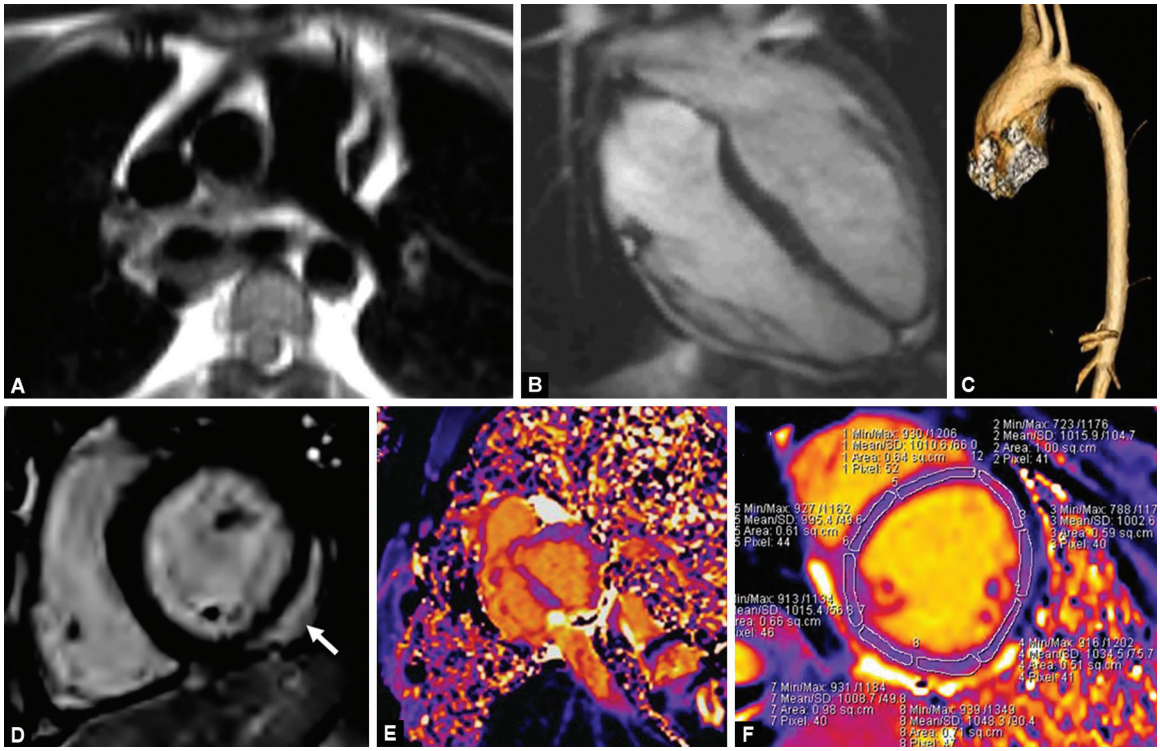
- **Balanced Steady-state Free Precision (bSSFP):** This is the work horse sequence of cardiac MRI where the blood pool is white and the tissue is black. The images obtained here are cine images and are in real time (Fig. 1B). This helps in assessing ventricular function, ejection fraction, valvular stenosis, and regurgitation. When there are artifacts and turbulent flow, conventional gradient recalled echo (GRE) cine sequences can be used.
- **Phase-encoded Flow Imaging:** This sequence is used to calculate flow measurements across the blood vessels (arteries, veins, and across the valves). This helps in calculating flow volumes, differential flow to each lung, pulmonary to systemic blood flow ratio (Qp:Qs), regurgitation fraction and peak gradients across the valves, collateral flow.
- **3D Gadolinium-enhanced Magnetic Resonance Angiography (3D-MRA):** This sequence needs intravenous administration of a gadolinium-based contrast agent. Images are simultaneously acquired in three planes and hence are very helpful to perform 3-D volume rendering and multiplanar reconstructions (Fig. 1C). It is extremely helpful in defining vascular anatomy and to measure accurate lengths with orthogonal measurements.
- **First-pass Perfusion Imaging:** This is a not so commonly used in the neonatal population. It is obtained after the administration of gadolinium and helps is identifying perfusion defects signifying infarction or fibrosis.
- **Late Gadolinium Enhancement Imaging (LGE):** Generally obtained 10 minutes after the administration of gadolinium, this sequence is useful in identifying edema, scarring, and fibrosis. The areas of scar and fibrosis have greater distribution of volume of contrast, and hence, there is delayed washout of the contrast. This makes the areas appear bright as compared to the normal myocardium (Fig. 1D).[39]
- **T1 and T2 Mapping:** It can provide data on tissue characterization like assessing presence of scarring/fibrosis and edema without administration of the contrast agent. In addition, it can provide extracellular volume (ECV) calculation if we obtain postcontrast T1 images (Figs 1E and F).

Cardiac CT in the Neonate

Cardiac CT is a complementary modality to echocardiography or cardiac MRI used in the evaluation of CHD in the neonates. It provides a fast approach to high-resolution images for defining the cardiac anatomy. Current CT scanners provide a spatial resolution of 0.24 mm and temporal resolution as low as 66 ms, for better visualization of complex congenital cardiac anatomy. They also have a high pitch rate with multidetector technology that can acquire full anatomic coverage of the heart and chest within 0.25 seconds freezing most of the cardiac and respiratory motion. This alleviates the need for sedation or cardiac anesthesia in most cases. The advancement of technology of current CT scanners with the short scan times also greatly reduces the radiation dose.

Radiation Exposure

The goal of every cardiac CT scan is to achieve the highest resolution images ensuring a radiation dose of as low as reasonably achievable (ALARA). However, cumulative radiation dose can be high particularly in patients with complex CHD, based on the different



Figs 1A to F: Representative images obtained by various sequences. (A) Image obtained with spin echo black blood imaging sequence where the blood pool is black; (B) Image obtained with balanced steady-state free precession (bSSFP) sequence where the blood pool is bright; (C) Volume-rendered image of aorta obtained with 3D-MRA; (D) Late Gadolinium enhancement image showing abnormal myocardium with arrow; (E) T1 parametric imaging in the short axis; (F) T2 parametric imaging in the short axis

tests that are undertaken in neonates that include chest X-rays, cardiac catheterization, cardiac CT and lung perfusion scans. This necessitates the need for inclusion of the most recent technology to minimize total radiation dose by prospective ECG-gated scans, modulation of tube voltage based on patient's size, high-pitched helical scanning, and iterative reconstruction techniques.[40,41] With today's third-generation multidetector-row computed tomography (MDCT) scanners, the radiation dose can be very minimal. We try to achieve submillisievert (less than 1 mSv) radiation dose exposure for most studies with excellent definition of their cardiac anatomy.[42] Most neonatal scans are limited to a dose of 70kVp and field of coverage is minimized based on the structure being imaged.

CT Angiogram Imaging Modalities

- Dual-source helical computed tomography scanner with ultra-fast, low dose, high-pitch scanners referred to as FLASH imaging (Siemens Healthcare, Forchheim, Germany). New scanners include the technology of dual-source computed tomography with prospectively ECG-triggered data acquisition by maintaining high pitch values. This helps minimize the total radiation dose by very short scan times with an approximate imaging window of 250 ms. Recent scanners with high pitch values of up to 3.4 when used can cover the entire volume of heart in one single cardiac cycle with high-resolution image quality and radiation doses in the submillisievert range.[43,44]
- Retrospective EKG-gated imaging: Prospective and retrospective gated cardiac imaging performed to minimize artifacts caused by cardiac motion. Prospective gated imaging performs well when acquired with heart rates less than 90 bpm and may be a limitation in the neonate. When detailed coronary anatomy or cardiac volumes need to be acquired, the usual technique is retrospective EKG-gated cardiac imaging. This involves acquiring images through various phases of the cardiac cycle. This uses a lower pitch with resultant increase in the radiation dose.[45]

Preparation of the Neonate for a CT Scan

Prior to any CT scan, it requires that the primary and cardiac teams review the necessity of advanced cardiac imaging and the direct outcomes for patient management weighing the risk of radiation exposure. Next would be to review renal function and allergies. If a neonate has abnormal renal function, other modalities of imaging should be explored or imaging delayed until the recovery of renal function. The next important step is evaluating the need for sedation. Most neonates can be calmed adequately using sucrose solution with a pacifier prior to the scan.[46] Motion can be minimized by feeding the baby prior to scan and comfortably swaddled in a blanket. However, for coronary artery delineation, the patient may need sedation with or without intubation, especially when breath holding is required to minimize motion artifacts, as in the case of a patient with d-transposition of the great arteries (TGA). A reliable peripheral intravenous catheter (PIVC) is required for contrast delivery. It can be of either a 24-G or a 22-G catheter. An umbilical venous or arterial catheter or a regular peripherally inserted central catheter (PICC) in general cannot be used for contrast delivery as they are not rated for power injections. This poses the risk of catheter tear and embolization during power injections. Routinely contrast in neonates is injected using a power injector with a pressure rating of 100–150 psi.

Contrast

For cardiac CT imaging, non-ionic iodinated contrast (iohexol, iopamidol, iopromide) is the standard of care. Dosing is usually limited to 2 mL/kg. Pediatric allergic reactions are rare, and management is similar to other allergic reactions with antihistamines, steroids, epinephrine, and bronchodilators.

Indications

The current indications for cardiac CT in neonates are listed below in order of what is more commonly used. Most of the acquisitions can be obtained by using a FLASH technique as described earlier (Fig. 2) and by retrospective gating for coronary imaging (Fig. 3).

Aortic Arch Anatomy

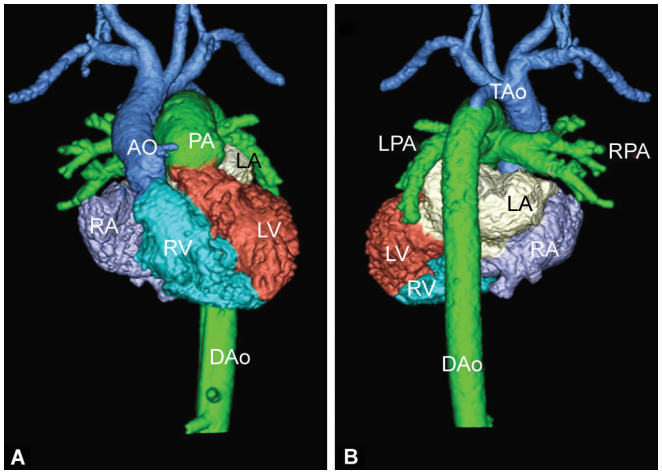
Various aortic arch anomalies can be present at birth (interrupted aortic arch, double aortic arch, vascular rings, supravalvar aortic stenosis) or develop within the first few days (coarctation of aorta). Diagnosis and delineation of aortic arch anomalies can be easily obtained by an ECG-gated or non-gated flash CT imaging of the chest. Usually, no sedation is required, and the patient can be fed and swaddled. Image acquisition is performed based on contrast opacification of left ventricle or aorta.

Pulmonary Artery Anomalies

Pulmonary artery anomalies are imaged with a similar technique as aorta. However, image acquisition timing may differ based on pulmonary arterial supply. With normal pulmonary arterial connections from the right ventricle outflow tract, image acquisition is timed based on opacification of the right ventricle. In patients with tricuspid or pulmonary atresia, usually pulmonary arteries are supplied by the patent ductus arteriosus, and timing of image acquisition is based on opacification of aorta.

Abnormalities of Great Vessels

Congenital heart defects like d-TGA (Fig. 2), congenitally corrected TGA, and tetralogy of Fallot (TOF) usually require advanced imaging in neonatal life if early surgical intervention is required. This may be to delineate the size of the pulmonary artery and aorta, coronary artery anatomy, or the location of the ventricular



Figs 2A and B: CT Flash images from a neonate at day of life 3 with d-transposition of the great arteries. Imaging performed to delineate the great vessel and aortic arch anatomy. Transverse aortic arch (TAo) was hypoplastic (A) Anterior view; (B) Posterior view. (LV, left ventricle; RV, right ventricle; RA, right atrium; LA, left atrium; AO, aorta; PA, Pulmonary artery; RPA, right pulmonary artery; LPA, left pulmonary artery; TAo, transverse aortic arch; DAo, descending aorta)

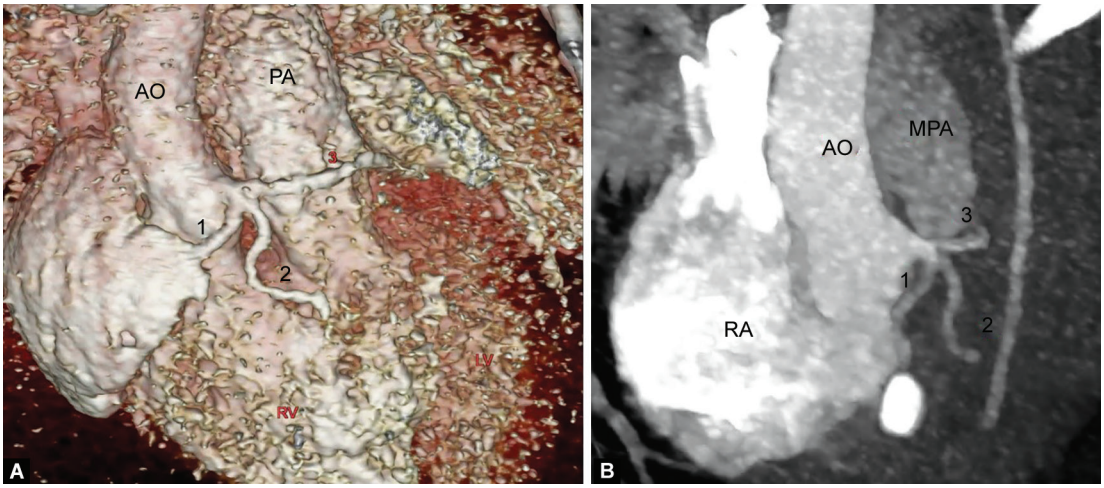


Fig. 3: Retrospective gated CTA in a neonate with d-transposition of the great arteries showing a single coronary artery that arises from the left anterior facing sinus of the aorta and divides into the right coronary artery (1), the left main coronary artery (2) and a prominent conal branch (3). (LV, left ventricle; RV, right ventricle; AO, aorta; MPA, main pulmonary artery). (A) It shows 3D reconstruction from the acquired imaging data; (B) MIP images of aorta with coronary arteries

septal defect. Usually, most of this can be accomplished by FLASH CT technique; however, if detailed coronary anatomy is required, it may necessitate retrospective ECG-gated coronary imaging. The scans are usually timed based on aorta opacification. Care should be taken to account for the anatomy and the presence of a ventricular septal defect. In TGA, based on the level of mixing between the right and the left side, the timing of contrast in the aorta could be quite variable. In neonates, this can be challenging given the small volume of contrast that can be used based on the body weight.

Congenital Coronary Artery Anomalies

Congenital coronary artery anomalies may exist in isolation or with other CHDs. Coronary arteries are quite small in infants with size ranging between 1 and 2 mm.[47] This combined with high heart rates and respiratory motion makes coronary imaging challenging. Imaging coronary origins in infants can mostly be achieved without sedation and FLASH CT technique. However, in cardiac lesions like TOF or TGA, clear delineation of coronary course is important prior to surgical repair. In these cases, patients may need sedation with intubation to perform breath holds and minimize respiratory motion. Some centers have also included the use of beta-blockers routinely to decrease heart rates prior to coronary scans.[48]

Evaluation of Pulmonary Veins

Evaluation of pulmonary venous anomalies will depend upon the suspected defect. Total anomalous pulmonary venous return (TAPVR) can be supracardiac, cardiac, intracardiac, or mixed type. Usually, the scan timing can be based on left atrial opacification. This scan range will depend upon the suspected drainage of the pulmonary veins. If infracardiac pulmonary venous drainage is suspected, the scan range should include subdiaphragmatic region up to the renal arteries. If anomalous venous drainage is suspected into the SVC draining to the right side, clear delineation of the anatomy may be accomplished by obtaining a peripheral intravenous route in the lower extremity for contrast administration.

Evaluation of Systemic Venous Anomalies

If systemic venous anomaly like interrupted inferior vena cava with azygous continuation or persistent left SVC is suspected, it may be required to image the systemic veins. This is usually accomplished by a delayed scan after contrast injection. The delay to image the venous recirculation can be based on the heart rate and presence of any intracardiac shunting. This generally can be accomplished by scanning between 30 and 45 seconds after contrast injection. If an arterial scan is required prior to the venous phase, it can be obtained with opacification of the aorta followed by a delayed scan 30–45 seconds later.

Evaluation of Single Ventricle Anatomy and Estimation of Ventricular Volume

Patients born with hypoplastic left or right ventricle, or an unfavorable anatomy that results in eventual single ventricle palliation, may need surgical intervention in the neonatal period. The usual CHD that undergo a single ventricle palliation include hypoplastic left heart syndrome, tricuspid atresia, pulmonary atresia with hypoplastic RV, unbalanced atrioventricular canal defect, and double inlet left ventricle. These patients may require delineation of their arterial anatomy and occasionally evaluation of their ventricular size prior to stage I palliation. The initial palliation in these cases may include a Blalock-Thomas-Taussig shunt, Sano shunt, or occasionally a hybrid procedure, which involves stenting of the patent ductus arteriosus and placement of branch pulmonary artery bands to limit the pulmonary blood flow. CT imaging for these patients can be accomplished usually by FLASH CT. Occasionally when coronary artery anatomy or ventricular volumes are required, a retrospective gated cardiac scan may be necessitated.

Image Analysis, 3D Image Reconstruction, and Printing

Many advanced software applications are available to further process the data sets obtained by cardiac MRI or cardiac CT. Cardiac MRI data can be post-processed to obtain three-dimensional images of aortic arch, pulmonary arteries, pulmonary veins, and coronary arteries. Further, the endocardial and epicardial borders can be traced out to

determine ventricular volumes and function. MRI provides a reference standard for the evaluation of cardiac function.[49]

Cardiac CT images can be further evaluated by processing to create high-resolution 3D images**.** Multiplanar reconstruction (MPR) images can be used to present structural anatomy and accurate measurements by orthogonal measurements by double oblique technique. Processing software can also determine vessel diameter in the areas of narrowing or stenosis. When imaged through the cardiac cycle, cine images can be created to demonstrate the changes in intracardiac structure and anatomy or extracardiac vessel diameters during the cardiac cycle.

From the acquired cardiac CT or cardiac MRI images, three-dimensional printing of the cardiac and extracardiac structures can be accomplished to further aid the understanding of the anatomy and surgical planning.[50] This is especially applicable in patients with double outlet right ventricle, aortic arch malformations, and patients with complex TOF, especially the cases with major aorto-pulmonary collaterals (MAPCAs).[51]

Conclusion

Neonatal cardiac imaging is a complex and exciting field that provides rapid and accurate diagnosis of neonatal anatomy and physiology to determine the optimal management. Fetal echocardiography and transthoracic echocardiography provide the initial tools in delineating the complex anatomy. For successful medical management and surgical intervention, some of these cardiac lesions require advanced cardiac imaging by cardiac MRI or CT. Indications for cardiac CT or cardiac MRI should be determined based on the requirement of anatomical or functional details in the presenting cardiac defect. With recent advances in both fields, this can be accomplished with minimal adverse effects. With the introduction of faster and efficient cardiac CT scans, the radiation dose is minimized. In the field of cardiac MRI, the use of specific neonatal magnetic coils and 3T scanners will help in getting images of better resolution in a shorter period. Ongoing research and advances in both fields with the complementary role of 3D models and virtual reality imaging project a more dynamic and exciting future in the field of neonatal cardiac imaging.

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REVIEW ARTICLE

The Potential Role of Maternal Periodontitis on Preterm Birth and Adverse Neonatal Neurologic Outcomes

Gregory Charles Valentine[1], Sandra E Juul[2]

Abstract

Periodontitis is an often overlooked but important risk factor for both preterm birth and adverse neonatal outcomes. With preterm birth being the leading cause of mortality for all children under the age of 5, any potentially modifiable risk factor associated with preterm birth must be fully evaluated. Periodontal disease is due to bacterial infection of the gingivae with resulting localized and systemic inflammation that can have profound effects in both nonpregnant and pregnant individuals. In pregnancy, several studies have demonstrated an association between periodontitis and preterm birth. Furthermore, extensive evidence demonstrates that fetal exposure to systemic inflammation during gestation predisposes to brain injury and neurodevelopmental delay. Thus, periodontitis and the resulting inflammatory cascade not only affect the pregnant individual but also have significant lifelong consequences on the development and well-being of future offspring. In this review, we will first discuss the epidemiology, prevalence, and pathophysiology of periodontitis. We will then explore the medical literature evaluating the association between periodontitis and preterm birth prior to delving into the potential for neurodevelopmental delay and brain injury among offspring. Finally, we will conclude by discussing future directions and unanswered questions related to periodontitis and its relationship with preterm birth and adverse neonatal outcomes.

Keywords: Inflammation, Neurologic impairment, Periodontitis, Preterm birth.

Newborn (2022): 10.5005/jp-journals-11002-0008

Introduction

Periodontitis is an often overlooked but important risk factor for both preterm birth and adverse neonatal outcomes. With preterm birth being the leading cause of mortality for all children under the age of 5, any potentially modifiable risk factor associated with preterm birth must be fully evaluated.[1–3]

Periodontal disease is due to bacterial infection of the gingivae with resulting localized and systemic inflammation that can have profound effects in both nonpregnant and pregnant individuals. In pregnancy, several studies have demonstrated an association between periodontitis and preterm birth.[4–9] Furthermore, extensive evidence demonstrates that fetal exposure to systemic inflammation during gestation predisposes to brain injury and neurodevelopmental delay.[10–13] Thus, periodontitis and the resulting inflammatory cascade not only affect the pregnant individual but also have significant lifelong consequences on the development and well-being of future offspring.

In this review, we will first discuss the epidemiology, prevalence, and pathophysiology of periodontitis. We will then explore the medical literature evaluating the association between periodontitis and preterm birth prior to delving into the potential for neurodevelopmental delay and brain injury among offspring. Finally, we will conclude by discussing future directions and unanswered questions related to periodontitis and its relationship with preterm birth and adverse neonatal outcomes.

Periodontitis

Epidemiology

Periodontitis is a noncommunicable disease of significant concern as it has a prevalence of 45–50% worldwide and is the sixth most common human disease.[14] Some studies even report periodontitis occurring in nearly 90% of certain populations.[15,16] Resource-limited settings have a substantially higher burden of periodontal disease and dental caries. For example, among nearly 400 pregnant or recently postpartum women in Malawi, the prevalence of dental caries was recently estimated to be 69.3% and composite dental disease (including dental caries and periodontal disease) was 76.7%.[17] Similar results have been found elsewhere with rates of gingivitis occurring in 47, 86, and 89% of pregnant women in Brazil, Thailand, and Ghana, respectively.[18–20]

Having a periodontal disease is associated with overall poorer health. Known risk factors for periodontitis include smoking, low socioeconomic status, low educational level, obesity, stress, diabetes, and increasing age.[21,22] Periodontal disease is known to be independently associated with other noncommunicable

[1]Department of Pediatrics, Division of Neonatology, University of Washington, Seattle, Washington; Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine at Baylor College of Medicine, Houston, Texas, United States of America

[2]Department of Pediatrics, Division of Neonatology, Center on Human Development and Disability, University of Washington, Seattle, Washington, United States of America

Corresponding Author: Gregory Charles Valentine, Department of Pediatrics, Division of Neonatology, University of Washington, Seattle, Washington; Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine at Baylor College of Medicine, Houston, Texas, United States of America, Phone: +2065433200, e-mail: gcvalent@uw.edu

How to cite this article: Valentine GC, Juul SE. The Potential Role of Maternal Periodontitis on Preterm Birth and Adverse Neonatal Neurologic Outcomes. Newborn 2022;1(1):81–90.

Source of support: Nil

Conflict of interest: None

diseases which have lifelong ramifications including diabetes mellitus, cardiovascular disease, chronic kidney disease, and chronic obstructive pulmonary disease.[23–26] Moreover, in individuals with multiple morbidities, having periodontitis is associated with decreased survival.[25] Thus, periodontitis is associated with overall poor health status and is a potential modifiable risk factor that can be targeted to potentially prevent further health decline or death.

Periodontitis Pathophysiology

Periodontitis has a multifactorial origin that begins with bacterial colonization of the gingival tissues. Initially, dental plaque develops, which consists of bacteria surrounding themselves within a protective biofilm that is resistant to antimicrobial agents.[27,28] The dental plaque is polymicrobial with Gram-positive, facultative bacteria such as *Streptococcus* and *Actinomyces* considered primary colonizers in an initially higher oxygen setting and Gram-negative, anaerobic bacteria such as *Fusobacterium* colonizing in more oxygen-depleted later stages.[29–31] In fact, the shift between aerobic to anaerobic conditions is a hallmark of the progression from gingivitis to periodontitis.[31,32]

Colonization with periodontopathic bacteria leads to a host response that is the main culprit behind the tissue destruction and local inflammatory reaction associated with periodontitis. The bacteria stimulate the innate immune response, which leads to inflammation and neutrophil migration.[33] Pro-inflammatory cytokines and other inflammatory mediators, such as prostaglandins, tumor necrosis factor-alpha (TNF-alpha), and interleukin 1-beta (IL-1 beta), are secreted.[33] Subsequently, cytokines stimulate the adaptive immune response, which leads to differentiation of T and B cells along with activation of the receptor activator of nuclear factor-kB (RANK).[34] While T and B cells lead to targeted tissue destruction, activation of RANK leads to osteoclast activation with resulting bone resorption and tooth loss.[34]

Gingivitis, or inflammation of the gingivae, is the initial sign of inflammation and tissue destruction and is a reversible process. Histopathologically, gingivitis does not involve any loss of bone or periodontal tissue support structures.[35,36] Bleeding, red, and/or swollen gums can occur and are clinical signs and symptoms of the acute inflammatory injury associated with gingivitis. In this earlier phase of periodontal disease, dental hygiene is paramount to prevent the progression of gingivitis into periodontitis.[35] If not reversed, the continued inflammatory injury and resulting tissue destruction of these events lead to periodontitis with the destruction of collagen fibers, loosening of teeth, bone resorption, and eventual loss of teeth.[37]

Pregnancy and Risk of Periodontitis

Pregnant women are at higher risk for periodontal disease.[15,16,38] It has been well-documented since the 1960s that there exists an association between gingival inflammation and pregnancy.[39–41] In women who had preexisting periodontal disease, pregnancy led to increased periodontal probing depths and worsening of bleeding gums which resolved after delivery.[42] While the exact mechanism for how or why increased gingival inflammation occurs during pregnancy is not known, there are key studies elucidating a likely role of circulating hormones such as estrogen and progesterone. These hormones are commonly elevated in pregnancy due to production by the corpus luteum and subsequent placenta.[43,44] Both estrogen and progesterone receptors are located in the periodontium including the periodontal ligament, the structure that connects teeth to the underlying alveolar bone which becomes eroded during periodontitis, further supporting the role of these hormones on oral health.[45,46]

One proposed mechanism for pregnancy-associated gingivitis and periodontitis is alteration of the oral, and specifically periodontal, microbiota. One study found increased levels of *Bacteroides intermedius* in the second trimester of pregnancy which decreased postpartum, which is believed to be due to the increased levels of estrogen and progesterone acting as growth factors for this bacteria.[42,47] *Porphyromonas gingivalis* and *Prevotella intermedia*, both periodontopathic bacteria leading to gingival inflammation, are also known to be associated with increased maternal hormone levels during pregnancy.[48] Another study demonstrated that pregnant women had higher levels of the periodontogenic bacteria *Campylobacter rectus* in unstimulated salivary samples compared to their nonpregnant counterparts.[49] These studies and others suggest a potential role of increased maternal hormone levels and alterations in the oral and periodontal microbiota including elevated levels of periodontopathic bacteria that increase the risk of periodontitis. Further studies are necessary to confirm these findings.

Alterations of the immune function of the gravida are another potential mechanism leading to an increased risk of gingival inflammation. During pregnancy, a state of relative immunosuppression occurs to prevent rejection of fetal tissues.[50] Resulting alterations in neutrophils and other innate and adaptive immune cells leads to an increased propensity for inflammation.[51–54] Specifically, neutrophil chemotaxis and adherence are diminished during pregnancy.[54] Moreover, pro-inflammatory cytokine production and secretion are increased during pregnancy; *in vitro* models demonstrate increased production of IL-6, IL-8, IL-1, TNF-alpha, and prostaglandin E2.[55–59] However, some *in vivo* human studies have not found clear differences in these pro-inflammatory cytokines comparing pregnant individuals to nonpregnant controls.[60,61] Thus, in pregnancy, there appears to be a predisposition toward impairment in neutrophil function with possible alterations in levels of pro-inflammatory cytokine levels.

Overall, there is evidence linking the increased levels of maternal estrogen and progesterone that occur during pregnancy with both worsening of preexisting gingival inflammation and further predisposition to new formation of gingivitis or periodontitis through likely alterations in the periodontal microbiota and the subsequent heightened, and potentially dysregulated, maternal inflammatory response. Thus, pregnant women represent a vulnerable population that are at higher inherent risk for the development of periodontitis with potential ramifications of the disease not only on the gravida but also the developing fetus(es).

Periodontitis and Risk of Preterm Birth

Periodontitis during pregnancy is associated with poor maternal and perinatal outcomes including gestational diabetes, preeclampsia, fetal growth restriction, low birth weight (LBW), preterm delivery, and perinatal mortality.[62–66] Here, we will specifically evaluate the literature surrounding periodontitis and its association with one of these outcomes—preterm birth.

Periodontitis and Preterm Birth: A Review of the Medical Literature

In 1996, Offenbacher et al. first published a case–control study of 124 pregnant or postpartum women evaluating rates of preterm low-birth-weight (PLBW) deliveries (defined as birth weight <2500 g

and one of the following: gestational age <37 weeks, preterm labor, or premature rupture of membranes). After multivariate logistic regression models were applied, periodontitis was significantly associated with PLBW delivery.[67] Similar findings were later reported in other studies.[68,69] For example, Jeffcoat et al. reported that pregnant women with generalized periodontal disease at 21–24 weeks of gestation had an increased risk for preterm delivery [adjusted odds ratio (AOR) 4.45, 95% confidence interval (CI) 2.16–9.18].[68,69] However, studies have not consistently demonstrated this strong association with even one study from the United Kingdom (UK), suggesting potential prevention of preterm birth in women with periodontitis.[70]

While some studies, like the UK study, have contradictory findings, a significant body of evidence supports an association between periodontitis during pregnancy and preterm birth, PLBW, or LBW neonates.[71–76] A meta-analysis published in 2016 evaluated published case–control studies evaluating pregnancy outcomes related to maternal periodontitis during pregnancy and reported a risk ratio of 1.61 (p <0.001) for preterm birth using data from 16 studies.[71] Furthermore, the risk ratio for having a neonate <2500 g at birth was 1.65 (p <0.001) and for PLBW was 3.44 (p <0.001).[71] Another systematic review reported 62 studies suggesting periodontitis as a potential risk factor for preterm birth or LBW neonates.[76] Thus, a large body of evidence supports maternal periodontitis as a likely modifiable risk factor for having preterm, PLBW, or LBW neonates.

While evidence supports the association between maternal periodontitis during pregnancy and preterm, LBW, or PLBW neonates, studies have subsequently assessed whether interventions during pregnancy to treat periodontitis can prevent these adverse outcomes. Two randomized controlled trials evaluated the impact of treating periodontitis with dental scaling and root planing on the prevention of preterm birth. Interestingly, neither trial demonstrated prevention of preterm birth, LBW, or fetal growth restriction with dental scaling and root planing of the mother in the second trimester.[77,78] Other findings have similarly not found improvements in birth outcomes related to periodontal treatment during gestation.[79] These results suggest that traditional methodologies for treating maternal periodontal disease during the second trimester of pregnancy do not likely have significant effects in the prevention of adverse offspring outcomes.

Biologic Plausibility and Potential Pathophysiological Explanation(s) for Association with Preterm Birth

While periodontitis appears to be associated with preterm birth, what are the possible pathophysiologic explanations? First, one must understand the current theories and hypotheses surrounding how preterm birth occurs prior to delving into how periodontitis may causally connect. While the mechanistic pathway for the development of preterm labor is not fully elucidated, one leading theory is the preterm parturition syndrome.[80] This theory proposes that birth, irrespective of if occurring at term or preterm, has a common terminal pathway leading to parturition that includes uterine myometrial contractions, membrane activation with eventual rupture, and cervical ripening. However, in preterm labor, there are multiple insults of varying strength that may lead to premature activation of this terminal pathway. These triggers can range from infection, inflammation, cervical disorders, hormonal disorders, allergic phenomena, uterine overdistension, uteroplacental insufficiency, gene–environment interaction, and stress.[80] Periodontitis likely leads to multiple insults leading to premature activation of the common terminal pathway including inflammation, infection, and potential alterations in the placental microbiota.

Preterm birth is well known to be associated with extrauterine maternal infections, such as malaria, pneumonia, and pyelonephritis, during pregnancy.[81–92] Furthermore, intrauterine infections are well known to be associated with preterm birth. In fact, intrauterine infection is considered the only firm causal link with preterm birth with a known mechanistic pathophysiologic understanding.[93–97] For example, when systemic administration of microbial products is provided to a pregnant animal or intrauterine infection develops, preterm labor and resulting birth occur.[95,98–107] Further supporting the role of maternal infection on the development of preterm birth, when antibiotics are administered for the treatment of intrauterine infections or asymptomatic bacteriuria, prevention of preterm birth can occur.[108–110] Ultimately, the association with preterm birth is the strongest with intrauterine infection but also linked with extrauterine infections. Thus, the infectious process of periodontitis has strong potential for leading to premature birth.

Part of the innate immune system includes pattern recognition receptors such as toll-like receptors (TLRs). Interestingly, TLRs are found in the maternal genital tract including on the vagina, cervix, endometrium, and fallopian tubes.[111] Ligation of TLRs leads to activation of downstream signaling cascade through nuclear factor-kB (NF-kB) and eventual production and secretion of cytokines and chemokines creating a pro-inflammatory milieu. In one mouse model of preterm birth, when TLR-4 contains a mutation that prevents proper signaling, these mice are less likely to deliver preterm when exposed to intrauterine inoculations of LPS compared to wild-type mice, supporting the mechanistic role of TLRs in signaling and activation of preterm birth.[107,112] Moreover, certain TLRs such as TLR-2 are known to promote apoptosis of trophoblastic cells, specialized cells that form the placenta and ensure proper uteroplacental vascular supply. As a consequence, when TLR-2 is stimulated by a pathogen, promotion of trophoblast apoptosis can occur leading to the potential development of intrauterine growth restriction of the fetus, preeclampsia in the mother, and/or miscarriage, all findings that have been associated with maternal periodontitis during pregnancy.[113–116] Thus, it is plausible that periodontitis activates the innate immune system and signaling cascade, which then likely plays an active role in the activation of preterm labor.

While infection itself is associated with the preterm parturition syndrome, maternal systemic and localized inflammation plays another potential mechanistic role in the early activation of the common terminal pathway. Specifically, pro-inflammatory cytokines, such as IL-1 and TNF-alpha, likely play a central role in the initiation of parturition. A body of evidence demonstrates that IL-1 causes uterine myometrial contractions. Systemic administration of IL-1 in animal models ultimately leads to preterm labor and birth.[117] Another pro-inflammatory cytokine TNF-alpha promotes the production and release of matrix metalloproteases that instigate membrane rupture and cervical ripening.[118–124] Blockade of both IL-1 and TNF-alpha through knockout and receptor antagonist murine models demonstrates decreased rates of preterm labor and resulting preterm birth, strongly supporting the role of these two cytokines as significant mechanistic contributors to the development of preterm parturition.[125–127] Evidence supports other pro-inflammatory cytokines (IL-6, IL-16, and IL-18) in the pathogenesis of preterm parturition, many of

which have been found to be elevated in periodontitis.[128–135] While pro-inflammatory cytokines are associated with preterm birth, the diminished production of anti-inflammatory cytokines (IL-10) likely also plays a pivotal role. Anti-inflammatory cytokines are known to decrease in the placenta at term, further promoting a pro-inflammatory state near the time of labor.[136] Additional evidence in animal models of infection demonstrates that when IL-10 is provided, less uterine myometrial contractility occurs along with less preterm birth.[137–139]

Another potential mechanism for how periodontitis may lead to preterm birth is through alterations in the oral and placental microbiotas. The traditional and long-taught notion that the "womb," including the placenta, amniotic cavity, and fetal tissues, is sterile is now uncertain.[140] Aagaard et al. demonstrated a unique, low-biomass placental microbiome that harbors unique microbes commonly found in the human oral cavity (i.e., *Prevotella tannerae*, nonpathogenic *Neisseria* species, *Bergeyella*, and *Fusobacterium*), urinary tract (i.e., *Escherichia coli*), and vagina (i.e., *Lactobacillus* species, *Ureaplasma* species, and *Streptococcus agalactiae*).[8,140] These findings suggest that while ascending spread from the vagina may occur, hematogenous spread and seeding from the oral cavity likely play another key role. In fact, the placental microbiome demonstrates greatest similarity to the oral microbiome.[140] Animal models in which food contaminated with periodontogenic pathogens such as *Porphyromonas gingivalis* is provided to pregnant animals demonstrate decreased fecundity and higher rates of inflammation within the placenta.[9] Findings in humans further support the hematogenous spread from the oral cavity to the placenta. For instance, when bacteria are detected in the amniotic fluid of women who have preterm birth, the bacteria are more commonly associated with the oral cavity rather than other regions such as the vagina.[141,142] Thus, dysbiosis of the placental microbiome due to hematogenous seeding of pathobionts from the oral cavity to the placenta may be an underlying etiology for the development of preterm labor that is associated with periodontitis.[67,143–145]

Overall, periodontitis has several potential methods for the activation of the terminal pathway leading to preterm parturition including extrauterine infection, potential hematogenous seeding leading to intrauterine infection, dysbiosis of placental microbiome, and establishment of a pro-inflammatory state all associated with increased uterine activity, cervical ripening, and ultimately preterm birth. Further research is necessary to determine causal pathways by exploring these potential pathways leading to preterm birth in association with periodontitis.

Periodontitis, Inflammation, and Potential Adverse Neurologic Complications in Offspring

While periodontitis is likely associated with an increased rate of prematurity, the subsequent maternal inflammation related to periodontitis can also have detrimental effects on offspring neurodevelopment. First, prematurity is associated with increased rates of neurodevelopmental delay compared to birth at term.[146–157] As periodontitis is associated with prematurity, this association is one reason for potential adverse long-term outcomes. Furthermore, fetal exposure to the resulting maternal inflammation, both local and systemic, due to periodontitis has the strong potential to injure a vulnerable, developing brain.

There exists a substantial body of evidence supporting the link between adverse neurologic outcomes with fetal exposure to maternal infection or its resulting inflammation.[10–13,158,159] Animal models across a large array of species (rat, mouse, sheep, rabbit, and piglet) consistently demonstrate a strong association between maternal inflammation and adverse neonatal neurologic outcomes. Specifically, increased numbers of macrophages and microglia within the white matter along with resulting white matter injury are well-known complications of maternal inflammation on the neonatal brain.[12,13,158,160–162] These findings suggest a role for inflammation leading to microglial activation, potential proliferation, and subsequent white matter damage.

Further exploring the potential pathophysiology of fetal neural injury associated with maternal infection and inflammation, studies have elucidated differential effects within specific structures within the brain. For example, in response to inflammation and cytokine signaling (i.e., IL-6), there is a proliferation of primitive neural precursors within the subventricular zone.[163] Cytokine signaling is associated with microglial activation and proliferation which are associated with neuronal injury.[164] However, the role of microglia in the development of neuronal injury is still unknown and not fully defined. While microglial proliferation occurs in the subventricular zone, exposure to prenatal inflammation leads to decreased neurogenesis within the hippocampal subgranular zone.[159,165,166] The hippocampus is critically important in memory formation and learning. Diminished neurogenesis during fetal development within the hippocampus may be one potential etiology for future neurodevelopmental impairments.

While fetal exposure to maternal inflammation leads to changes in the developing fetal brain, the timing of exposure is also of paramount importance as the immature fetal brain undergoes critical windows of development *in utero*. Exposure to inflammation during these periods may potentiate adverse effects. It is well documented in the medical literature that certain maternal infections, such as Zika virus, toxoplasmosis, or cytomegalovirus, have increased risk of transmission or worse prognosis for offspring if infection occurs during certain time periods during gestation.[167–169] Zika virus, for example, is known to preferentially lead to adverse offspring outcomes in a murine model if maternal infection occurs on embryonic day 8 as opposed to day 4 or 12.[169] Thus, critical windows of inherent vulnerability to infection and related inflammation occur in the developing fetus.

In times of maternal inflammation, the human placenta upregulates conversion of tryptophan to serotonin (5-HT), an important hormone in fetal neurogenesis and future neurocognitive disorders. Normally, placental-derived 5-HT reaches the fetal brain. In times of maternal inflammation, the subsequent increase in 5-HT within the placenta leads to increased concentration within the fetal brain leading to significant potential for alterations in neurogenesis.[170]

5-HT plays a critical role in neural crest stem cell survival, growth, migration, and proliferation as well as overall synaptogenesis.[171–176] With 5-HT being one of the first neurotransmitters to emerge during embryogenesis, and with 5-HT neurons proliferating from gestational weeks 5–10, any dysregulation of 5-HT signaling during this crucial developmental window has the potential to cause lasting long-term, detrimental effects on neurodevelopment. 5-HT is a neuromodulator and intricately connected to future mood and anxiety disorders and even autism.[177] Thus, maternal inflammation and subsequent derangements in neuromodulators

during the periods of neurogenesis and synaptogenesis during the fetal neurodevelopment may play a pivotal role in the eventual development of adverse neurodevelopmental outcomes.

Consistent with this theory, researchers evaluated 1,791,520 children born over a 41-year period in Sweden and evaluated the association of hospitalization with any maternal infection, severe maternal infection, or a urinary tract infection with neuropsychiatric offspring outcomes including autism, depression, bipolar disorder, or psychosis.[10] While no associations were found increasing the risk for bipolar disorder or psychosis among offspring, fetal exposure to maternal infection during hospitalization increased the risk for both autism [hazard ratio (HR) 1.79, 95% CI 1.34–2.40] and depression (HR 1.24, 95% CI 1.08–1.42).[10] Thus, maternal infection and related inflammation have significant potential to lead to lifelong neurodevelopmental impairment in offspring.

Overall, periodontitis, an extrauterine maternal infection, is associated with both localized and systemic inflammation.[178] With a substantial body of evidence linking maternal inflammation with poor neurodevelopmental outcomes of offspring, these findings provide biologic plausibility for adverse neurologic outcomes of offspring exposed to maternal periodontitis.

Future Directions

While a plethora of evidence has demonstrated an association between periodontitis and preterm birth, there exist also some conflicting evidence that suggests no association may be present.[70,179] However, research tends to focus on high-income settings rather than lower income settings where higher rates of preterm birth more commonly occur. In low- and middle-income countries, causes of preterm birth are oftentimes unknown. It is in these same settings that rates of periodontitis may exceed 80–90% of pregnant or recently postpartum women. Therefore, research exploring the association of periodontitis, its treatment, and any association with preterm birth would be well suited in these settings where the magnitude of effect will lead to increased power for detection.

Furthermore, while randomized controlled trials have explored the effects of dental planing and root scaling on pregnant women with periodontitis during pregnancy compared to after pregnancy and did not find an effect on prevention of preterm birth, other prevention or treatment strategies targeting periodontitis need to similarly be vigorously explored. One possibility is the evaluation of fluoridated water sources. Studies have reported that exposure to fluoridated water sources provides protection against periodontal disease in adults.[180–182] This low-cost strategy has the potential for far-reaching effects within communities. In fact, in a murine model of preterm birth, pregnant mice that were exposed to low-dose fluoride supplementation postponed preterm birth, increased the rate of live births, and decreased perinatal brain injury in offspring.[183] However, further studies are needed to determine any potential adverse effects, optimal dosing, use in varying geographical and cultural contexts, and other aspects prior to larger scale-up of this affordable and accessible option.

Another potential strategy is the evaluation of certain sugar alcohols within the polyol family (e.g., sorbitol, xylitol, or erythritol) that are known to prevent dental caries and periodontal disease. These polyols prevent periodontitis via multiple mechanisms that include disruption of periodontopathic bacterial energy production processes, reduction of adhesion of microorganisms to the teeth, and diminishing gingival inflammation via inhibiting LPS-induced inflammatory cytokine expression and signaling (TNF-alpha, IL-1 beta, and NF-kB).[184–189] These sugar alcohols have the potential for preventing maternal periodontitis and further studies are needed on the effects on the maternal–neonatal dyad and associated outcomes.

Conclusion

Preterm birth is the leading cause of neonatal mortality, morbidity, and poor neurodevelopmental outcomes worldwide. Efforts seeking innovative methods to prevent preterm birth are critically important to attempt to prevent the 15 million preterm deliveries occurring every year globally.[1,190] Substantial evidence links maternal periodontitis during pregnancy with adverse pregnancy outcomes including preterm birth, PLBW, and LBW offspring. With up to 90% of pregnant women suffering from poor oral hygiene in some resource-limited settings, periodontitis is likely an overlooked, important contributor to preterm birth. While no randomized controlled trials have reported the prevention of these adverse outcomes, these interventional studies have largely been limited to dental scaling and root planing. Further randomized controlled trials are needed evaluating other strategies to both treat and prevent periodontitis on offspring outcomes, preferentially in settings where periodontitis is highly prevalent. Moreover, fetal exposure to inflammation secondary to periodontitis and/or alterations in the developing neonatal microbiota are potentially modifiable risk factors for adverse neurodevelopmental outcomes in offspring. Therefore, these further studies should evaluate the impact not only on prevention of preterm, PLBW, or LBW neonates, but also on adverse long-term neurologic and neurodevelopmental outcomes of offspring.

Orcid

Gregory Charles Valentine https://orcid.org/0000-0002-3055-2987

Abbreviations

5-HT: Serotonin
IL: Interleukin
LBW: Low birth weight
PLBW: Premature low birth weight
PTB: Preterm birth
RANK: Receptor activator of nuclear factor-kB
TLR: Toll-like receptor
TNF-alpha: Tumor necrosis factor-alpha

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REVIEW ARTICLE

Let's Talk about Dex: When do the Benefits of Dexamethasone for Prevention of Bronchopulmonary Dysplasia Outweigh the Risks?

Thuy Nguyen[1], Brian K Jordan[2]

Abstract

Bronchopulmonary dysplasia (BPD) is the most common complication of extreme prematurity and carries increased respiratory morbidity into childhood and adulthood. Systemic administration of dexamethasone during the preterm period has been shown to decrease the incidence of BPD in this population. However, enthusiasm about its use has been tempered by early evidence that suggested potential adverse neurodevelopmental outcomes. More recent studies suggest that the timing, dosing, and duration of therapy may have a significant impact on the safety and efficacy of dexamethasone administration and that side effects and harms may be minimized if its use is appropriately targeted. Focusing on studies published since the 2010s American Academy of Pediatrics (AAP) statement on dexamethasone, this review seeks to examine the evidence from recent clinical trials to present the current state of knowledge regarding the systemic dexamethasone administration to prevent BPD in extremely premature infants and how dose, duration, and timing might impact its safety and efficacy in this vulnerable population.

Keywords: Bronchopulmonary dysplasia, Corticosteroids, Dexamethasone, Prematurity.

Newborn (2022): 10.5005/jp-journals-11002-0009

Introduction

Bronchopulmonary dysplasia (BPD) is the most common complication of extreme prematurity and carries increased respiratory morbidity into childhood and adulthood, including increased risk of chronic obstructive pulmonary disease (COPD) in later adulthood.[1–4] As has been shown for caffeine[5] and vitamin A,[6] systemic administration of corticosteroids (primarily dexamethasone) decreases the incidence of BPD in extremely premature infants.[7] The precise mechanism by which postnatally administered dexamethasone confers its protection against BPD is not fully known. It has been postulated that the benefits of dexamethasone are mediated by its potent anti-inflammatory effects.[8] Alternatively, the benefit may derive from the fact that short-term improvement in lung function[9] includes increased compliance[10] and functional residual capacity[11] facilitating earlier extubation to noninvasive ventilation. However, balancing the potential benefits of systemic corticosteroid therapy to decrease the incidence of BPD with its potential harms has been an evolving challenge in neonatology. The clear short-term beneficial physiologic effects on lung function that facilitates extubation and reduces BPD in the most at-risk infants must be balanced against concerns for adverse events including intestinal perforation and hypertrophic cardiomyopathy[12] as well as adverse long-term neurodevelopmental outcomes such as cerebral palsy (CP).[13,14]

Interpretation of data on the long-term neurodevelopmental outcomes after systemic dexamethasone therapy is complicated by the fact that clinical trials have employed different dosing regimens, with different timing of administration, and different durations of treatment. Some studies have suggested that adverse neurodevelopmental outcomes (e.g., CP) occur more commonly with higher dosing and longer courses of dexamethasone. For example, while one early study did find a benefit in long-duration/high-dose dexamethasone (cumulative dose 7.98 mg/kg) in reducing BPD, follow-up studies of these patients raised concerns about possible impairment of motor development.[14] These concerns led to the American Academy of Pediatrics (AAP) and Canadian Pediatric Society (CPS) jointly to release a statement in 2002 recommending dexamethasone therapy be limited in its use. Subsequently, a 2005 meta-regression analysis of published dexamethasone studies showed that for infants with >50% risk of BPD, the risk/benefit ratio favors the use of dexamethasone, an early indication that the harm/benefit ratio is favorable for at least some at-risk infants.[15] Even so, in the most recent update of this statement released in 2010, the AAP revised its statement, concluding the data were still insufficient to recommend routine use of glucocorticoid therapy in ventilator-dependent neonates, but that "the clinician must use clinical judgment when attempting to balance the potential adverse effects of glucocorticoid treatment

[1]Department of Pharmacy, Oregon Health & Science University, Portland, Oregon, United States of America

[2]Department of Pediatrics, Oregon Health & Science University, Portland, Oregon, United States of America

Corresponding Author: Brian K Jordan, Department of Pediatrics, Oregon Health & Science University, Portland, Oregon, United States of America, e-mail: jordabr@ohsu.edu

How to cite this article: Nguyen T, Jordan BK. Let's Talk about Dex: When do the Benefits of Dexamethasone for Prevention of Bronchopulmonary Dysplasia Outweigh the Risks? Newborn 2022;1(1):91–96.

Source of support: This manuscript was supported by the National Institutes of Health's National Heart, Lung, and Blood Institute under award number K23 HL144918. The funders had no role in the analysis, decision to publish, or preparation of the manuscript.

Conflict of interest: None

with those of BPD."[16] Importantly, the resulting relative decrease in dexamethasone use at this time, stemming from concerns about its side effects and caveats from the AAP and CPS, is temporally related to a relative increase in BPD rates over the same time period.[17] This tension between the benefits and harms of dexamethasone therapy continues to complicate clinical decision-making at the bedside in neonatal intensive care units (NICUs) around the world. To address this ongoing knowledge gap, we have reviewed the relevant studies (Table 1) published in the decade since the last AAP statement to assess what is currently known regarding dose, duration, and timing of systemic dexamethasone administration to prevent BPD in extremely premature infants.

What's New? Updated Evidence from Clinical Trials

Two closely related meta-analyses published in the Cochrane Database describe the benefits of corticosteroids (primarily dexamethasone) in extremely premature infants. The first, summarizing dexamethasone administration initiated within the first week after birth, showed a decrease in BPD [relative risk (RR) 0.7 (0.61, 0.81)] as well as a decrease in the composite outcome of BPD or death [RR 0.87 (0.80, 0.94)]. Additionally, this study showed a decrease in extubation failure and in need for repeated administration of dexamethasone later in an infant's course. However, these benefits were accompanied by significant harms including an increased incidence of gastrointestinal bleeding [RR 1.87 (1.35, 2.58)], intestinal perforation [RR 1.73 (1.20, 2.51)], hypertrophic cardiomyopathy [RR 4.33 (1.4, 13.4)], and CP [RR 1.75 (1.20, 2.55)] as well as increased incidence of hypertension, hyperglycemia, and growth failure. The authors conclude that although there are clear benefits to the early administration of dexamethasone, these benefits "may not outweigh the adverse effects of this treatment."[12]

In a second meta-analysis of dexamethasone administered after 7 days of age, dexamethasone was shown to decrease BPD [RR 0.77 (0.67, 0.88)], composite of BPD or death [RR 0.77 (0.70, 0.86)], and the need for oxygen at discharge [RR 0.71 (0.54, 0.94)]. Importantly, these benefits were accompanied by far fewer adverse events than in those with early exposure to dexamethasone. Of note, there was no increase in CP [RR 1.16 (0.82, 1.64)] or a composite outcome of death or CP [RR 0.95 (0.78, 1.15)] in this analysis of >900 infants.[7] Further, these studies have shown no increase in short-term risk such as necrotizing enterocolitis (NEC) [RR 1.03 (0.61, 1.74)] or spontaneous intestinal perforation [RR 1.60 (0.28, 9.31)] when dexamethasone is given after the first week of life. This meta-analysis concludes that the use of dexamethasone should be limited to infants who cannot be weaned from the ventilator after 7 days of age and that both dose and duration should be limited as much as possible. Taken together, these studies show the clear benefits of dexamethasone on rates of BPD, and they suggest that harms might be minimized by delaying administration until after the first week after birth. However, given the considerable heterogeneity in dose and duration, even these important meta-analyses leave critical questions incompletely answered.

Optimal Dosing of Dexamethasone

In light of the data from this meta-analysis and the conclusion that the dose and duration should be limited, especially in the context of the cautionary statements from the AAP, the relatively low-dose regimen described in the DART trial[18] has become one of the most commonly used. The DART trial aimed to determine whether low-dose dexamethasone (0.89 mg/kg over 10 days) would lead to a reduction in BPD by facilitating extubation. This study found a 34.3% extubation rate by day 3, 51.4% by day 7, and 60% by day 10 [p <0.01; number needed to treat (NNT) = 2 by day 10]. However, there was no difference in either oxygen dependence at 36 weeks of postmenstrual age (PMA) [85 vs 91%; odds ratio (OR) 0.58 (0.13, 2.66)] or in mortality rate [11 vs 20%; OR 0.52 (0.14, 1.95)]. No cases of intestinal bleeding or intestinal perforation were reported. In a follow-up study, no difference in CP was noted at 2 years of age.[19] Importantly, though the target sample size was intended to be 814 infants to ensure sufficient power to detect the primary outcome, difficulty in recruitment caused enrollment of the DART trial to be prematurely halted after only 70 participants were enrolled. Thus, this study may be significantly underpowered to detect true differences in either benefits or harms.

In the last 5 years, there have been several new clinical trials reexamining the use of systemic dexamethasone at widely differing cumulative dosages ranging from 0.72 mg/kg over 10 days[20] to 7.98 mg/kg over 42 days[20–22] to try to maximize benefits and minimize harms. To explore the minimum effective dose in ventilator-dependent infants born at <29 weeks, Cuna et al. compared two dexamethasone regimens: 27 patients received the DART regimen (0.89 mg/kg over 10 days) and 32 patients received a reduced version of the DART regimen (0.72 mg/kg over 7 days). Similar successful extubation rates (defined as extubation within 14 days of starting therapy and remaining extubated for more than 72 hours) were reported in both groups: 56% in 7-day group and 67% in 10-day group. The average time to successful extubation was also similar: 5 days in 7-day course and 6 days in 10-day course.[20] This study suggests that relatively low doses of dexamethasone are effective in facilitating beneficial short-term outcomes including extubation. Long-term outcomes, however, were not assessed.

A significantly larger cumulative dexamethasone dosing regimen was reported from a prospective, single-center, randomized study in 59 infants ≤27 weeks of gestational age (GA) and ~14 days of postnatal age at randomization.[22] Infants were randomized to either a 42-day course (cumulative dexamethasone dose of 7.98 mg/kg), or a 9-day course (cumulative dose of 2.63 mg/kg—allowing for repeat courses if necessary). Importantly, this study was designed to evaluate the long-term impact of dexamethasone on neurodevelopment rather than focusing on the more clearly established short-term benefits such as BPD rates. As such, the primary outcome measure was intact survival, defined as survival to 7 years of age without severe neurologic, cognitive, or academic handicap [IQ >70 and with no need for Individualized Education Program (IEP)]. There were no differences between groups for height, weight, or head circumference at 7 years of age—as had been reported in prior studies of a similar dosing regimen, but started within the first week after birth.[14] Significantly, more children in the 42-day group were alive without neurodevelopmental impairment (NDI) compared to those in 9-day group (93% vs 66%, p <0.02). More children in 42-day group received regular classroom without IEP (75 vs 38%, p <0.01) with an NNT of 4. Overall intact survival with IQ >70 was significantly greater for children in 42-day course (75 vs 35%, p <0.005) with an NNT of only 3. Regarding secondary outcomes, successful extubation rates were earlier (median 23 vs 35 days of age, p <0.01) and higher (50 vs 15% after 1 week, p <0.005) in the 42-day group. Successful extubation continued

Table 1: Compilation of recent studies of dexamethasone in premature infants

Study and location	*Method*	*Inclusion criteria*	*Dexamethasone regimen*	*# of participants*	*GA at birth (weeks)*	*BW (g)*	*Age @ treatment (days)*	*Findings*
Doyle et al. (2006) International	RCT; 11 centers	GA <28 weeks BW <1000 g Ventilator dependent after 1 week of life	0.89 mg/kg over 10 days	70	24 IQR 24–26	680 IQR 605–785	13–34	Extubation by day 3: 34.3%, p <0.01 Extubation by day 7: 51.4%, p <0.01 Extubation by day 10: 60%, p <0.01 No difference in BPD and mortality Less weight gain −76 g, $p = 0.006$
Cuna et al. (2017) Kansas, MO	Retrospective Single center Two dosing regimens	GA <29 weeks Mechanical ventilated	• 0.89 mg/kg over 10 days • 0.72 mg/kg over 7 days	27 32	24.9 ± 1.0 25.4 ± 1.3	762 ± 141 740 ± 148	33 ± 9 36 ± 13	Extubation within 14 days of treatment: 67% in 10-day and 56% in 7-day groups No difference between two groups in rates of severe BPD, tracheostomy, days on O_2, and days on mechanical ventilation
Cuna et al. (2018) Kansas, MO (same cohort from Cuna et al., 2017)	Retrospective Single center Late (DOL 14–28) vs Delayed (DOL 29–42) therapy	GA <29 weeks Mechanical ventilated	• 0.89 mg/kg over 10 days • 0.72 mg/kg over 7 days	55	Late: 24.9 ± 1.4 Delayed 25.2 ± 1.2	Late 728.5 ± 190.4 Delayed 750 ± 135.4	Late 22.8 ± 4.1 Delayed 35.1 ± 3.9	Delayed treatment group had significantly longer LOS, intubation days, days on oxygen. No difference between two groups in rates of severe BPD, mortality, IVH grade III or IV
Marr et al. (2019) Syracuse, NY	RCT Single center Two dosing regimens	GA <28 weeks DOL 10–21	• 7.98 mg/kg over 42 days • 2.625 mg/kg over 9 days	30 29	25 ± 1.2 25.2 ± 1.1	769 ± 149 785 ± 167	14 ± 4 13 ± 3	9-day course: 17% received two courses, 17% received three courses (mean 4.04 mg ± 0.07 mg/kg) Extubation rates were higher in 42-day group at weeks 1, 2, 3, and 4 (p <0.005) 42-day group had earlier extubation (median 23 vs 35 days), less frequent needs for re-intubation (7 vs 25%), and shorter ventilation duration (25 vs 37 days) No difference in rates of BPD, NEC, and LOS 7-year outcomes: • No difference in height, weight, head circumference, re-hospitalization rate • 42-day group had higher survival rate without neurodevelopmental impairment (93 vs 66%, p <0.05), more attended school without IEP (75 vs 38%, p <0.01), higher intact survival rate (75 vs 35%, p <0.005)

(*Contd...*)

Table 1: (*Contd...*)

Study and location	Method	Inclusion criteria	Dexamethasone regimen	# of participants	GA at birth (weeks)	BW (g)	Age @ treatment (days)	Findings
Harmon et al. (2020) NICHD Multicenter	Retrospective cohort 25 centers Early (by DOL 28) vs Late (after DOL 28)	GA <27 weeks	Various regimens Two main agents Dexamethasone: 73% Hydrocortisone: 27%	951 total Early: 420 Late: 951	24.9 ± 1.0 24.9 ± 1.0	669 ± 132 687 ± 136* *$p = 0.04$	21 (16–25) 43 (35–54)	Early group had shorter ventilation days (47.9 ± 23.4 vs 53.8 ± 23.5, $p < 0.001$), shorter supplemental oxygen days (99.4 ± 22.8 vs 103.8 ± 21.6, $p < 0.01$), fewer patients on oxygen upon discharge (55.4 vs 68%, $p < 0.001$) Higher aOR for severe BPD in patients started therapy DOL ≥ 50 (week 8) Higher aOR for death or BPD in patients started therapy DOL 15–21, and DOL ≥ 64 Higher aOR for NDI at 18–26 months in patients started therapy DOL 8–14 and DOL 36–49

BPD, bronchopulmonary dysplasia; DOL, day of life; GA, gestational age; IEP, Individualized Education Program; IVH, intraventricular hemorrhage; LOS, length of stay; NDI, neurodevelopmental impairment; NEC, necrotizing enterocolitis

to be significantly higher for infants in the 42-day group at weeks 2, 3, and 4 ($p < 0.005$ for all time points). The need for re-intubation was lower in 42-day group (7 vs 25%, $p < 0.001$), but there was no difference in BPD (defined as the need for supplemental oxygen at 36 weeks of PMA) between the two groups: 93% in 42-day group and 89% in 9-day group.[22] Due to significant outcome differences in the 6-month preliminary data evaluation, study enrollment was terminated early (after 59 of the intended, 72 patients were enrolled). As with any individual study, the conclusion must be interpreted with caution, especially given the surprisingly large effect sizes and resulting NNTs. However, this study supports that notion that rather than causing harms, high-dose dexamethasone for infants born at ≤27 weeks of GA may support improved neurodevelopmental outcomes at 7 years of age. Prior to the publication of this study, a 2017 meta-analysis found that compared with moderate-dose dexamethasone regimens, high-dose regimens were associated with a lower risk of BPD and lower risk of adverse neurodevelopmental outcomes.[23] However, due to concerns about the degree of heterogeneity among the studies, the authors refrained from formally recommending any particular dosing regimen. Further study of this question is urgently needed.

Timing of Treatment: Is There an Optimal Window?

Meta-analysis of recent clinical trials suggests that timing of systemic dexamethasone administration may have a significant modifying effect on benefits and adverse outcomes,[7,12] perhaps greater than the effect of the cumulative dose. Amid the conflicting data on dosing and duration, one clear signal that has emerged is that early administration of systemic dexamethasone (within the first 7 days), though effective in reducing the incidence of BPD, confers more harm than benefit. As noted above, early administration is associated with an increased risk of intestinal perforation, gastrointestinal hemorrhage, hypertrophic cardiomyopathy, and CP; thus, it should be avoided.[12] Dexamethasone administration after the first week reduces the incidence of BPD while minimizing the harms.[7] But beyond this, what else can be gleaned from recent studies about optimal timing of dexamethasone administration? Is there a window of optimal benefit?

In a retrospective cohort study of preterm infants treated with dexamethasone (0.72 mg to 0.89 mg/kg over 7–10 days) for BPD prevention, infants were grouped by timing of dexamethasone exposure into two cohorts: moderately late [14–28 day of life (DOL) when therapy started, $n = 25$] and delayed (29–42 DOL, $n = 30$). Baseline demographics were similar, except that there were more male patients (84 vs 57%; $p = 0.03$) and more patients on high-frequency ventilation (96 vs 47%, $p < 0.0001$) in the moderately late group. The average postnatal age and PMA were 23 days (28.2 weeks) for moderately late group compared to 53 days (30.2 weeks) in the delayed group. Despite having a greater burden of comorbidities, those in the moderately late group had fewer intubation days (46 ± 18 days vs 77.4 ± 67 days, $p = 0.02$), fewer days of supplemental O_2 (114.3 ± 40.8 vs 149.8 ± 57 days, $p = 0.005$), and fewer hospital days (125.5 ± 33 vs 157 ± 57.6 days, $p = 0.02$) that the those in the delayed group. However, rates of the composite outcomes of BPD or mortality were similar, as well as rates of tracheostomy, BPD-associated pulmonary hypertension, retinopathy of prematurity (ROP), intraventricular hemorrhage (IVH) grade III or IV, and periventricular leukomalacia (PVL).[21] This small study suggests that dexamethasone may have beneficial

effects when initiated up to 6 weeks after birth, though the benefits may be greater with earlier administration. Importantly, neurodevelopmental outcomes were not assessed in this study.

Harmon et al.[24] reported a retrospective cohort study of 863 infants born at <27 weeks of GA with steroid exposure dichotomized to either the early group (started ≤28 DOL) or the late group (started >28 DOL). Of these, 73% received dexamethasone and 27% received hydrocortisone (HC). Total doses and duration of courses were not reported. The adjusted Odds Ratio (aOR) of NDI (cognitive composite score <70, or motor composite score <70, or moderate-to-severe CP, or visual impairment, or permanent hearing loss) at 18–26 months was only statistically significant when therapy was started 36–49 DOL. The aOR for severe BPD was significantly higher in those who received therapy between DOL 50–63 and older. Interestingly, the aOR for death or BPD is higher in those who received therapy between DOL 15–21 and DOL ≥ 64. The early group was less likely to be discharged on O_2 (55 vs 68%, p <0.001), less likely to have moderate or severe BPD (84 vs 92%, p <0.001), and statistically shorter duration of ventilation and supplemental O_2 (p <0.001 and <0.01, respectively). Though the interpretation of this study is complex, its findings suggested that postnatal steroid therapy starting between DOL 8 and 49 is associated with no greater risks of neurodevelopmental delay and may potentially minimize severe BPD risks compared to later therapy.[24] In a recent systematic review and meta-analysis involving 5,559 extremely premature infants, Ramaswamy et al. simultaneously evaluated the effects of dosing and timing of dexamethasone on the prevention of BPD.[25] This study concludes that moderate-dose dexamethasone courses (cumulative dose of 2–4 mg/kg) initiated at 8–14 days carried the greatest protection against BPD with an RR of 0.61 (0.45, 0.79). High-dose dexamethasone courses (cumulative dose of >4 mg/kg) initiated within the same time window conferred a similar but slightly smaller benefit with an RR of 0.64 (0.48, 0.82). Importantly, this study notes that none of the regimens studied was associated with an increased risk of NDI. Taken together, these data support the conclusion that there may be an optimal time frame to consider initiating dexamethasone in ventilator-dependent premature neonates between the second and third weeks after birth, and its use up to DOL 49 is less likely to result in greater risks of neurodevelopmental delay.

Hydrocortisone and Methylprednisolone

Though this review is primarily focused on the use of dexamethasone to minimize the risk of BPD, it is worth noting that other steroids, namely HC and methylprednisolone (MP), have been studied for this purpose as well. In a recent retrospective, single-center, cohort study of 98 intubated preterm infants ≤34 6/7 weeks and >7 postnatal days, Nath et al. compared three steroid regimens including dexamethasone starting at 0.2 mg/kg/day, HC starting at 4–8 µg/kg/day (equivalent to dexamethasone 0.15–0.3 mg/kg/day), and methylprednisolone (MP) 2.4 mg/kg/day (equivalent to dexamethasone 0.4–0.5 mg/kg/day) over an average 10-day course.[26] In this study, the decrease in the respiratory severity scale (RSS) was different only between the dexamethasone group (58.6% decrease) and HC group (19.4% decrease, p <0.002). The rates of extubation at day 3 and at day 7 were higher for dexamethasone (44 and 59%), than for either HC (40 and 44%) or MP (23 and 41%). Given concerns about potential undesirable neurodevelopmental side effects of dexamethasone, a multicenter randomized controlled trial (RCT) of 800 premature infants at <30 weeks of GA to investigate the efficacy of HC in facilitating extubation and increasing survival without BPD was recently completed. In this as-yet-unpublished study, although HC was found to increase the rate of extubation in this population, no difference in survival without BPD or survival without NDI was found.[27] These recent studies underscore the notion that a superior alternative to dexamethasone has yet to be identified.

Additional Potential Risks

As mentioned in recent individual studies above, NEC, culture-proven sepsis, ROP, IVH, and PVL have been consistently similar and in dexamethasone-treated neonates regardless of dosing regimen, exposure duration, and timing of therapy. These rates have not been found to be increasing compared to previous studies. Though no causal link has been shown, ROP has been associated with systemic steroids used during the first 96 hours of life[28] and after 3 weeks.[29] However, a more recent study specifically looking at the association between dexamethasone and betamethasone administration (via insulin growth factor-1 and vascular endothelial growth factor expression) and ROP showed that his apparent association became insignificant after regression model was applied.[30] Importantly, retrospective studies such as these latter two cannot demonstrate causation and may simply identify late dexamethasone administration as a marker of greater illness severity, a known risk factor for ROP.

Conclusion and Remaining Knowledge Gaps

Despite almost 50 years of study regarding systemic dexamethasone therapy to treat or prevent BPD, significant questions remain unanswered. Meta-analyses of clinical trials have demonstrated clear short-term benefits of dexamethasone in reducing the incidence of BPD in extremely premature infants, especially those who have difficulty being weaned from mechanical ventilation. But studies show conflicting data on the simple but critical question of whether its effects on neurodevelopmental outcomes are beneficial or deleterious.[7,12,22,31] A potential explanation for these disparate findings could be that the timing of administration of dexamethasone may modify the harm/benefit ratio. If there is a clear signal that emerged from repeated analyses, it is that the harms outweigh the benefits when dexamethasone is administered during the first week after birth. Beyond that, there is no definitive consensus on optimal dosing or duration of systemic dexamethasone that maximizes benefits while limiting harms. Could there be an optimal window of timing for administration in the second or third week as some research has suggested? More RCTs examining the relationship between timing, dosing, and duration with primary endpoints involving long-term outcomes like survival without NDI are urgently needed.

Abbreviations

BPD: Bronchopulmonary dysplasia
COPD: Chronic obstructive pulmonary disease
CP: Cerebral palsy
DOL: Day of life
GA: Gestational age
IVH: Intraventricular hemorrhage
NDI: Neurodevelopmental impairment

NICU: Neonatal intensive care unit
NNT: Number needed to treat
PMA: Postmenstrual age
PVL: Periventricular leukomalacia
ROP: Retinopathy of prematurity
RR: Relative risk
OR: Odds ratio
aOR: adjusted odds ratio

Orcid

Brian K Jordan https://orcid.org/0000-0002-1553-8503

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REVIEW ARTICLE

Iron Deficiency in Newborn Infants: Global Rewards for Recognizing and Treating This Silent Malady

Robert D Christensen[1], Timothy M Bahr[2], Diane M Ward[3]

Abstract

Iron deficiency can exist at birth. Even if iron is sufficient at birth, deficiency can develop during the neonatal period, or during infancy, or during childhood. Iron deficiency can exist despite a normal hematocrit and a normal blood hemoglobin concentration, because anemia is a very late manifestation of iron deficiency. It is likely that adverse neurodevelopmental consequences occur during perinatal biochemical iron deficiency, despite a normal hematocrit and hemoglobin. Consequently, measuring those parameters is a very insensitive method for perinatal iron deficiency screening. This review focuses on potentially better practices for diagnosing perinatal iron deficiency, including recent advances in understanding the pathogenesis of this condition, and also on practical means of treatment, and on global rewards of so doing.

Keywords: Anemia, Erythropoiesis, Erythroferrone, Diagnosis, Hepcidin, Iron, Treatment.

Newborn (2022): 10.5005/jp-journals-11002-0021

Preamble

Saving lives of babies who would otherwise die is a magnificent aim. However, insuring that survivors have full functionality, the best possible health, and maximal potential intellect must also be part of that aim. One "silent malady" that sometimes thwarts best possible health outcomes of neonatal survivors is a deficiency in the element iron during critical perinatal periods. Survivors of premature birth are particularly susceptible to this problem, as are survivors who were born small for gestational age, or were infants of diabetic mothers, or were born to a mother with obesity, because each of those conditions impedes fetal/neonatal iron accretion in unique and often unseen ways.[1,2]

As this global society endeavors to find new ways to improve neonatal survival, and also strives to produce the best possible long-term outcomes for babies, efforts are needed to assure perinatal iron sufficiency. Global dividends will result from improved methods to recognize neonates who are at-risk, and to diagnose and adequately manage them so as to see that their future is not jeopardized by the silent malady of perinatal iron deficiency.

Central to understanding perinatal iron deficiency is the realization that it is a spectrum, not a dichotomous variable of iron deficiency vs iron sufficiency.[3,4] Table 1 groups the spectrum of perinatal iron deficiency into three recognizable entities. These can be considered as early, mid-, and late manifestations of iron deficiency, or perhaps mild, moderate, and severe iron deficiency. Another terminology scheme, which is the one we used in Table 1, is biochemical iron deficiency, iron-limited erythropoiesis, and iron deficiency anemia.

[1–3]Divisions of Neonatology and Hematology, Department of Pediatrics, and the Center for Iron and Heme Disorders, University of Utah Health, and Women and Newborn's Research, Intermountain Healthcare, Salt Lake City, Utah, United States of America; Division of Microbiology and Immunology, Department of Pathology, and the Center for Iron and Heme Disorders, University of Utah Health, and Women and Newborn's Research, Intermountain Healthcare, Salt Lake City, Utah, United States of America

Corresponding Author: Robert D Christensen, Divisions of Neonatology and Hematology, Department of Pediatrics, and the Center for Iron and Heme Disorders, University of Utah Health, and Women and Newborn's Research, Intermountain Healthcare, Salt Lake City, Utah, United States of America, e-mail; Robert.christensen@hsc.utah.edu

How to cite this article: Christensen RD, Bahr TM, Ward DM. Iron Deficiency in Newborn Infants: Global Rewards for Recognizing and Treating This Silent Malady. Newborn 2022;1(1):97–103.

Source of support: This work was supported in part by grant U54DK110858 from the U. S. Public Health Service.

Conflict of interest: None

Table 1: One categorization of the spectrum of perinatal iron deficiency

Category of iron deficiency	*Iron metrics are below the lower reference interval*	*Erythrocyte size and Hemoglobin content are below the lower reference interval*	*Hematocrit and Hemoglobin are below the lower reference interval*
Biochemical iron deficiency	YES	NO	NO
Iron-limited erythropoiesis	YES	YES	NO
Iron-deficiency anemia	YES	YES	YES

Note: Reference intervals for iron metrics, erythrocyte size and hemoglobin content, and hematocrit and hemoglobin differ for women vs neonates, and in neonates, they differ on the basis of gestational age at birth and postnatal age. For reference ranges for women, see "Anemias during Pregnancy and the Postpartum Period," Chapter 43, RT Means, Jr. in Wintrobe's Clinical Hematology, 14th Edition, Wolters Kluwer, Philadelphia, 2019. For reference ranges for neonates, see "Reference Intervals in Neonatal Hematology," Chapter 24, RD Christensen, in Neonatal Hematology, 3rd edition, Cambridge University Press, New York, 2021

Biochemical iron deficiency exists when metrics indicate that the iron supply is low. This means the biochemical iron measurements are below the lower reference interval (5th percentile) for age. However, in biochemical iron deficiency, the red blood cell size and hemoglobin content are normal, and the subject is not anemic. In contrast, the next phase is iron-limited erythropoiesis, which exists when biochemical iron deficiency is present and there is evidence of erythrocyte microcytosis and hypochromia. Perhaps the subtlest such evidence is an elevation in the Micro-R% and the HYPO-He%.[5,6] These parameters equate to the percent of erythrocytes that have a mean corpuscular volume (MCV) below 60 fL and have a mean corpuscular hemoglobin (MCH)below 16 pg/dL, respectively. As iron deficiency worsens further, the MCV and MCH fall to a point where they both are below the 5th percentile lower reference interval, and microcytosis and hypochromia are recognizable on a stained blood film. However, at this intermediate phase of iron deficiency, the hemoglobin and hematocrit remain within the reference range; thus, anemia does not exist. Only with more severe iron lack will the hemoglobin and hematocrit fall below their lower reference intervals, revealing iron deficiency *anemia*, accompanying biochemical iron deficiency and iron-limited erythropoiesis. Thus, iron deficiency anemia is not a subtle sign of low iron, but rather is an extreme condition where iron is in such short supply that erythropoiesis is failing and anemia has resulted.[4,7]

One unknown, but critically important aspect of perinatal iron deficiency involves the issue of iron-limited *neurodevelopmental impairment*. Specifically, exactly when during the spectrum of worsening iron deficiency does a human fetus or newborn infant begin to have inadequate iron to support normal neurodevelopment? Does this point occur only once iron deficiency *anemia* has developed, or is the fetus/neonate at risk when biochemical iron deficiency or iron-limited erythropoiesis has occurred? Although more work must be done to define this clearly in humans, animal experimentation suggests that once iron-limited erythropoiesis is present, impaired neurodevelopment has already occurred.[8–10]

The Iron Endowment at Birth

The *iron endowment* is a concept for the sum of all the iron a newborn baby has at birth. This includes all the iron in storage, such as that in ferritin and hemosiderin, plus the iron within heme-containing molecules like hemoglobin and myoglobin, plus that in the circulation, generally bound to transferrin, plus that in the hundreds of different iron-containing enzymes and cofactors. In fact, it is estimated that 6–7% of all human enzymes are iron dependent.[11] Obviously, a newborn baby's entire iron endowment is derived from transplacental passage of iron from the mother during gestation. One of the falsehoods previously dogmatically taught was that a fetus, being a parasite, invariably takes sufficient iron from its mother, even at her own peril, so the fetus will be iron sufficient at birth. That statement is not true. Pregnant women who have critically low iron reserves deliver babies who have critically low iron reserves.[12–14] Moreover, some pregnant women have defective mechanisms for transferring iron to her fetus.[15] Also, preterm birth virtually always results in a lower-than-normal iron endowment. Unfortunately, an inadequate iron endowment is not rare, and two common reasons for it are the perennial problem of preterm birth and the relatively new problem of obesity in pregnancy.[16–19]

Relevance of the Perinatal Erythropoietin/Erythroferrone/Hepcidin Axis

Iron deficiency in newborn infants can result in substantial and persistent neurocognitive dysfunction.[1,2,8–10,20,21] Consequently, efforts are needed to prevent or to promptly and adequately treat this deficiency. However, enteral iron supplementation will not always prevent or treat neonatal iron deficiency. In fact, the success of enteral iron dosing depends, in part, on the integrity of the patient's iron homeostatic mechanisms. Those mechanisms are well described in adults, but studies are only beginning in neonates.[22–24]

The iron-regulatory hormonal axis includes erythropoietin (Epo), erythroferrone (ERFE), hepcidin, and ferroportin. In brief (Fig. 1), when Epo binds to cognate receptors on the surface of erythroid progenitors, erythrocytic clonal proliferation occurs. The Epo-stimulated erythroid progenitors rapidly produce ERFE and ERFE blood levels consequently rise.[25] High circulating levels of ERFE suppress hepcidin production by the liver.[26] Elevated hepcidin levels trigger the degradation of ferroportin, the iron exporter that moves iron from the enterocyte or macrophage to plasma, thereby inhibiting the absorption of enteral iron and preventing the mobilization of iron from storage.[12,13] In contrast, low hepcidin levels (seen with high ERFE levels) foster the absorption of enteral iron through ferroportin-mediated iron transport.

Premature Birth Cheats the Iron Endowment

The iron endowment at birth is needed to support development and growth throughout the neonatal period and infancy. A term "neonate" has a relatively high hematocrit at birth that gradually falls over the first weeks following birth, yielding some additional iron bioavailable for early iron needs. An adequate iron endowment plus this extra iron stored in the "excess" erythrocytes typically constitutes a sufficient iron supply despite a relatively iron-poor diet during the first several months of neonate life.[27]

When birth occurs prematurely, the iron endowment is compromised. Premature delivery deprives the fetus of the iron that should be transferred during the final weeks of pregnancy. Since 60–70% of the total body content of iron in a term "fetus" is obtained during the last trimester of pregnancy, premature birth can result in a very low iron endowment. If not made up for in some way, this low endowment may be inadequate to fully support neurodevelopment and other iron-dependent functions over the coming months. At birth preterm neonates generally do not have the high hematocrits typical of term neonates. As shown in Figure 2, the more preterm at birth, the lower the hematocrit will be.[28,29] Moreover, preterm neonates, particularly those who require neonatal intensive care unit (NICU) care, are sometimes subjected to repeated blood testing for clinical management, which further diminishes their iron supply. Since 1 mL of blood typically contains about 0.5 mg of iron, the removal of 30 mL/kg of blood or more, over the first weeks in a NICU, will diminish the iron supply by 25–30%.

The Epidemic of Obesity and its Effects on Maternal and Fetal Iron

The widespread consumption of a "western style" diet, plus a sedentary lifestyle, is leading to a global epidemic of obesity.[30,31]

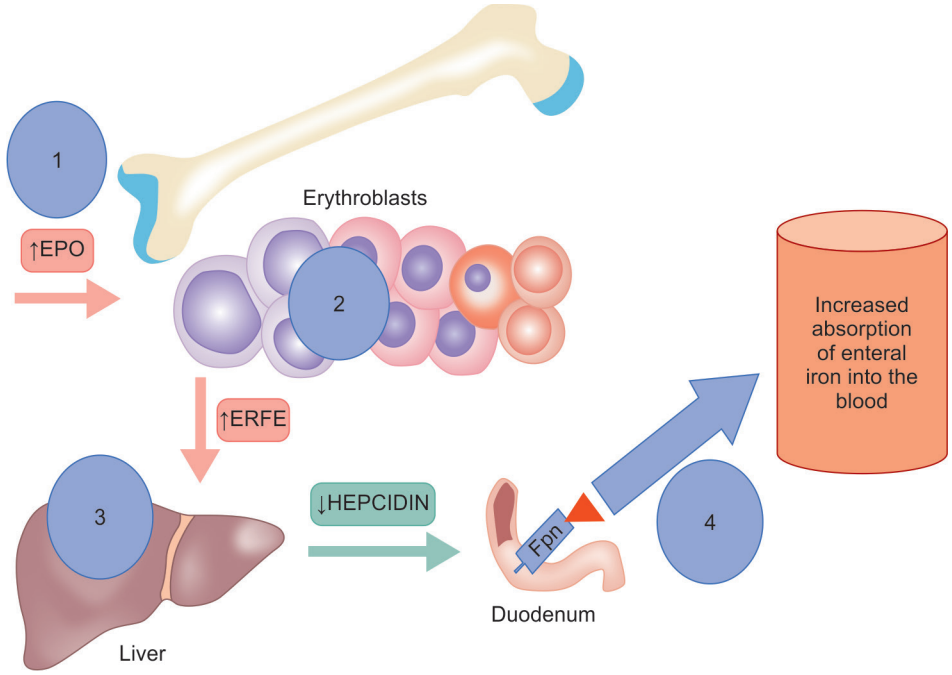

Fig. 1: The erythropoietin axis. Schematic representation of the erythropoietin (EPO), erythroferrone (ERFE), hepcidin, and ferroportin (Fpn) axis, as it pertains to the regulation of intestinal iron absorption. (1) High EPO levels; (2) Stimulate erythroblasts to produce ERFE; (3) High ERFE levels reduce hepcidin production in the liver; (4) Low hepcidin levels facilitate the absorption of enteral iron through ferroportin[24]

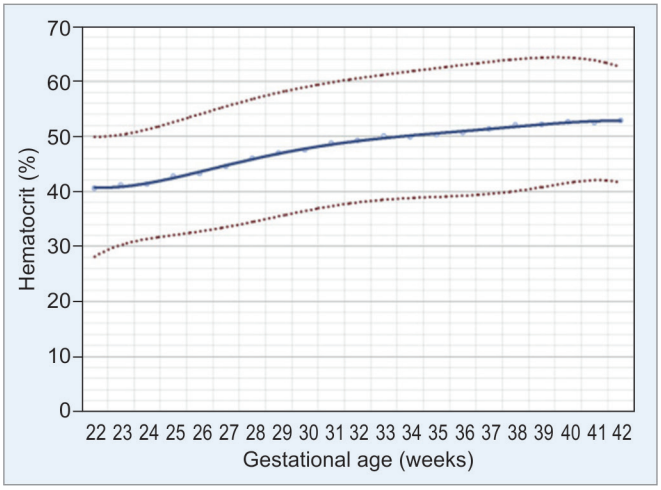

Fig. 2: Hematocrit on the day of birth according to gestational age, from 22 to 42 weeks. The figure was produced using over 350,000 hematocrit values from Intermountain Healthcare. The lower and upper dotted lines represent the lower reference interval (5th percentile) and the upper reference interval (95th percentile), and the middle solid line is the median[28,29]

Iron deficiency is particularly frequent in obese patients, as a result of adiposity-associated inflammation, and the consequent high blood levels of hepcidin.[32,33] During healthy pregnancy, maternal hepcidin levels are low, which is essential to increase the absorption of iron from the maternal diet, and to mobilize iron from maternal storage sites to produce the hemoglobin needed for increasing maternal red blood mass during pregnancy, and for the iron needed for the fetus. As shown in Figure 3, high maternal levels of hepcidin bind to ferroportin on syncytiotrophoblasts, thereby blocking maternal-to-fetal iron transfer. Consequently, high levels of hepcidin can interfere with maternal absorption of dietary iron and also block the transfer of maternal iron to the fetus.[23]

Phillips et al. demonstrated that women with a pre-pregnancy body mass index ≥30 kg/m^2, or excessive gestational weight gain, delivered offspring with lower serum ferritin concentrations, compared to non-obese women or those without excessive gestational weight gain.[16] Recent studies in pregnant animal models suggest that maternal hepcidin levels determine embryo iron endowment. Specifically, Sangkhae et al. showed that higher levels of maternal hepcidin caused maternal iron restriction, resulting in lower embryo weight, increased incidence of embryo anemia, and increased embryo mortality.[23] These observations support the idea that obesity, through the mechanism of elevated hepcidin levels, can render fetuses at risk of reduced iron delivery.

Iron Lack and Perinatal Brain Development

Iron plays an important role in many neurodevelopmental processes, and animal studies indicate that iron sufficiency in pregnancy and infancy is particularly important for neurodevelopment.[34] Even so, many questions remain regarding how iron deficiency in the human fetus and neonate impacts neurodevelopment, and how the timing and severity of the iron lack result in subsequent specific developmental problems. Available studies support improved neurodevelopmental outcomes with either iron supplementation or delayed umbilical cord clamping at birth, which is associated with a larger iron endowment.[35] However, it is not clear, from human studies, whether a prompt and effective treatment of perinatal iron deficiency completely reverses the adverse neurodevelopmental effects of iron lack.[36]

It is clear that iron sufficiency during the neonatal period is important for erythropoiesis, mitochondrial respiration, nucleic acid replication, and immune function.[37] Iron sufficiency is particularly

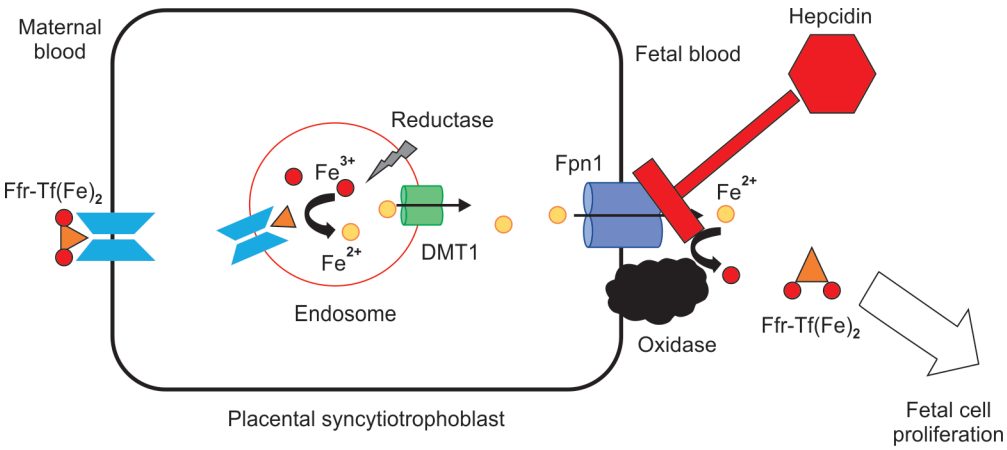

Fig. 3: Placental Iron Transport. Maternal iron is delivered to the placenta by transferrin (Tf)-mediated endocytosis. Iron is released from an intracellular compartment by divalent metal transport (DMT1). Iron can then be stored as ferritin or utilized for heme synthesis in the placental cells. Iron is transported out of the placental cells by ferroportin (Fpn1) providing iron to fetal Tf for delivery throughout the fetus. However, high levels of hepcidin will block the movement of iron from the syncytiotrophoblast into the fetal circulation

crucial for neonates receiving treatment with recombinant erythropoietin because inadequate iron availability during accelerated erythropoiesis can deplete iron stores and precipitate multiorgan iron deficiency.[38,39] Moreover, due to the prioritization of iron stores to support erythropoiesis over the iron needs of other organs, it is possible that even moderate iron limitation could result in deficient brain iron.[40] Neonatal animal models suggest that deficient brain iron can cause neurological damage that persists even after the iron deficiency is corrected.[8,10,20,34,41] Consequently, avoiding iron deficiency in neonates who are receiving erythropoietin treatment is an important facet of assuring their optimal neurodevelopment.

Developing Practical Ways to Identify Neonatal Iron Deficiency

As a screening method to detect neonatal/infant iron deficiency, measuring the hematocrit or blood hemoglobin level will not do. Why? Because, by the time iron deficiency has caused neonatal *anemia*, iron-deficient *neurodevelopmental damage* has probably already occurred.[1,8,40] Thus, screening for iron deficiency by looking for anemia is like closing the barn door after the horse already escaped. The basis for the insensitivity of anemia as a screen for iron deficiency is due to the natural prioritization of iron trafficking to support erythropoiesis above the priority to support normal neurodevelopment.[40] Perhaps nature decided that when iron deficiency is present, it is better for the baby to be alive with some degree of neurodevelopmental impairment than to be dead from severe anemia with an intact brain.

Iron deficiency can be confirmed in neonates in a sophisticated but expensive battery of tests, including serum iron, transferrin, transferrin saturation, serum ferritin, soluble transferrin receptor, zinc protoporphyrin-to-heme ratio, Micro-R and HYPO-He values, and reticulocyte hemoglobin content (RET-He).[6,42] However, the large volume of blood required and the costs of the combined tests dictate that typically just one screening test is used. Serum ferritin might be the most common single test for assessing iron sufficiency in NICU patients; however, serum ferritin has the disadvantage that it requires phlebotomy, and some clinical laboratories require 1 mL of serum for ferritin testing. Moreover, the serum ferritin can be artifactually elevated by inflammation, rendering the test less informative during inflammatory states, which are not uncommon in NICU patients. Non-invasive tests for iron status, such as urine ferritin, have inherent advantages over serum-based testing, but might also be flawed by artifactually elevated urinary ferritin levels during inflammation.

Though serum ferritin level is commonly used to screen neonates for iron deficiency,[43] it is not well validated in extremely preterm neonates. A more recent method used to assess iron stores is the RET-He, which measures the hemoglobin within reticulocytes. The RET-He serves as a metric of the iron available for hemoglobin production during the previous several days.[6,7,44] The validity of RET-He as a marker of iron status has been well studied in adult and pediatric patients.[45] However, like ferritin, limited data validate RET-He as a marker of iron status in preterm neonates.[45–48]

Ishikawa et al. reported that healthy adults in Japan had urine ferritin levels about 5% of their serum ferritin levels, with a correlation coefficient of 0.79.[49] On that basis, we speculated that measuring urinary ferritin might be a useful non-invasive way to screen NICU patients for iron deficiency. Specifically, we hypothesized that a low concentration of ferritin in the urine might identify neonates who are iron deficient. In a pilot study, we measured paired serum/urine ferritin from healthy adults, healthy term neonates, growing preterm neonates, and children with very high serum ferritin levels from liver disorders or iron overload.[50] In that study, we detected ferritin in every urine sample and found a correlation with serum ferritin (correlation coefficient 0.78). Those findings led us to further evaluate urinary ferritin as a potential screen for iron deficiency.

In a subsequent study, we found[51] (1) that it was highly feasible to obtain urine samples from NICU patients; (2) ferritin was measurable in every urine sample collected, with the same laboratory method used for measuring serum ferritin; (3) dividing the urinary ferritin concentration by the urinary creatinine improved the correlation with serum ferritin; (4) a low urine ferritin (either <10 ng/mL or <12 ng/mL) performed well statistically in identifying iron-limited erythropoiesis; and (5) some affirmation of the association between maternal obesity and neonatal iron deficiency. These findings encourage us to pursue using urine to assess a neonate's iron status, without phlebotomy. Such monitoring is particularly relevant

among NICU patients receiving erythropoiesis-stimulating factors, because of the potential to cause iron-limited neurodevelopmental delay if their iron supplementation is inadequate.

New, inexpensive, rapid, reliable, and non-invasive means are needed to identify iron deficiency in neonates. Urinary methods are particularly attractive, especially if they could be as simple as a dip-stick method.[49–51] Other creative potential methods might involve testing saliva.

Developing More Effective Treatments for Neonatal Iron Deficiency

Advances are needed to generate enteral iron preparations for neonates that are better absorbed and have fewer adverse effects. In most investigations, neonates seem to tolerate ferrous sulfate fairly well, but just as in older children and adults, constipation and other gastrointestinal symptoms (e.g., emesis) have been reported.[52] In one of our studies of iron dosing in a multihospital collaborative group, we did not find emesis to be a common problem in preterm neonates receiving ferrous sulfate as early as 10–14 days after birth.[44] In adults with iron deficiency, vitamin C given along with the enteral iron improves the iron absorption.[53] This has not been formally investigated in neonates but should be. Also, other preparations of enteral iron should be tested in neonates, looking for better absorption, fewer adverse effects, lower costs for large underserved populations, and greater effectiveness.

Some iron-deficient neonates seem to require very high doses of enteral iron in order to increase their serum ferritin or RET-He levels.[38] Perhaps some of this refractoriness is on the basis of high hepcidin levels accompanying inflammatory conditions.[54] This theory is consistent with one of our pilot studies.[44] Identifying iron-deficient neonates who have high hepcidin levels, or who have other mechanisms causing refractoriness to enteral iron treatment, could permit the use of intravenous iron for those unlikely to respond to high enteral dosing. Clearly, new oral preparations of iron and also improvements in intravenous iron administration are needed for neonates as are better non-invasive means of monitoring the efficacy of iron treatment, as are ways to make these improvements practical and inexpensive.[55]

Studies in infant rhesus monkeys, classified as iron deficient on the basis of hematological values, affirm the importance of iron for normal brain development.[56] Neuroimaging studies indicated that a history of iron deficiency was associated with smaller total brain volumes, primarily due to significantly less total gray matter. These brain differences were evident even after iron treatment and recovery from the iron-deficiency anemia. These experiments highlight the importance of early detection and preemptive supplementation to limit the neural consequences of neonatal iron deficiency.

Global Rewards for Doing this Better

Posit 1: Iron deficiency during the neonatal period results in an average diminution in the adult IQ of five points. The truth here is unknown. However, in one study, the mean IQ of iron-deficient children was 91.5 ± 2.3 compared to 97.5 ± 3.2 in controls.[57] Individual readers can decide whether they believe the assumption in Posit 1 is plausible or not. We submit it is reasonable, or perhaps even an underestimate of the harm that iron deficiency during that critical period of neurodevelopment can produce.

Posit 2: At least 10% of the world's population have had iron deficiency during their neonatal period. Although we know of no clear data, the world health organization (WHO) estimates that 80 percent of the world's population has insufficient iron and that 30 percent have iron deficiency anemia.[58] So what do you think about Posit 2? Plausible or not?

Posit 3: Prevention, or timely detection and treatment, of neonatal iron deficiency will avert a 5-point IQ drop. If you think all three posits are ridiculous, you can stop reading this section, because you will not agree with the Results and Conclusions below. However, if you think these assumptions are at least theoretical possibilities with some merit, keep reading.

Result 1: About 790 million people alive today (10% of the world's population as of May 2021) each have five fewer IQ points than they "should have" on the basis that they had neonatal iron deficiency. Thus, the world's population right now has 3,950,000 fewer IQ points because of neonatal iron deficiency.

Result 2: Each one-point increase in IQ is associated with $500 more income per year. A report on global economy and intelligence, published in 2016, calculated that one IQ point is associated with measurably higher adult productivity, wages, and capital and that this is the case for individuals in poor and rich countries.[59] In the USA, one IQ point was associated with a $500/year higher income.

Conclusion

If these estimates are even somewhat close to accurate, prevention or timely treatment of neonatal iron deficiency would enrich the world by a minimum of 2 trillion dollars every year. However, having more money and a higher gross world product are not the only reasons we should work to eliminate neonatal iron deficiency. Might *you* have had iron deficiency as a neonate? How would *you* like to have five more IQ points? How would their quality of life be enriched if the people you know with a low IQ had five more points each?

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REVIEW ARTICLE

Oral Feeding of Preterm Infants in the NICU: Interventions and Outcomes

Leslie-Anne Juarez Dietrich[1], Cynthia Blanco[2]

Abstract

Preterm infants spend much of their time in the neonatal intensive care unit (NICU) learning to orally feed. Attempts to support the preterm infant in acquiring oral skills have evolved greatly over the past decades, including the increasing involvement of speech, physical, and occupational therapists. Interventions have included modified positioning, specialized nipples, external pacing, sensorimotor exercises, oral motor skills programs, and cue-based feeding programs. While many infants seem to have benefited from these methods, a subset of babies continues to require supplemental feeding methods via nasogastric or gastrostomy tube. In particular, infants with aerodigestive complications are at high risk for needing supplemental feeding methods. Additionally, the neurodevelopmental implications of having significant feeding difficulties early on is not fully known. Studies have brought about concerns that children with early oral feeding difficulties may be at risk for the presence of neurodevelopmental delays and continued feeding issues later in childhood. Further research is needed to better understand which infants will struggle with oral feeding, as well as identify appropriate therapeutic options and optimal time periods of implementation.

Keywords: Feeding disorder, Gastrostomy tube, Nasogastric feeding, Neurodevelopment, Oral feeding, Preterm.

Newborn (2022): 10.5005/jp-journals-11002-0010

Introduction

With advances in neonatal care younger infants, as early as 22 weeks gestational age (GA), are surviving to discharge. Approximately 450,000 preterm infants are born a year in the United States and up to 80% of preterm infants will struggle with oral feeding during the neonatal intensive care unit (NICU) stay.[1,2] Of children being referred to specialized clinics for feeding or swallowing disorders up to 40% are born prematurely.[3] Preterm infants admitted to the NICU can have a variety of barriers to overcome (i.e., respiratory distress, feeding intolerance), but for many they spend their last weeks in the NICU learning to feed orally, the so called "feeder and growers." With more research being done on neurodevelopmental care strategies to support the infant, so too have feeding strategies changed over the last 2 decades. But much remains unanswered in the realm of feeding in the NICU and preterm infants continue to be at high risk for short- and long-term oral-feeding difficulties.

Oral Physiology and Feeding Difficulties

The process of eating or drinking by mouth is complex requiring a coordinated progression of sucking, swallowing, and breathing with the goal of moving food from mouth to stomach without disrupting the airway. A non-nutritive suck (NNS) is thought to be present *in utero* as early as 12 weeks GA.[4] The NNS consists of immature and short sucks in which liquid is not consumed. This is followed by the development of a nutritive suck at approximately 33–34 weeks GA which requires the infant to (1) generate sufficient suck for milk expression from bottle or breast, (2) pass the bolus smoothly to the back of the oropharynx, (3) move the bolus to the esophagus while rapidly clearing airway structures, and (4) transport the milk from the esophagus to the stomach,[5,6] Any of these steps may be compromised by poor tongue movement, sphincter closure, epiglottic closure, esophageal muscle peristalsis, and breathing patterns leading to coughing, choking, gagging, laryngeal penetration, or aspiration.

Terms such as "feeding difficulty," "feeding disorder," and "swallowing disorder" have been used in the literature to describe infants and children struggling with oral feeding. However, definitions can vary from study to study. Definitions for "feeding difficulty" have included any number of the following: infants unable to tolerate oral feeding, presence of nasogastric (NG) or gastrostomy tube (GT) at discharge, difficulty swallowing, coughing, gagging, or presence of aspiration. Studies will also define feeding difficulty based on a range of oral intake volumes attained at various GA cutoffs. These differences within the literature can make it difficult to draw comparisons among interventions and truly understand the deficits which preterm infants face when feeding. The purpose of this review is to examine the evidence for current feeding strategies and feeding outcomes of preterm infants, as well as the neurodevelopmental outcomes in this specific population.

[1,2]Pediatrics, Division of Neonatology, University of Texas Health San Antonio, San Antonio, Texas, United States of America

Corresponding Author: Leslie-Anne Juarez Dietrich, Pediatrics, Division of Neonatology, University of Texas Health San Antonio, San Antonio, Texas, United States of America, Phone: 210-787-8818, e-mail: DietrichL3@uthscsa.edu

How to cite this article: Dietrich LJ, Blanco C. Oral Feeding of Preterm Infants in the NICU: Interventions and Outcomes. Newborn 2022;1(1):104–108.

Source of support: Nil

Conflict of interest: None

Current Interventions in the NICU to Promote Oral Feeding

Initial Interventions

Throughout the past decades many have studied potential interventions for improving infant oral feeding in the NICU (Table 1). Common first line interventions for oral feeding support include non-nutritive sucking, modified positioning, use of slow flow nipples, and external pacing by the feeder. In two 2016 Cochrane Reviews, among the randomized trials included studies suggested that oral stimulation decreased length of hospital stay and days of parenteral nutrition.[7] One review found days to full oral feeding shortened in the NNS group, while the other found no difference.

Modified positioning may include placing the infant in side-lying position or use of swaddling for improved containment to facilitate improvement in state and organization during the feeding.[8] The benefits of providing modified positioning for all infants are unclear. Pacing consists of the feeder intermittently stopping the flow of milk through the nipple to allow the infant to breath when the infant does not independently coordinate the suck–swallow–breath pattern. Pacing may be done by tilting the nipple upward such that it is not filled with milk or by completely removing the nipple from the infant's mouth. Typically, the feeder paces after a given number of sucks such as every two to three sucks. Pacing is particularly useful in infants with abnormal sucking patterns to prevent undue stress during the feed including resulting episodes of desaturations and bradycardia.[9] Pacing is further helped by a variety of available nipples with varying flow rates.

Oral Physical Therapy and Motor-based Interventions

Other researchers have sought to develop therapy programs based on stimulation of the oral motor muscles. The underlying hypothesis being these maneuvers affect and train underlying neuronal and musculoskeletal structures that overall improve suck, swallow, and respiration coordination.[10] Lau and Fucile examined various oral and tactile/kinesthetic sensorimotor interventions performed prior to oral feeding attempts in preterm infants off continuous positive airway pressure (CPAP). Maneuvers were performed two times a day for 10 days. Sucking, swallowing, and respiration were all positively impacted by the sensorimotor interventions. Oral interventions improved nutritive sucking and tactile/kinesthetic maneuvers seemed to improve swallow–respiration coordination possibly secondary to improving infant's head, neck, and trunk posture. Similar studies based on Lau and Fucile's program have found those receiving oral stimulation vs standard care achieved oral feeds significantly earlier (8.3 days) and spent significantly less time in the hospital (6.9 days).[11]

Table 1: Current NICU practices to support oral feeding

Facilitate Non-nutritive Suck (breast or pacifier)
Modified positioning
Pacing
Slow flow nipples
Sensorimotor interventions: massage, kinesthetic maneuvers
Early introduction of oral stimulation
Cue-based feeding programs
Supplemental feeding devices at discharge (NG, GT)
Multidisciplinary feeding-focused teams

A second program that has also been studied is the premature infant oral motor intervention (PIOMI) created by Dr Brenda Lessen and is based off Beckman's Oral Motor Intervention.[12,13] The PIOMI consists of eight steps including sucking, stretch, and massage maneuvers of the oral structures. Infants treated with the PIOMI vs standard of care achieved full oral feeds earlier and were discharged sooner. These studies have been conducted with small sample sizes and further research of these interventions is needed.

Cue-based Feeding: Does it Work?

With the advent of neurodevelopmental programs such as NIDCAP (Newborn Individualized Developmental Care and Assessment Program) emphasizing the interpretation of infant's positive and negative cues, so too began a movement toward cue-based feeding. Cue-based feeding or infant-driven feeding consists of feeding infants based on hunger and satiation cues as opposed to oral feeding at predetermined scheduled intervals. Cue-based feeding encourages the caregiver to truly understand the infant's more subtle communication of stress or stability during the feeding and react accordingly.[14] A focus on volume consumption at an early age may in fact promote negative feeding experiences that lead to adverse compensatory behaviors and increased long-term feeding problems. A focus on feeding quality should lead to increased positive feeding experiences for the infant and in turn, long-lasting feeding skills.

A 2016 Cochrane Review found in nine randomized control trial (RCTs) comparing cue-based feeding policies with scheduled interval feeding in preterm infants may reduce time to transition from enteral tube to oral feeding, but did not consistently show a decrease in length of hospitalization.[15] Evidence was low quality in small trials. Davidson et al. found cue-based feeding beneficial in particular for infants with bronchopulmonary dysplasia (BPD).[16] Time to full oral feeds was earlier than the standard of care in each BPD severity group. No adverse events occurred in any infants.

Recent studies have investigated the effects of introducing small quantities of milk into the mouth of preterm infants from birth to provide early exposure to smell and taste during gavage feeds until bottle or breast feeds are initiated.[17,18] A small amount of milk is typically introduced with a cotton swab into the infant's mouth and can be done in the presence of most respiratory support modalities. These early steps are more frequently being integrated into the initial steps of cue-based feeding protocols. However, such trials have not consistently shown a decrease in time to full oral feeds nor have the effects on feeding quality been evaluated.[19] It is also not known whether benefits are strictly in the presence of mom's breastmilk vs donor milk or formula.

Invasive Therapies: Supplemental Feeding Tubes

Given the desire to shorten length of NICU stay, it has led to the consideration of NG tube use upon discharge for those infants who have recovered and solely are working on oral-feeding skill. The use of home enteral feeds has been used to varying degrees among NICUs in the United States and with few studies examining risk and benefit. Some institutions have documented success in implementing a structured program for home enteral feeding support. White et al. found with construction of a home enteral feeding program and follow-up clinic overall GT placement did not decrease before and after discharge.[20] Nevertheless, of those discharged with an NG tube 40% no longer needed it within 2 weeks after discharge and by 8 weeks post discharge 65% were without the use of an NG tube. They found no increase in

complications, emergency room visits, or hospital admissions post discharge.

Children's Hospital of Wisconsin found infants discharged home with NG feeds had shorter hospital stays and less hospital utilization for complications than infants with GTs at NICU discharge.[21] Of 35 infants discharged with NG feeds, 27 (77%) reached full oral feeds within 3 months. Such programs may need to be considered as an option to potentially conserve health care costs while improving the family's experience as their infant learns to feed.

Outcomes of Infants Requiring a Gastrostomy Tube

As more infants of younger GA are surviving to discharge with various comorbidities, they are at high risk of developing feeding difficulties to the degree that they require GT dependence. In a comprehensive study conducted through the Neonatal Network database a cohort of 4549 ELBW infants from 25 centers was analyzed.[22] Approximately 7% required GT placement with 75% requiring GT placement after NICU discharge. Of these infants, 77% had BPD, 29% Grade III or IV intraventricular hemorrhage (IVH) or periventricular leukomalacia (PVL) and 7% had necrotizing enterocolitis (NEC). Sex and race were not associated with the need for a GT. In infants with surgical NEC and subsequently short bowel syndrome, 45% were GT dependent. At 18–22 months follow-up, GT placement was associated with feeding difficulties, cerebral palsy, poor growth, and chronic breathing issues. Thirty-two percent of infants with GTs were taking full oral feeds by the time of follow-up.

When looking at whether feeding method at discharge could be a predicting factor for neurodevelopmental outcomes in infants <37 weeks, Jadcherla et al. found that infants leaving the NICU with a GT had lower cognitive, communication, and motor scores on the Bayley Scales of Infant Development (BSID)-III at 18–24 months even after accounting for gestation and comorbidities.[23] In that population of 194 infants, 77 (40%) were discharged with a GT. At 18–24 months, 40% of these babies continued to require a majority GT feeds and 22% progressed to all feeding by mouth. Interestingly, in this study GT placement before a median post menstrual age (PMA) of 49.3 weeks was associated with reduced odds of cognitive, language, and motor delay. The reason for this is unclear, but one may speculate infants discharged home sooner may have then been surrounded by a more optimal environment at home to engage in developmental activities. If that is the case, perhaps there is an ideal time for the GT procedure that promotes the best neurologic outcome.

The population of infants requiring GTs remains a heterogeneous one with varying degrees of complexities from degree of oxygen support (tracheostomy vs nasal cannula) to degree of oral skill and neurological maturation. Further studies are required to better characterize which subset of these infants is not only at higher risk for significant neurodevelopmental delays, but also persistent feeding problems.

Long-term Developmental and Feeding Outcomes

Recently more interest has arisen around a potential connection between oral-feeding delays and developmental outcomes in preterm infants. Researchers have speculated whether language and feeding are regulated by the same neural pathways in the brain.[24] This has led to the question—does an infant's oral feeding skill in the NICU predict developmental and feeding outcomes?

Investigators have found when feeding difficulties are present at follow-up, so too are various neurodevelopmental delays and certain comorbidities (Table 2). Adams-Chapman et al. found in a group of preterm infants born <26 weeks GA, 13% reported dysfunctional feeding at 18 months.[25] Interestingly, 50% of those with feeding abnormalities did not have a motor impairment. Severe language delays occurred in 47% of children with dysfunctional feeding compared with 11% of children with normal feeding patterns. Findings of language delay in the presence of feeding issues was again seen at 30 months.[26] Infants with <34.5 ventilator days had a decreased incidence of dysfunctional feeding (27 vs 7%). Cognitive and language scores on the BSID-III were significantly lower in infants with feeding difficulties. Infants with feeding difficulties were of lower GA and birth weight. They had increased presence of comorbidities including BPD, NEC, late onset sepsis and IVH/PVL, and cerebral palsy.

Crapnell and colleagues evaluated children born at <30 weeks GA at 2 years of age and similarly found children with feeding difficulties (18 of 80; 23% of patients) were more likely to have lower scores in motor, language, and cognitive outcomes.[27] Importantly, parents of children born "very preterm" with feeding difficulties reported increased stress and difficulties with behavior including depression and anxiety.

Medoff-Cooper et al. reported on sucking behavior of preterm infants while in the NICU as a potential predictor for neurodevelopmental outcomes within the first year.[28] In preterm infants (28–34 weeks GA), the number of sucks, mean number of sucks per burst, and mean sucking pressure peak at the 40-week PMA assessment were significantly associated with BSID-II outcomes of psychomotor and mental developmental indices at 12 months corrected age (CA), but not 6 months outcomes.

Studies have found preterm infants with abnormal sucking patterns as they near 37–40 weeks PMA had significantly lower performance on neurodevelopmental testing from 6 months up to 18–24 months.[29,30] Lainwala et al. examined time to full per os i.e., by mouth (PO) feeds and outcomes at 18–26 months in a group of infants <32 weeks GA.[31] Of 372 infants, 77% reached full oral feeds by 40 weeks PMA and 23% reached full oral feeds at >40 weeks PMA. The incidence of IVH, BPD, NEC, PDA, and sepsis was higher and number of ventilator days longer in infants achieving full oral feeds >40 weeks PMA. Thirty-nine percent of infants reaching full oral feeds at >40 weeks were discharged home or transferred from the NICU with a GT. At 18–26 months follow-up, cognitive, language, and motor scores on the BSID-III were significantly lower, and incidence of cerebral palsy higher in those who took longer to learn oral feeding skills in the NICU.

Patra and Greene studied infants <28 weeks GA diagnosed with feeding difficulty during the NICU stay.[32] Of 218 babies, 59 (27%)

Table 2: Risk factors for feeding challenges in preterm infants

Younger gestational age
Bronchopulmonary dysplasia
Number of ventilator days
Necrotizing enterocolitis
Grade III and IV intraventricular hemorrhage
Periventricular leukomalacia
Sepsis

had feeding difficulties, many of whom had BPD. At 8 months CA, infants with feeding difficulties had significantly lower cognitive and motor scores, with no differences in language scores on the BSID-III. However, at 20 months CA there were no significant differences between groups of infants with and without feeding difficulties in the NICU. Age at which oral feeds was started was an independent predictor of lower cognitive and fine motor outcomes at 8 months CA. Infants with feeding difficulties began oral feeds at an average PMA 36.9 + 3.9 vs 34.9 + 1.8 weeks in those without feeding difficulties. Feeding difficulty was also a strong predictor of cognitive and gross motor outcomes. However, BPD was not predictive of outcome and unlike prior studies this may be due to the lower gestation age group this study examined overall.

Conclusion

Unfortunately, for many providers the exact time as to when a preterm infant will learn to orally feed remains nonspecific. Most anticipate oral feeds to start at 33–34 weeks PMA with achievement of full oral feeds by 37–40 weeks PMA. However, each infant is unique with varying complexities and differences in neurodevelopment progression. Many patients do not fit the expected standard. This leaves us with the difficult question of how long to wait before considering alternative means for enteral nutrition to facilitate discharge home. Currently, practices vary by institution with some undergoing GT placement at 44 weeks PMA and others up to 52 weeks PMA. Hospitals also continue to vary in the practice of discharging infants with NG feeds, as not all have the resources to train and safely support families with such interventions at home. More information is needed as to the feeding trajectories of infants using such supplemental feeding devices to better determine who benefits most from such interventions. This will help prevent infants from receiving invasive procedures they may not require if achieving oral feeds is expected within weeks to a couple of months.

It is becoming more clear that the area of oral feeding for sick infants is a challenging one. For complex infants, many of whom have aerodigestive complications, an approach involving not only the medical team, but health professionals from every area may be needed. A few institutions have begun implementing multidisciplinary type feeding teams in the NICU.[33,34] This model allows for neonatologists, specialists, dieticians, nurses, and therapists to collaborate in determining diagnostic studies used, root cause, and feeding plans for infants. Increased success in oral feeding at discharge and follow-up at 1 year has been experienced in units with such programs. But it is recognized that not all hospitals have the resources and staff to construct these teams.

Additional studies are needed on the proposed interventions discussed to determine optimal initiation and frequency. While many focus on quantitative parameters such as days to oral feeding and length of hospital stay, we must remember to also draw attention to the quality of feeding as this may be more telling of a child's future feeding abilities. Furthermore, the neurodevelopmental track of these patients is likely intertwined with feeding capabilities. We need to continue assessing the link between feeding behaviors in the NICU and neurodevelopmental outcomes long term. This in turn can help us refine current and create new therapeutic interventions that can be implemented early and ideally, positively enhance an infant's feeding and developmental outcomes.

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REVIEW ARTICLE

Group B Streptococcal Infections in Neonates

Kirtikumar Upadhyay[1], Ajay Talati[2]

Abstract

Despite significant advances in preventive and therapeutic approaches, Group B streptococcus (GBS) still remains one of the most common causes of sepsis and meningitis in neonates. There is considerable variability in the immune responses that is related to microbial virulence, bacterial load, and immaturity of immune response system of the host. In this review, the mechanisms of GBS invasion and host–pathogen interactions are described. Understanding the host immune response to various bacterial components of GBS could help in refining our future strategies to mitigate the immune response and improve neonatal outcomes due to GBS sepsis.

Keywords: Antibiotics, Bacterial components, Group B streptococcus, Group B streptococcus immune response, Group B streptococcus vaccine, Host–pathogen, Inflammation.

Newborn (2022): 10.5005/jp-journals-11002-0022

Introduction

Group B streptococci (GBS, *Streptococcus agalactiae*) were first reported as a disease-causing pathogen in humans in 1935.[1] These have been identified as a leading cause of early and late sepsis in neonates across the world (Fig. 1).[2–4] GBS are encapsulated Gram-positive bacteria that can colonize the genitourinary and gastrointestinal tracts of 10–30% of healthy women.[5–7] During pregnancy, these bacteria have been implicated as a primary/contributing cause in preterm labor, urinary tract infections, chorioamnionitis, endometritis, pelvic thrombophlebitis, and endocarditis.[8] Data from the Center for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance System, a network of 10 sites across the United States that conducts active, population-based surveillance, show GBS to cause about 1,000 cases of neonatal sepsis/invasive disease per year. About 70% of these cases are full-term infants born at ≥37 weeks' gestation.[9] These microorganisms may sometimes cause serious invasive infections in non-pregnant adults, who are often immunocompromised or elderly with multiple associated morbidities. The total burden of invasive GBS disease in the population is approximately 9.9 per 100,000 with a mortality rate of 0.55 per 100,0000 population.[10]

Maternal colonization with GBS is an important risk factors for neonatal sepsis.[11] The risk of vaginal GBS colonization in women is known to increase in several biological and socioeconomic conditions. The biological risk factors include premature rupture of membranes (PROM), gastrointestinal GBS colonization, and increased maternal age.[12–15] High rates of vaginal carriage have also been associated with specific ethnic groups, obesity, low vitamin D intake, hygiene, sexual activity, specific healthcare occupations, and illiteracy.[13,16–18] The identification of GBS colonization prior to the onset of labor and intrapartum antibiotic prophylaxis is an important preventive strategy for early-onset GBS sepsis.[19] The implementation of these strategies by both the American College of Obstetricians and Gynecologists (ACOG) and the American Academy of Pediatrics (AAP)[20] has helped in reducing the incidence of early-onset GBS disease, although the frequency of late-onset GBS disease has not changed. The most recent CDC active bacterial surveillance data show the incidence of late-onset GBS sepsis in the United States to be approximately 0.28 per 1,000 live births. Following widespread implementation of intrapartum antibiotic prophylaxis in 1999, early-onset GBS infections decreased to 0.25/1,000 live births, but mortality continues to be high in premature infants.[20]

In GBS-colonized mothers, bacterial carriage >10 colony-forming units per milliliter have been associated with increased risk of vertical transmission to infants. Vertical transmission from

[1]Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington, United States of America

[2]Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America

Corresponding Author: Kirtikumar Upadhyay, Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington, United States of America, e-mail: kupadhya@uw.edu

How to cite this article: Upadhyay K, Talati A. Group B Streptococcal Infections in Neonates. Newborn 2022;1(1):109–119.

Source of support: Nil

Conflict of interest: None

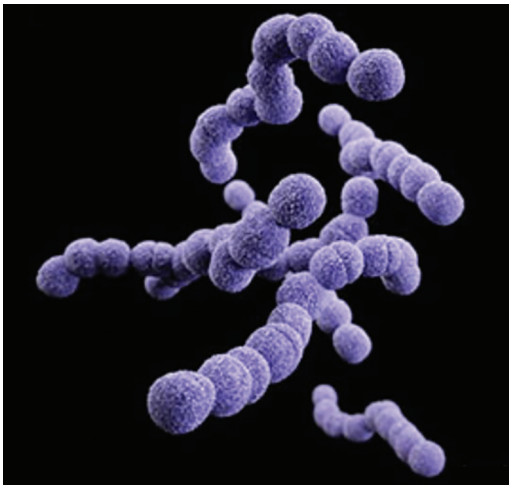


Fig. 1: Group B streptococci

colonized mothers to their neonates is seen in 41–72% of cases (mean 50%). About 1–12% of colonized infants (mean, 5%) are born to non-colonized mothers. The severity of colonization in infants can increase the risk of early- or late-onset GBS disease,[21] which typically presents with pneumonia, bacteremia, meningitis, and sometimes, septic arthritis and osteomyelitis.[7]

Since Austrian and Gold first demonstrated the therapeutic efficacy of penicillin in adults with streptococcal infections more than 50 years ago, GBS still remains the drug of choice for these infections.[22] However, even though timely and successful treatment of maternal GBS infection using ampicillin or penicillin may correct maternal colonization and reduce the risk of neonatal infections, it may not always alter medium/long-term neonatal outcomes.[23,24] These infants need close clinical follow-up after discharge.[25]

GBS Infection

Invasive fetal/neonatal GBS disease begins with the migration of these bacteria across the epithelial barrier in the mucus membranes or the skin. Most infants can successfully control GBS invasion, but some aspirate maternal secretions containing GBS into the lungs. These bacteria can proliferate to enormous densities (10^9–10^{11} colony-forming units per gram lung tissue).[26]

Doran and Nizet[27] have described four stages of fetal GBS infection: (a) adherence of bacteria to the lung mucosa followed by transepithelial migration; (b) proliferation in lung tissue and evasion of local innate immune defenses; (c) migration into the bloodstream, where these circulating bacteria escape elimination by mononuclear phagocytes; and finally (d) widespread dissemination to cause a systemic inflammatory response syndrome.

From the host's perspective, the phagocytic efficiency of innate immune cells such as neutrophils and monocyte/macrophages are important.[28] The ability of these cells to eliminate bacteria without an unduly intense/dysregulated inflammatory response is a key determinant of outcome. These defenses can be studied as host recognition of the pathogens, directed cellular movements (chemotaxis), engulfment (phagocytosis), and finally, the destruction of the microorganism.

Types of GBS and its Disease-causing Components

GBS normally resides as a commensal in maternal genital and lower gastrointestinal tracts but can acquire pathogenic characteristics and infiltrate many tissues following changes in the variable fraction of the genome. The Lancefield classification of GBS is based on the cell wall polysaccharides and describes nine serotypes, including Ia, Ib, and II–VIII.[28] More recently, a serotype IX was added. Type III is frequently seen in GBS meningitis, whereas serotype V is a leading cause of invasive disease in adults.[29,30] Overall, 96% of the invasive GBS infections are caused by serotypes Ia, Ib, and III. The maternal isolates included the serotype variant I (35%), III (21%), Ib (13%), and Ia (11%). Frequent sequence types were ST1 (32%), ST12 (22%), and ST23 (15%).[31] A surface antigen, the C protein with its α and β components, is seen in all Ib, 30% of type Ia, 60% of type II, and in some type IV, V, and VI strains.

Genome analysis of the five major disease-causing capsular serotypes (Ia, Ib, II, III, and V) indicates that there is a "core" genome comprised of nearly 80% of all genes.[32] The remaining 20% of the genome is relatively variable and contains many virulence factors such as pore-forming toxins and the sialic acid–rich capsular polysaccharides, which are involved in adherence and invasion of host cells and evasion of host immunity.[33]

The GBS cell wall is a network of cross-linked peptidoglycans, surface proteins, polyanionic teichoic acid, and lipoteichoic acid. The best-characterized proteins in the cell wall are shown clockwise in Figure 2 and the characterized are summarized in the following.[34] We have also provided brief descriptions of carbohydrates and lipids known to be present in the GBS cell wall.

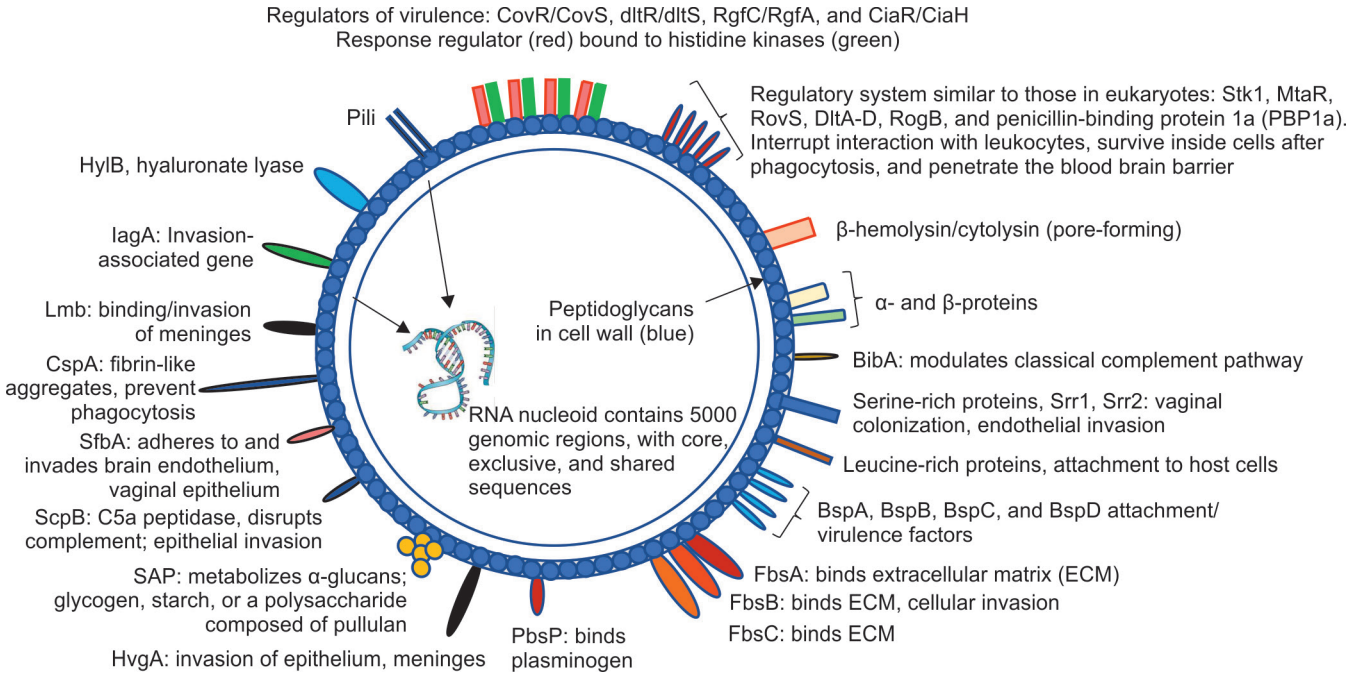


Fig. 2: Best-characterized GBS cell wall proteins involved in its pathogenic effects

Regulatory Signaling Systems

GBS are known to contain up to 107 regulatory signaling systems, 17–20 have been associated with pathogenicity and 4 have been studied closely.[35] These four response regulators CovR, dltR, RgfC, and CiaR stay bound to specific histidine kinases: CovR/CovS, dltR/dltS, RgfC/RgfA, and CiaR/CiaH.[35] Upon exposure to an external signal, the kinases phosphorylate conserved aspartate residue(s) in target proteins to alter the function.

GBS encodes for several regulatory enzymes that resemble those in eukaryotes. One example is the serine-threonine kinase Stk1 and its cognate phosphatase Stp1; the pair regulates the expression of pore-forming toxins.[36] Three other regulators, MtaR, RovS, and RogB have also been identified.[37] MtaR, methionine transport regulator regulates methionine transport/uptake and seems to be critical for GBS survival.[38,39] Another tyrosine kinase, CpsD and its cognate phosphatase, CpsB, are also being investigated; these proteins may be immunization targets.[40]

RovS (regulator of virulence in *S. agalactiae*) can activate superoxide dismutase and detoxify reactive oxygen species (ROS).[37] It is important for the adhesion of GBS to eukaryotic cells and increases the expression of other known and putative virulence genes and of hemolysin.

DltA-D proteins, the RogB protein, penicillin-binding protein 1a (PBP1a), and pilus island proteins PI-2a and PI-2b can modify reactions to other antimicrobial peptides.[37] The fibronectin-binding proteins, (FBP)-A and FBP-B, can also alter GBS adherence to host cells.[41]

Pore-forming toxins such as β-hemolysin/cytolysin (β-H/C, CylE) may trigger host-cell lysis.[42] The toxins are activated by the serine/threonine protein kinase Stk1 through CovR, or directly by CovS. The Christie Atkins Munch Peterson (CAMP) factor Cfb also forms pores in host cell membranes and is activated by CovR/CovS.[43]

Alpha-like proteins (alps) expressed on the bacterial surface bind glycosaminoglycans on epithelial cells and facilitate entry into the host epithelial cells.[44] Alp1 is expressed on serotypes Ia, Ib, and II; alp2 on serotypes Ia, III, and V; and Alp 3 on V and VIII. The Alp family also includes the Rib proteins expressed in type III, several type II, and some type V strains.

Beta proteins on the surface of serotypes Ia, Ib, II, and V can bind (a) the Fc moiety of human IgA and inhibit its function; (b) the complement inhibitor factor H and block phagocytosis; and (c) human Siglec-5, a leukocyte cell-surface receptor, to inhibit phagocytosis, oxidative burst, and extracellular trap production, promoting bacterial survival in the host.[45]

BibA, GBS immunogenic bacterial adhesion protein is a cell wall protein that binds human C4-binding protein (C4BP), a modulator of the complement classical pathway.[46]

Serine-rich repeat (Srr) proteins are a group of cell wall–anchored proteins; the best characterized members are Srr1 and Srr2.[47] Srr1 gets glycosylated and is then displayed on the cell wall in a configuration resistant to proteolysis. Srr1 glycosylation alters binding to epithelium; binding to cytokeratin 4 and keratin promotes attachment to vaginal epithelial cells.

Leucine-rich repeat (LRR) proteins contain leucine-rich repeats and are involved in enzyme inhibition, cell adhesion, trafficking, and signal transduction.[46] These are virulence factors; a leucine-rich repeat protein of GBS (LrrG) promotes attachment of bacterium to host cells.

BspA, BspB, BspC, and BspD, Group B Streptococcus surface proteins encode for four GBS attachment/virulence factors that bind host proteins or other surface components.[46]

FbsA, FbsB, and FbsC, Fibronectin-binding surface proteins are seen in nearly all serotypes with variable number of repeats.[41] These adhesins promote attachment to epithelial cells and protect against opsonophagocytosis. There may be a variable degree of binding to fibrinogen and platelets. FbsA may activate TLR2. FbsC contains immunoglobulin-like tandem repeats, which might promote attachment to epithelial cells.[48]

PbsP, plasminogen-binding surface protein is a cell wall–anchored serotype III protein expressed in some concentrations by almost all clinical GBS isolates. It binds/activates plasminogen.[49]

HvgA, hypervirulent GBS adhesin is seen in the hypervirulent GBS clone ST-17 associated with severe late-onset disease.[50] GBS strains expressing HvgA adhere avidly to epithelial and blood-brain barrier endothelial cells.

SAP, S. agalactiae pullulanase: SAP metabolizes α-glucans.[51] It can degrade glycogen, starch, or a α-glucan polysaccharide composed of repeating maltotriosyl units known as pullulan.[52] Sap is a conserved protein comprised of five conserved domains: (a) an N1 unit encoding two carbohydrate-binding motifs; (b) N2 pullunase unit; (c) N3 isoamylase; (d) a glycoside hydrolase; and (e) a C-terminal β-sandwich domain.

ScpB, Streptococcal C5a peptidase B is expressed in all GBS serotypes and functions as a surface protease and adhesin/invasin.[53] Some naturally occurring variants maintain the ability to interact with fibronectin by inhibiting C5 peptidase function.

SfbA, Streptococcal fibronectin binding adhesin binds and invades the microvascular endothelial cells in the brain. It also contributes to GBS invasion of vaginal and cervical epithelium and hence may take part in GBS niche establishment in the vagina.

CspA, cell surface-associated protein A is expressed in highly virulent type III GBS isolates.[54] It promotes the formation of fibrin-like aggregates and protects these bacteria from phagocytosis by neutrophils. It can also inactivate CXC chemokines to block leukocyte chemotaxis.

Lmb, laminin-binding protein promotes GBS adherence to host cells.[55] It binds the extracellular matrix and is an important determinant of pathogenicity.

Invasion-associated gene (*iagA*) contributes to GBS meningeal infection and virulence by facilitating invasion of blood-brain barrier and other host cells. The gene product is a glycolipid anchor for lipoteichoic acid and interacts directly with host cells.[56]

HylB, hyaluronate lyase cleaves hyaluronan and promotes spread of GBS during infection.[57] Both bacterial hyaluronan and hyaluronan lyases play a role.

Pilus island proteins (PI-2a and PI-2b): These proteins can alter the reaction to other antimicrobial peptides.[58,59]

Capsular Polysaccharides

Capsular polysaccharides contribute to GBS virulence by interfering with C3 opsonization through inhibition of the alternative complement pathway in the absence of type-specific capsule antibodies.[60,61] Group-specific GBS polysaccharides seem to be more potent inflammatory stimuli than the type-specific ones.[27,62] Both categories are covalently linked to peptidoglycans and, possibly, to other cell wall components of GBS. Sialylation of these polysaccharides can help immune evasion through molecular mimicry of glycoconjugates on the host cell surface.[63] Sialylation can also prevent opsonophagocytosis through inhibition of alternative complement pathway activation.

Capsular Lipids: GBS produce a pigmented, cytotoxic lipid, known as granadaene (ornithine *rhamnopolyene)*, which confers

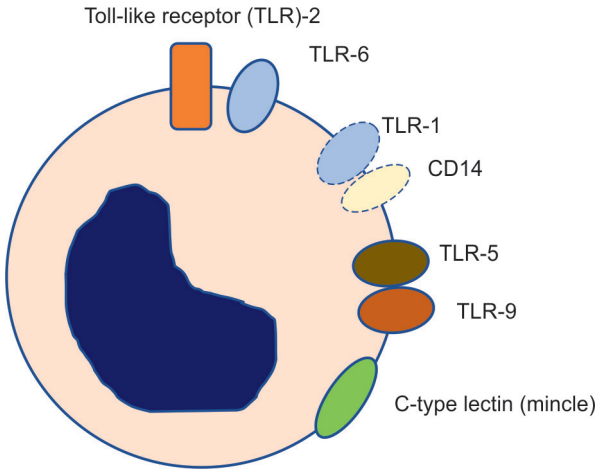


Fig. 3: Cell surface receptors that may play a role in recognition of GBS and induction of downstream signaling

pigmentation and hemolytic activity, and is a major contributor to most of the inflammatory manifestations of GBS disease. Hemolytic and hyper-hemolytic GBS strains are associated with increased virulence.[64]

Host Recognition of GBS

The innate immune system may utilize several receptor systems in timely recognition of GBS and the induction of appropriate local/systemic defense responses (Fig. 3). Toll-like receptors 2 (TLR2) are the primary pattern-recognition receptors (PRRs).[65,66] GBS shows sialic acid O-acetylation, and therefore, C-type lectin receptors (mincle) also merit investigation. However, nucleotide-binding oligomerization domain-containing receptors (NLRs) may not play a major role in GBS immunity. At the cellular level, the innate immune system is comprised of monocytes, granulocytes, macrophages, and the complement system. In a healthy host, targeted local immune responses destroy invading bacteria without undue inflammation. However, if the local immunity is inadequate, a systemic inflammatory response syndrome may be seen.[67]

TLR-2 and its analogues are membrane-spanning, non-catalytic PRRs that are most highly expressed in sentinel cells, such as macrophages and dendritic cells.[68] These receptors, in conjunction with TLR6, bind GBS peptidoglycans and lipoteichoic acid. The binding may be stronger to secreted components of GBS components than to those present in the bacterial cell wall.[69] There may also be some species differences. In human cells, but not in mice, lipoteichoic acid–mediated TLR2 activation may involve CD14 and TLR1.[70,71] In conjunction with TLR6 or TLR1, TLR2 can recognize bacterial products, such as peptidoglycan, lipoproteins, capsular polysaccharide, and glycolipids.[72] TLR5 and TLR9 can bind bacterial flagellin and bacterial DNA, respectively.[73]

TLRs typically contain an extracellular leucine-rich repeat domain that binds specific pathogen-associated molecular patterns, and adaptors containing an intracellular Toll/IL-1R (TIR) domain that can activate downstream signaling. MyD88 is one of the best known of these adaptors; it recruits IL-1R-associated kinase (IRAK), followed by the tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6). The activation of TGF-β-activated kinase (TAK1) and downstream signaling stimulates mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappa B (NF-κB). These events stimulate the expression of inflammatory cytokines/chemokines.[74]

TLR2 and MyD88 play a synergistic defense role against GBS.[75] TLR2 can activate cytokine responses to extracellular products of GBS, although this does not happen upon exposure to whole bacteria. MyD88 can activate inflammation upon exposure to both types of stimuli.[76] MyD88 and downstream IRAK1 stimulate the protein kinase D1 (PKD1) and related inflammatory mediators, such as the mitogen-activated protein (MAP) kinases, p38 and c-Jun kinase (JNK), and the transcription factors NF-κB and activator protein 1.[77] Kenzel et al.[65] showed that JNK evokes cytokine expression by activating AP-1 and NF-κB. Human studies have confirmed elevated plasma interleukin (IL)-1 and CXC ligand/IL-8 concentrations (57). GBS-infected neonates develop systemic inflammation with increased TNF and IL-1.[75] In contrast, for unclear reasons, purified TLR2 and -4 do not consistently activate cord blood mononuclear cells to the same extent.[78–81]

TLR8, an endosomal sensor of RNA degradation products, can sense GBS and activate the interferon regulatory factor 5 (IRF5) to increase the expression of interferon-β, IL-12p70, TNF, and IL-12. TLR8 activates IRAK-1, by forming a Myddosome, filamentous structures composed of MyD88 oligomers.[82] TLR9 binds CpG DNA and can play an important role in macrophage expression of TNF, IL-6, and IL-12 upon exposure to GBS. This pathway may not be so important to upregulate NO, iNOS, or IFN-β production.[83]

To summarize, TLR-2 is the key receptor for detecting GBS. TLR6 and TLR1 can assist in detecting bacterial products, such as peptidoglycan, lipoproteins, capsular polysaccharide, and glycolipids.[72] TLR5 can bind bacterial flagellin. TLR8 and TLR9 might play important roles in the detection of bacterial RNA and DNA, respectively.

Fetal/Neonatal Adaptive Immune System

The fetal adaptive immune system is relatively immature due to the limited exposure to antigens *in utero*.[84,85] Transplacentally acquired maternal anti-GBS antibodies provide some protection prior to and after birth. *In vitro*, maternal anti-capsular IgG concentrations >1 μg/mL mediated GBS killing and were predicted to reduce the risk of early-onset GBS Ia and III disease by 81% [95% confidence interval (CI): 40–100%] and 78% (95% CI: 45–100%), respectively.[86] In other studies, infants born to mothers with anti-GBS type III IgG antibody ≥10 μg/mL has a 91% lower risk for early-onset disease than those born to mothers with levels <2 μg/mL.[87] Infants with GBS sepsis had lower levels of antibodies against the capsular polysaccharide than those who were recently colonized with these bacteria, suggesting that these antibodies are rapidly consumed.[88] The neonatal adaptive immune system took a few weeks to start functioning with synthesis of immunoglobulin G and expansion of the appropriate V_H gene repertoire.[89] GBS rapidly activated NK cells in the innate immune response to encapsulated bacterial infection by inducing the release of IFN-γ.[90]

Both the maternal and fetal immune systems show a bias toward producing T helper-2 (T_H2)-cell-polarizing cytokines.[91] After birth, the neonatal immune responses can rapidly shift toward a T_H1 prominent proinflammatory cytokine response following exposure to certain antigens,[67,92] although there is some evidence that infections such as those with GBS can overwhelm these changes and suppress such rise in T_H1-polarizing cytokines.[93] GBS infections can bias the Th differentiation program of neonatal $CD4^+$ T cells and promote proinflammatory Th1 and Th17 phenotypes in Tregs. GBS-stimulated neonatal neutrophils may drive proinflammatory T-helper (Th) cell programming. GBS-stimulated neonatal

neutrophils can also induce the expression of the canonical nuclear transcription factors for Th1 (Tbet) and Th17 (IL-17) cells in $CD4^+$ T cells. These activated neutrophils and neutrophil-derived mediators can also alter the Tregs to acquire Th1 and Th17 characteristics.[94]

Immunity or Inflammation: Host Responses to GBS

The elimination of GBS from tissues and the bloodstream involves a sequence of events, where resident macrophages, monocytes, and circulating neutrophils recognize the pathogen, release cytokines to activate peers in the vicinity, chemokines to recruit other circulating phagocytes, and finally, to promote phagocytosis and killing of the pathogens that have been internalized or are in close vicinity.[82] Many of these events are not fully matured in newborn infants.

Chemotaxis is focused leukocyte movement that is directed toward pathogens or their components. It is often still immature in preterm and term neonates; possible reasons may involve lower total neutrophil cell mass and sFcRIII concentration;[34] poor rolling and adhesion to endothelium due to less L-selectin expression,[60,61] inefficient formation of lamellipodia, and/or reduced movement toward stimulus.[62] Neutrophils exposed to GBS recruit peer phagocytes by releasing/expressing chemokines, such as CXC ligand 8/interleukin-8 and its analogues, leukotriene B4, and complement factors C3b and C5a.[69] To counteract the concentration gradients of these factors, GBS express a C5a peptidase on its surface that contributes to immune evasion by reducing chemoattractant C5a.[70]

Phagocytosis involves recognition of the pathogen by cell surface receptors, actin polymerization under the membrane at the site of contact, and the formation of actin-rich membrane extensions to engulf the pathogen. The phagosome matures via a series of membrane fusion and fission events and eventually fuses with a proximate lysosome to become a phagolysosome. This phagolysosome is an acidic, hydrolytic compartment in which the pathogen is killed and digested in preparation for antigen presentation.[95]

In the lungs, resident macrophages are the first to encounter newly aspirated GBS, and neutrophils are recruited to enhance the protective responses. Unfortunately, both macrophages and neutrophils in neonates seem to be less efficient in killing GBS. The TLR2 receptors are not particularly important because wild-type and genetically altered macrophages lacking TLRs or MyD88 internalize GBS at similar speeds.[95] The chronological age is important; the efficiency of phagocytosis is low in preterm infants and increases with development (64). These findings may be explained by the requirement for the CD11b/CD18 (Mac-1) receptor and opsonization with complement.[96,97] Neonatal neutrophils also contain less lysozyme.[98] Reactive oxygen species are critical for killing of GBS;[99] neutrophils from very-low-birth weight (VLBW) infants show a less-intense intracellular oxidative burst than in those from older subjects.[100] GBS possess a Mn-cofactored superoxide dismutase that serves as a virulence factor by counteracting intracellular killing in macrophages.[101]

Animal models of GBS sepsis and meningitis show an intense, dysregulated inflammatory response. There is excessive production of inflammatory mediators, especially TNF and nitric oxide (NO), which have been associated with more severe disease and increased mortality.[102–104] Excessive NO during sepsis appears to be largely responsible for the refractory hypotension that is seen in septic shock.[102] Indeed, the clinical symptoms of GBS sepsis are related to the host–pathogen interaction and cytokine production during the process.

Early- and Late-onset GBS

Early-onset disease begins within the first 6 days after birth.[105] However, most infants (61–95%) become symptomatic within the first 24 hours (median, 1 hour). The most frequent presentation is with respiratory distress, apnea, tachypnea, grunting respirations, and cyanosis. Many patients may show lethargy, poor feeding, abdominal distention, pallor, jaundice, tachycardia, and hypotension. Term infants may be febrile, although preterm infants may be hypothermic.

Bacteremia is the most common form of early-onset GBS disease, accounting for approximately 80% of cases. Pneumonia and meningitis, although not uncommon, are less likely presentations in early-onset disease, accounting for 15% and 5–10%, respectively.

Late-onset disease is defined as GBS infections manifesting between postnatal days 7–89 (median 37 days). The clinical presentation resembles early-onset disease.[105] Bloodstream infections remain the most common presentation of the late-onset disease. However, meningitis occurs in about 30% of cases, as opposed to 5% in early-onset disease.

Late-onset disease may also present with focal diseases, such as osteomyelitis, pyogenic arthritis, and cellulitis-adenitis syndrome.[106] The proximal humerus is frequently affected in infants with osteomyelitis, whereas pyogenic arthritis typically affects the hip and/or knee joints. GBS cellulitis-adenitis syndrome is generally unilateral, involving facial or submandibular sites. It can also involve inguinal, scrotal, and prepatellar regions. The cellulitis-adenitis syndrome presents with swelling of the affected area and local lymphadenopathy. Aspiration of the affected area of cellulitis can yield GBS. Blood cultures can be positive.

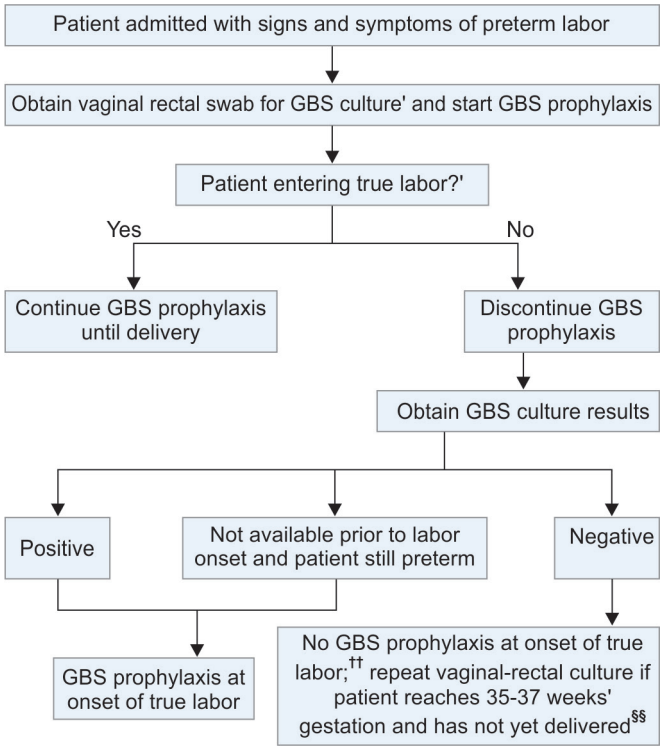
Delayed late-onset GBS disease manifests after 3 months following birth. Most cases occur in premature/VLBW infants. In term infants, delayed late-onset GBS disease can be associated with HIV infection or immunodeficiency. The clinical manifestations are similar to those in patients with typical late-onset infections; bacteremia without a focus and meningitis are the most common clinical features.[21]

Intrapartum Antibiotic Prophylaxis

The US CDC recommend universal screening of pregnant women for vaginorectal colonization in weeks 35–37 of gestation and intrapartum (intravenous) antibiotic prophylaxis (IAP) to GBS-positive mothers (Flowcharts 1 and 2). IAP can reduce vaginal colonization with GBS to 47% within 2 hours of administration and 12% after 4 hours of administration.[107] It also reduces the likelihood of neonatal colonization.[107–109] Importantly, the maternal vaginal flora, including GBS, does not appear to develop selective antibiotic resistance after IAP.[110]

IAP has lowered the incidence of early-onset neonatal GBS sepsis. The possibility of negative effects of IAP including increased infections with Gram-negative bacteria, such as ampicillin-resistant *Escherichia coli* remains unclear.[104,111] A recent epidemiological study found increased late-onset GBS disease during the periods 1997–2001 and 2002–2010, but we do not know if these shifts reflected changes GBS pathogenicity, increased survival of preterm infants, or delay in disease onset from IAP.[112] Some data suggest that maternal vaginal flora may be altered due to IAP with increased susceptibility of both the mother and the child to fungal infections during the postpartum period. Infants born to women treated with antibiotics for spontaneous preterm labor showed increased

Flowchart 1: Algorithm for GBS intrapartum prophylaxis for women with preterm labor



*At <37 weeks and 0 days' gestation; †If patient has undergone vaginal-rectal GBS culture within the preceding 5 weeks, the results of that culture should guide management. GBS-colonized women should receive intrapartum antibiotic prophylaxis. No antibiotics are indicated for GBS prophylaxis if a vaginal-rectal screen within 5 weeks was negative; Patient should be regularly assessed for progression to true labor; if the patient is considered not to be in true labor, discontinue GBS prophylaxis; **If GBS culture results become available prior to delivery and are negative, then discontinue GBS prophylaxis; ††Unless subsequent GBS culture prior to delivery is positive; §§A negative GBS screen is considered valid for 5 weeks. If a patient with a history of PTL is re-admitted with signs and symptoms of PTL and had a negative GBS screen >5 weeks prior, she should be rescreened and managed according to this algorithm at that time (From: Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. Prevention of perinatal group B streptococcal disease—revised guidelines from CDC, 2010. MMWR Recomm Rep 2010;59(RR-10):22. PMID: 21088663.)

risk of cerebral palsy.[113] Exposure to antibiotics prior to, or during early infancy, is associated with increased risk of childhood obesity, asthma, and thicker aortic intima-media layer on echocardiography, an early marker for risk of cardiovascular disease.[114–116]

Alternative Therapies Against GBS

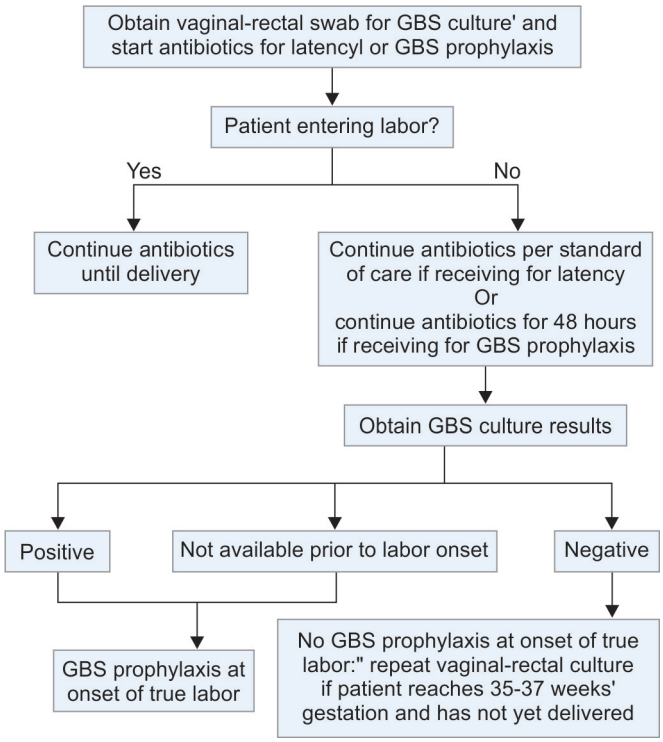
Since Austrian and Gold[22] showed that penicillin was an effective treatment for adults with streptococcal infections, and penicillin or other β-lactam agents have been considered to be the treatment of choice for most GBS-infected infants.[117] Currently used bactericidal antibiotics may cause a rapid release of bacterial components in infants with high bacterial loads and induce a SIRS with morbidity and mortality.[118]

There is a need for alternative therapies with immunomodulatory and bacteriostatic effects, such as macrolides such as azithromycin. These antibiotics show a ribosomal-targeted mechanism to inhibit the expression of production of inflammatory toxins and other virulence factors. There have been encouraging results in pneumococcal infections.[23,24,119] The combination of a β-lactam with a macrolide may have benefits. Some *in vitro* and animal models have been used to compare antibiotics, such as rifampin, clindamycin, ampicillin, azithromycin, and cefotaxime, in various combinations.[77,120,121] The role of various inflammatory molecular pathways involving TLR2, MyD88, IRAK, and PKD1 also needs attention.[77]

GBS Vaccine

Vaccination may be a useful strategy to stimulate the production of active antibodies that could cross the placenta and prevent GBS disease. Humans generate serotype-specific IgG antibodies against the GBS capsular polysaccharides,[122] which show a concentration-dependent protective effect.[87,123] The first human clinical trials were conducted with purified native type Ia, II, or III polysaccharides injected in healthy adult volunteers, including pregnant women.[122] These vaccines were safe but were not adequately immunogenic.[124] Conjugation with protein carriers enhanced the immunogenicity of polysaccharide vaccines.[125,126] A second generation of GBS vaccines was developed using glycoconjugates. A trial showed the conjugates of serotype III with tetanus toxoid in pregnant women showed increased titers of protective IgG to type III CPS. After glycoconjugate vaccination, the titers were also increased.[127,128] Monovalent conjugate vaccines representing the disease-causing serotypes Ia, Ib, II, III, and V are being tested in phase I and phase II trials.[129] Multivalent capsular conjugate vaccines are also being developed.[130] There may be promising vaccine candidates in the core genome but there is a risk of losing proteins that are not

Flowchart 2: Algorithm for screening for group B streptococcal colonization and use of intrapartum prophylaxis for women with preterm premature rupture of membranes



*At <37 weeks and 0 days' gestation; †If patient has undergone vaginal-rectal GBS culture within the preceding 5 weeks, the results of that culture should guide management. GBS-colonized women should receive intrapartum antibiotic prophylaxis. No antibiotics are indicated for GBS prophylaxis if a vaginal-rectal screen within 5 weeks was negative; §Antibiotics given for latency in the setting of pROM that include ampicillin 2 g intravenously (IV) once, followed by 1 g IV every 6 hours for at least 48 hours are adequate for GBS prophylaxis. If other regimens are used, GBS prophylaxis should be initiated in addition; **GBS prophylaxis should be discontinued at 48 hours for women with pROM who are not in labor. If results from a GBS screen performed on admission become available during the 48-hour period and are negative, GBS prophylaxis should be discontinued at that time; ††Unless subsequent GBS culture prior to delivery is positive; §§A negative GBS screen is considered valid for 5 weeks. If a patient with pROM is entering labor and had a negative GBS screen >5 weeks prior, she should be rescreened and managed according to this algorithm at that time (From: Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. Prevention of perinatal group B streptococcal disease—revised guidelines from CDC, 2010. MMWR Recomm Rep 2010;59(RR-10):22. PMID: 21088663.)

essential for bacterial growth but may be important virulence factors. Hence, the glycoconjugate generation vaccine remains the best hope.

There are challenges in conducting efficacy clinical trials due to the low incidence of neonatal diseases. The establishment of maternal CPS-specific antibody levels at the time of delivery above a quantified threshold, which can be predicted to confer high level of protection against early-onset GBS disease.[87,131,132]

Orcid

Kirtikumar Upadhyay https://orcid.org/0000-0002-5951-8686

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REVIEW ARTICLE

Non-coding RNAs in Neonatal Necrotizing Enterocolitis

Keyur Donda[1], Benjamin A Torres[2], Akhil Maheshwari[3]

Abstract

The incomplete understanding of the etiopathogenesis of necrotizing enterocolitis (NEC) contributes to the lack of timely diagnosis and limited therapeutic options. Non-coding RNAs (ncRNAs) have emerged as key regulators of gene expression in various pathways that can modulate various physiological and pathological processes. Despite several studies revealing the role of ncRNAs in intestinal inflammatory diseases in adults, these remain largely unexplored in NEC. In this article, we review the information on ncRNAs that have been specifically identified in NEC or have been noted in other inflammatory bowel disorders that share some of the histopathological abnormalities seen frequently in NEC. We have assimilated the most current research findings on ncRNAs in intestinal diseases. This is an attempt to explore a novel field that has immense potential for future translational and clinical research in preventing, detecting, and treating NEC.

Keywords: Genetic predisposition, Intestinal inflammation, Necrotizing enterocolitis, Neonates, Non-coding RNA, Spontaneous intestinal perforation.

Newborn (2022): 10.5005/jp-journals-11002-0012

Impact

- Current information categorizes NEC as a multifactorial, inflammatory bowel necrosis of newborn infants.
- Non-coding RNAs (ncRNAs) may influence the risk of occurrence of NEC.
- ncRNAs may modulate the severity of intestinal injury and consequently the clinical outcome of NEC.
- ncRNAs have been linked with inflammatory intestinal diseases of adults that share histopathological findings with neonatal NEC and, hence, need to be explored.

Introduction

Necrotizing enterocolitis (NEC), an inflammatory necrosis that may involve parts of the small and the large intestine, is one of the most common and serious diseases in premature infants causing significant morbidity and mortality. The etiopathogenesis of NEC in neonates is multifactorial. Prematurity is the prime risk factor for NEC development. In addition, various prenatal and postnatal factors contribute to the disease development and progression such as antenatal steroids, type of feeding, gut dysbiosis, hypoxic-ischemic injury, severe anemia requiring packed red cell transfusion. Clinically, the presenting features include abdominal distension, hematochezia, emesis, and feeding intolerance, which can be associated with subtle changes in vital signs including temperature instability, tachycardia, and lethargy. Abdominal radiography remains the diagnostic tool of choice with pathognomonic sign of *pneumatosis intestinalis*. The disease usually involves ileocolic region and colon; histopathologically, NEC is characterized by exaggerated inflammation, coagulative necrosis, *pneumatosis intestinalis*, intestinal hemorrhage, and reparative changes.[1] The treatment is currently limited to supportive care in an attempt to prevent further injury to the intestine. Despite major advances in neonatology, the options to diagnose and treat NEC are few, and the available strategies have not made a significant impact bringing down the prevalence and improving outcomes. There is a need for more research to explore novel biomarkers and potential therapeutic targets.

[1,2]Department of Pediatrics, University of South Florida Health Morsani College of Medicine, Tampa, Florida, United States of America

[3]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Akhil Maheshwari, Global Newborn Society, Clarksville, Maryland, United States of America, Phone: +1 708 910 8729, e-mail: akhil1uic@gmail.com

How to cite this article: Donda K, Torres BA, Maheshwari A. Non-coding RNAs in Neonatal Necrotizing Enterocolitis. Newborn 2022;1(1): 120–130.

Source of support: NIH awards HL133022 and HL124078 (to AM)

Conflict of interest: None

Recently, rapidly growing interest in genetic research along with the availability of in-depth transcriptome sequencing techniques has exponentially expanded our understanding of gene expression and its regulation. This added knowledge has introduced the possibility of a complex interaction between clinical risk factors and genetic susceptibility explaining inter-individual variability of NEC susceptibility, progression, and prognosis. Non-coding RNAs (ncRNAs) have been unraveled recently as one of the key regulators of gene expression. In this review, we aim to provide currently available evidence from human and animal studies on role of ncRNAs in the pathogenesis of NEC. We also present the evidence for ncRNAs in other intestinal diseases that share similar histopathological characteristics with NEC for future direction. We have extensively searched in the databases PubMed, EMBASE, and Scopus after short-listing the keywords to describe the histopathological and clinical features of NEC.

Non-coding RNAs

The ncRNAs, as the name suggests, are RNA molecules that are not translated into proteins. Since first discovered in eukaryotic

cells in 1989, ncRNAs have gained tremendous visibility. About 80–90% of living cell genome is transcribed, however, only less than 2% of that transcribed RNA encodes for protein. Thus, RNAs can be categorized into coding and ncRNAs. ncRNA molecules are further categorized based on function into (1) housekeeping ncRNAs, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs), and (2) regulatory ncRNAs. The ncRNAs can also be categorized into two groups based on their nucleotide size, (1) small ncRNAs (<200 nucleotides), and (2) long ncRNAs (>200 nucleotides). The most studied small ncRNAs are <50 nucleotides long and therefore, to better categorize 50–200 nucleotide-long ncRNAS, a term "mid-size" ncRNAs have been proposed which includes snoRNAs, promoter-associated small RNAs (PASRs), transcription start site-associated RNAs (TSSa-RNAs), and promoter upstream transcripts (PROMPTs).[2,3] Circular RNAs (circ-RNAs) are another variant, which are comprised of a covalently closed continuous loop that lacks the 5′ cap and the 3′ tail.[4] Similarly, pyknons are recognizable non-random sequences that may be repeated mainly in the non-coding genomic DNA.[5] Different types of ncRNAs are depicted in Figure 1. So far, microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), lncRNAs, and circular RNAs (circRNAs) have been studied.

MicroRNAs

MicroRNAs (miRNAs) are endogenous, conserved, 21–23 nucleotide-long ncRNAs involved in posttranscriptional silencing of gene expression.[6] Approximately 1–3% of the mammalian genome is now known to code for miRNAs. MiRNA genes are distributed throughout the genome and can be seen in intronic sequences of protein-coding genes, within intronic or exonic regions of ncRNAs, and even between independent transcription units (intergenic). MiRNAs may carry specific promoters for independent transcription, share promoters with host genes, or could be cotranscribed as a single primary miRNA transcript.[7]

The DNA sequences encoding for mRNAs are first transcribed in the nucleus by the RNA polymerase II-producing primary RNAs (pri-miRNAs). The pri-miRNA is then processed in a stepwise manner by nuclear as well as cytoplasmic endoribonucleases forming mature miRNA. Drosha, a type III ribonuclease located in the nucleus, processes pri-miRNA into ~70 nucleotide-containing precursor (pre-) miRNAs. These oligonucleotides are then translocated into the cytoplasm by the exportin-5 shuttle.[8] In the cytoplasm, this pre-miRNA is further processed by another type III ribonuclease, dicer, into a mature miRNA. The 3′-end of the miRNA binds the Argonaute protein in a specialized oligonucleotide/oligosaccharide-binding fold to form an RNA-induced silencing complex (RISC).[9] RISCs bind approximately complementary sequences in the 3′-untranslated region (UTR)s of target mRNAs and regulate protein output by either promoting mRNA degradation and/or inhibiting translation.[10] Genomic analyses of miRNA-target interactions show conserved complementarity for approximately 6–8 base pairs from position II of the miRNA. This region (nucleotides 2–7 at the 5′ end of the miRNA) is often termed the "seed" sequence for computational miRNA target prediction.[11]

The function of most miRNAs is still unclear. A single miRNA can regulate hundreds of genes, because only a few RNA nucleotides (2 through 7 or 8) are needed to recruit RISC and bind the seed sequence of a target mRNA for repression.[12,13] Many miRNAs are now believed to modulate cellular differentiation, proliferation, apoptosis, inflammation, and stem cell maintenance and may also indicate the timing of various events during development.[6] These features, together with the observation that miRNAs can be secreted and stay stable in plasma, make them prominent, accessible biomarkers as well as therapeutic targets.[14]

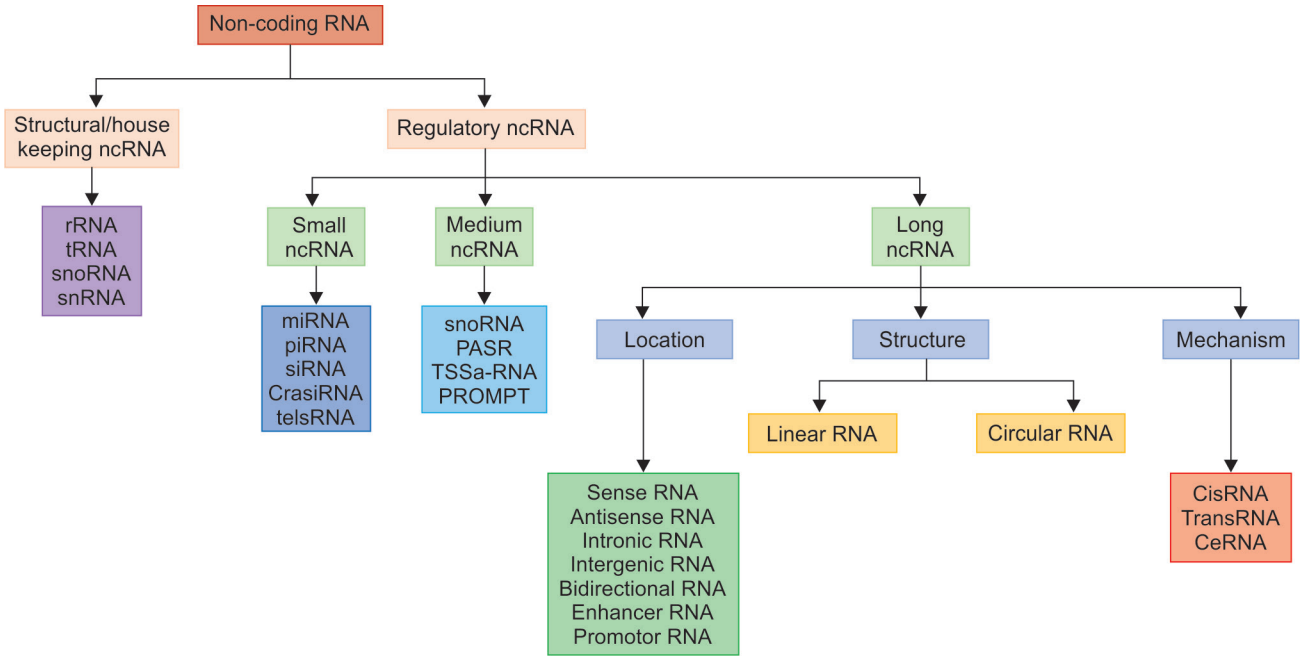


Fig. 1: Classification of non-coding RNAs. ceRNA, competing endogenous RNA; cisRNA, cis-acting RNA; crasiRNA, centromere repeat-associated small interacting RNA; miRNA, microRNA; ncRNA, non-coding RNA; PASR, promoter-associated small RNA; piRNA, piwi-interacting RNA; PROMPT, promoter upstream transcripts; rRNA, ribosomal RNA; siRNA, small interfering RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA; telsRNA, telomere-specific small RNA; transRNA, trans-acting RNA; TSSa-RNA, transcription start site-associated RNAs

Piwi-interacting RNAs

Piwi-interacting RNAs (piRNAs) are 26–31 nucleotide-long ncRNAs that interact with the piwi family of proteins. The transcription process of piRNA is dicer-independent and is activated in the piRNA gene clusters on heterochromatin. Pre-initiation complex (PIC) is formed after recruitment of RNA polymerase II, and other transcription factors that in turn initiate piRNA transcription and eventually produce pre-piRNA. Once formed, pre-piRNA is translocated into the cytoplasm. In the cytoplasm, 5′-end of pre-piRNA binds to the piwi protein to form a piRNA-induced silencing complex (piRISC).[15] Processed piRISC is transported back in to the nucleus through nuclear pores where it inhibits the transcription of transposon elements.[16] Transposon elements have been identified to have a role in gene mutation leading to various diseases including cancers and infertility.[17,18]

Small Interfering RNAs (siRNAs)

Small interfering RNAs (siRNAs) are double-stranded, 21–25 nucleotide-long RNAs with two nucleotide overhangs at each hydroxylated 3′-end and phosphorylated 5′-end.[19] Once in the cytoplasm, RNAse III dicer enzyme cleaves the long double-stranded RNA into siRNA. The siRNA is incorporated into RISC, which consists of Argonaute (Ago) protein, Dicer, and transactivating response RNA-binding protein (TRBP), leading to separation of double-stranded siRNA into the sense and antisense strand within the RISC complex. The antisense strand, with more stable 5′-end, forms the activated RISC complex, which in turn, targets mRNA through complementary base pairing.[20–22]

Small Nucleolar RNA (snoRNA)

Small nucleolar RNAs (snoRNAs) are 60–300 base-pair-long unique RNAs found only inside the nucleolus. There are two types of snoRNAs: (1) C/D box containing snoRNAs and (2) H/ACA box containing snoRNAs.[23] Acting as a guide, snoRNAs direct selective chemical modification of nucleotides on other small housekeeping RNAs such as rRNAs. C/D box containing snoRNAs regulate sequence-specific 2′-O-methylation while H/ACA box snoRNAs regulate posttranscriptional isomerization of a uridine to a pseudouridine in rRNA.[24]

Circular RNAs (circRNAs)

Circular RNAs (circRNAs) are a large class of ncRNAs that originate from pre-mRNAs by a non-canonical splicing event called back-splicing. Consequently, loss of the terminal structures of a 5′-cap and a 3′-polyadenylation (poly-A) tail makes the circRNAs a covalently closed continuous ring structure.[25] The unique configuration of circRNAs confers protection from exonuclease-mediated degradation and makes them remarkably stable molecules.[4] Based on the sequence of origin, the circRNAs are categorized into exonic circRNAs (EcircRNA), intronic circRNAs (ciRNAs), exon-intronic circRNAs (ElciRNAs), intergenic circRNAs, and fusion circRNAs (f-cir-cRNAs). The EcircRNAs are the most abundant circRNAs predominantly located in the cytoplasm. The EcircRNAs function as miRNA sponge, modulate gene expression, regulate cell development and proliferation, as well as interact with RNA-binding proteins (RBPs).[26] CiRNAs and ElciRNAs are predominantly present in the nucleus and regulate transcription and translation.[27,28]

Advances in genetic technologies and bioinformatics have shown that circRNAs may be generated from intergenic, intronic, coding regions, as well as untranslated regions of the DNA. The biosynthesis of circRNAs is explained by three proposed models based on splicing event orders: (a) lariat-driven circularization, also known as exon-skipping model; (b) intron interaction-driven circularization, also known as the direct back-splicing model; and (c) re-splicing-driven circularization.[4,25] The biogenesis of circRNAs is illustrated in Fig. 2.

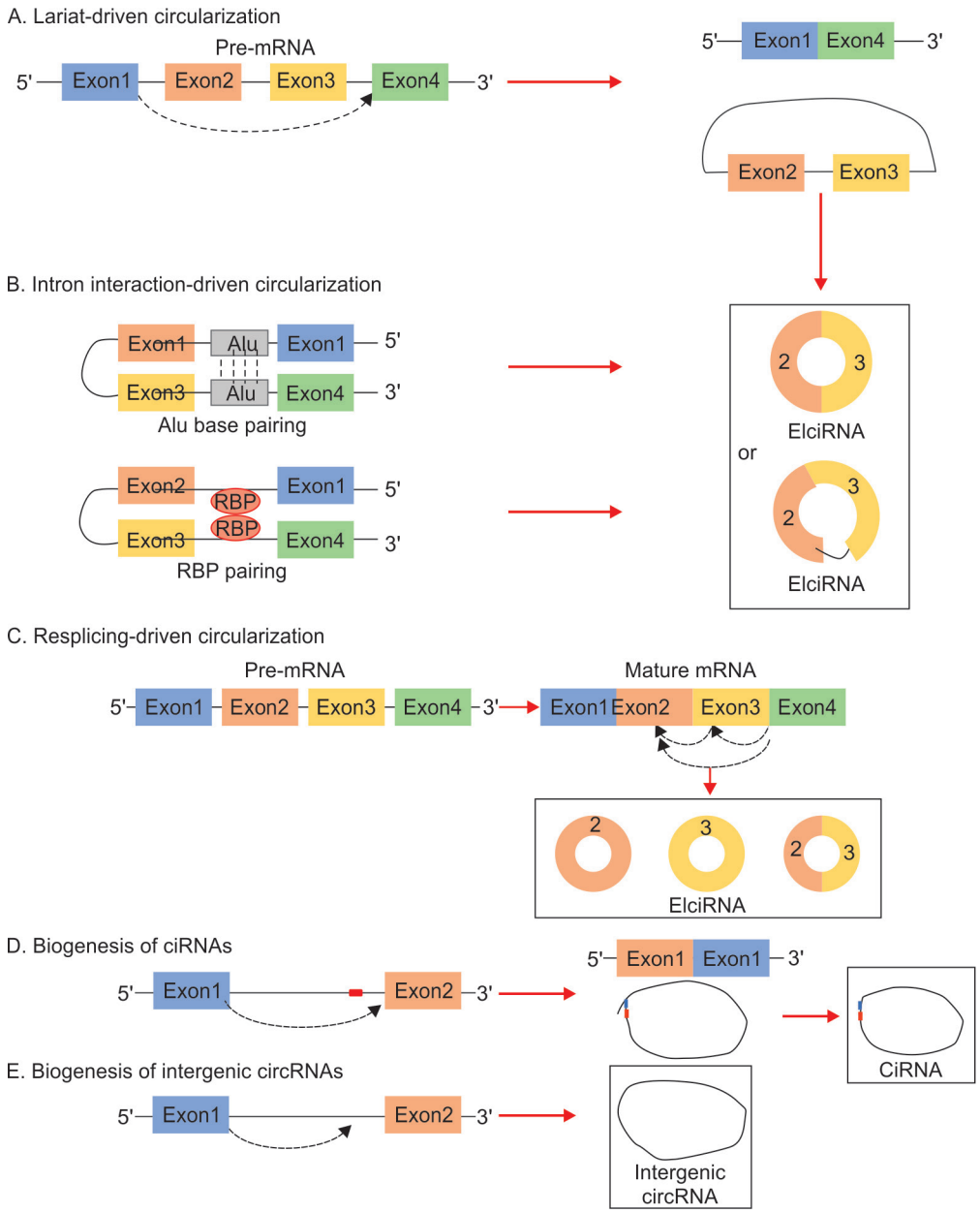
Long Non-coding RNAs (lncRNAs)

lncRNAs affect many cellular processes at transcriptional, posttranscriptional, and translational levels. Most lncRNAs are located within intergenic stretches and are usually comprised of two-exon transcripts.[29] These are interlaced, complex networks of overlapping sense and antisense transcripts that may also include protein-coding genes. Most ncRNAs, by definition, do not show protein-coding capacity, but some lncRNAs are now being identified to contain cryptic reading frames that may be translated into short, unstable micropeptides.[30] Some sequence elements in lncRNAs may show conserved structure, but these do not show conserved functions. In other regions, some lncRNAs that are derived from syntenic regions and presumably have shared evolution, no longer show any similarity in sequences.[31] These features suggest that many lncRNAs could possibly be non-functional or may have evolved from species-specific adaptive selection. The lncRNAs do seem to be important components of the address codes, which regulate directed trafficking, activation, and deactivation of protein complexes, genes, and chromosomes.[32]

Several types of lncRNAs have been identified. Based on proximity to the conventional protein-encoding mRNAs, lncRNAs can be classified as sense-, antisense-, or bidirectional lncRNAs.[33] Sense lncRNA regions may overlap one or more exons of another coding transcript. In other instances, antisense lncRNAs can extend into coding genes. LncRNAs have also been classified by the genomic location as intronic- or long intervening/intergenic-ncRNAs (lincRNAs). Intronic lncRNAs are encoded in non-coding DNA sequences.[34] The lincRNAs seem to be universal—these have been documented in plants, yeast, prokaryotes, and viruses, but the nucleotide sequences are not as well-conserved. Many lincRNAs are non-coding, autonomously transcribed long (>200 nucleotides) sequences that do not overlap with coding genes. Other classification group these into same-strand, isolated, convergent, or divergent categories, based on the location vis-à-vis the nearest protein-coding RNA.[35] In terms of function, lincRNAs may regulate cellular processes such as the p53-mediated transcriptional responses to DNA damage.[36]

ncRNA Associated with Prematurity that may Influence NEC

NEC is mainly a disease of premature neonates. Gestational age (GA) is inversely related to the incidence and severity of NEC. The intricate process of pregnancy maintenance and parturition necessitates a fine balance between many coordinated, consequential changes in hormones, tissue remodeling, metabolism, and immune system. Genetic factors, in conjugation with clinical and environmental variables, can alter the maternal-fetal interface and cause preterm birth. ncRNAs may play a regulatory role in gene expression controlling the process of pregnancy and birth. Since inflammation is a common theme for both, premature labor and NEC, various studies have evaluated miRNAs as well as lncRNAs that upregulate pro-inflammatory pathways and found that miRs-494, 142, 223, 15a, 329, 23a and lncRNAs-BF328678, BG258490, AA451649, BF667001, ENST00000423797, AX474492, BC107431, BX483760, DN918055,



Figs 2A to E: The biogenesis of circRNAs. (A) Lariat-driven circularization also known as exon skipping. Exon skipping during canonical splicing forms lariats containing the skipped exons as well as mRNAs. The exon-containing lariats undergo back-splicing yielding EcircRNAs (intronic sequence removed) or ElciRNAs (intronic sequence retained); (B) Intron interaction-driven circularization. Direct base pairing between cis-acting; splicing regulatory elements (Alu repeats) or trans-acting factors (RBPs) couples flanking introns, followed by back-splicing and exon circularization. (C) Resplicing-driven circularization. Exons on mature RNA can undergo back-splicing and produce EcircRNA; (D) Biogenesis of ciRNA. The GU-rich (near 5, splice site, blue box) and the C-rich (near 3, splice site, red box) sequences can escape the debranching and degradation and form ciRNAs; (E) Biogenesis of intergenic circRNAs

ENST00000437593 were associated with preterm labor (Luo 2013, Luo 2015).[37–42] Similarly, chorioamnionitis increases the risk of NEC in premature infants due to deleterious effect of chronic inflammation on fetal cell programming.[43] MiRNAs have been studied as modulators of chorioamnionitis, and consequently, its effects on fetal development and premature birth. Lee et al.[44] examined autopsy samples of fetuses exposed to chorioamnionitis and noted increased expression of miR-223-3p in fetal thymus (2.55-fold), lung (1.93-fold), and liver (1.7-fold). This is an important finding as thymus plays a critical role in T cell development and aberrant T-helper cell response may cause inflammation.[45,46] Montenegro et al.[47] evaluated miRNA expression with advancing gestation and with chorioamnionitis in 39 pregnant women. Compared to controls, pregnant women with preterm labor and chorioamnionitis had increased expression of miR-223 (37-fold) and miR-338 (24-fold). In another study, 48 Korean pregnant women with chorioamnionitis and preterm birth had decreased expression of miR-548, but increased HMGB1 and inflammatory cytokines.[48] These data

suggest a need for further study of ncRNAs in the pathogenesis of premature birth and neonatal morbidities.

Maternal preeclampsia is an important cause of preterm birth. Qian et al. noted increased expression of hsa_circRNA_100782, hsa_circRNA_102682, and hsa_circRNA_104820 in human placental tissues from mothers with preeclampsia.[49] Small for gestational age (SGA) neonates may be at increased risk of NEC.[50,51] Wang et al.[52] evaluated circRNAs in maternal and neonatal umbilical cord blood from SGA neonates and demonstrated that Hsa_circRNA15994-13, hsa_circ_0001359, and hsa_circ_0001360 were differentially expressed between SGA and AGA groups. The study also identified the target, hsa-miR-3619-5p, which plays an important role in the Wnt signaling pathway. These studies did not evaluate the neonatal outcomes, and future studies may be needed to examine neonatal outcomes.

ncRNAs Associated with Specific Histopathological Findings Seen in NEC

NEC is characterized by exaggerated inflammation, coagulative necrosis, and hemorrhagic necrosis.[1] In the following sections, ncRNAs that may be associated with the characteristic histopathological NEC features have been described.

ncRNAs Associated with Bowel Necrosis

The pathological process of NEC begins with intestinal epithelial cell (IEC) apoptosis, which results in mucosal defects and consequently, bacterial translocation from the gut lumen into the intestinal wall.[53–59] This triggers an overwhelming inflammatory response and mucosal necrosis, and can ultimately lead to NEC.[53,58,59] Apoptosis, programmed cell death, is immunologically a silent event, but NEC is characterized by exaggerated inflammation. To describe this unique pathoanatomical combination of NEC, a novel term, necroptosis, has been coined. Necroptosis may be caspase-independent in certain situations and can be triggered by death receptors such as transferrin-independent receptor-1 (NTFR1), interferon-production regulator (IFNR), Toll-like receptor (TLR) 3/4, Fas, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). The ligation of death receptors activates the necrosome, a complex of the receptor-interacting serine/threonine kinases 1-3 (RIPK1-3) that in turn phosphorylates the mixed-linkage kinase domain-like protein (MLKL) to promote necroptosis.[60,61]

Werts et al.[62] examined IEC necroptosis and noted TLR-induced activation of RIPK1, RIPK3, and MLKL, and the protective effect of human breast milk. Li et al.[63] identified miR-141-3p as one of the agents that could possibly protect RIPK1 by downregulating RIPK1-MLKL-mediated necroptosis pathway. Chen et al.[64] identified motor neuron and pancreas homeobox (MNX) 1, also known as HB9 or HLXB9, as another binding target of miR-141-3p, by showing that it suppresses the MNX1 gene. MiR-141-3p may also alleviate inflammation, apoptosis, and oxidative stress damage by regulating MNX1 expression. Wu et al.[65] studied miR-431 in Chinese infants with stage 3 NEC (10 infants with NEC and an equal number of matched controls, and noted higher expression of miR-431 in the NEC group leading to suppressed forkhead box A1 (FOXA1) expression, and a significant effect downstream of miR-431-FOXA1 axis with exaggerated inflammation (increased expression of TNF, IL-6, IL-8, IL-10, NFKB2, and PLA2G2A), apoptosis (increased LGR5, decreased estrogen-related receptor gamma-ESRRG expression), and dysregulated tight junctions (decreased hepatocyte nuclear factor (HNF) 4A and PRKCZ expression). In another study, Ng et al.[66] searched for novel NEC biomarkers. After studying 301 episodes (36 episodes of NEC, 265 episodes of non-NEC) in Chinese infants, they identified three potential early biomarkers, miR-1290, miR-1246, and miR-375. MiR-1290 was most accurate in the detection of NEC (sensitivity of 0.83, specificity of 0.92, PPV of 0.6, and NPV of 0.98 with a cutoff of >220 copies/μL). When they combined miR-1290 level of >650 copies/μL measured on day 0 and CRP level of >15.8 mg/L measured on day 1, they were able to correctly recognize 30/36 (83%) NEC cases. MiR-1290 has been studied in colorectal cancer and inflammatory bowel diseases (IBDs) and is noted to modulate inflammation, cell renewal, and apoptosis via FOXA1 pathway.[67,68]

ncRNAs Associated with Intestinal Inflammation in NEC

NEC is marked by an acute inflammatory response to microbial invasion. However, the determinants of the severity of inflammation are not fully understood.[69] The pattern-recognition receptors (PRRs) are known to differentially recognize pathogens from other antigens and modulate the consequent immune responses. TLRs are one class of pattern-recognition receptors; TLR4 recognizes Gram-negative bacterial cell wall components such a lipopolysaccharide that may be involved in the pathogenesis of NEC. The activated TLRs recruit the myeloid differentiation (MD) factor and trigger downstream signaling to activate the nuclear factor-κB (NF-κB) and its related inflammatory responses.[70] Therefore, the role of ncRNAs in the regulation of TLR-mediated pathways may be important in NEC pathogenesis.

The role of miR-124 on TLR-mediated inflammation and apoptosis via myosin phosphate target subunit 1 (MYPT1) and rho-associated coiled-coil-containing protein kinase 1 (ROCK1) was evaluated by Yin et al.[71] using neonatal rat models of NEC. The study reported that miR-124 may protect against NEC by suppressing MYPT1, ROCK1, and TLR-9. Xu et al.[72] evaluated the regulatory interactions of miRNAs and lncRNAs in NEC pathogenesis. They reported upregulation of the lncRNA MSTRG.42950 and MSTRG.104993 and downregulation of lncRNAs MSTRG.61378 and MSTRG.8198. There are recognizable binding patterns: lncRNA MSTRG.42950 with miR181a-5p; lncRNA MSTRG.104993 with miR-124-3p; and miR-194-5p with lncRNA. MSTRG.61378 may bind miR-362-3p, and lncRNA MSTRG.8198 binds miR-124-3p. These interactions likely modulate the TLR4 signaling pathway, TORC2 complex, notch signaling pathway, the p53 signaling pathway, and the mTOR pathway and, consequently, determine the severity of inflammation in NEC. More recently, Sun et al. studied the role of let-7d-5p/LGALS3/TLR4/NF-κB axis in the inflammatory cascades known to be active in NEC lesions. They noted decreased let-7d-5p and increased LGALS3 (galectin) in such lesions, possibly pointing to anti-inflammatory and protective roles of let-7d-5p.

TLR pathways also activate macrophages, and these cells, in turn, control gene expression and immune response modulation. Ng et al.[73,74] evaluated the regulatory role of mcircRasGEF1B in the TLR4/LPS pathway. They identified increased mcircRASGEF1B in macrophages after LPS-induced activation. Depletion of mcircRasGEF1B dysregulated the TLR4/LPS pathway and caused macrophage dysfunction. Together, these findings provide future directions for large clinical studies with infants of different genetic backgrounds.

The nucleotide-binding oligomerization domain-containing (NOD) 2 is another cytosolic PRR that binds bacterial peptidoglycans and promotes pro-inflammatory cytokine production, inflammation, and innate immune defenses.[75] MiRNAs also interact with the NOD2 pathway in adults with IBD. MiRNAs including miR-122, miR-192,

miR-495, miR-671, miR-320, and miR-10a influence NOD2 expression, modulating inflammation and injury in IECs.[76–81] Such studies are needed to evaluate the role of these miRNAs in NEC.

Mannose-binding lectin (MBL) is a circulating PRR that opsonizes pathogens and activates the lectin pathway of the complement system. It is an important regulator of inflammation, in a variety of conditions such as neonatal sepsis, pneumonia, NEC, and IBD.[82–85] Prencipe et al. studied 107 neonates with NEC and showed that an SNP in the MBL2 gene increased serum levels of MBL in severe NEC.[86] MiRNA regulation of MBL levels has been previously examined in hepatocellular carcinoma; miR-942-3p has been noted to bind MBL2.[87] Studies are needed to evaluate how miRNAs may modulate the MBL pathway in NEC.

Pro-inflammatory cytokines are upregulated in NEC. The pathophysiological role of cytokines is unresolved; we still are unsure whether many of these cytokines are the cause, or the effect of inflammation in various conditions. Chen et al.[64] showed that increasing the expression of miR-141-3p can reverse the overexpression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in intestinal injury models. In another study, Wu et al.[65] investigated miR-431 effects on TNF, IL-6, IL-8, and IL-10 and found it to increase IL-6 and TNF expression. Findings in these studies warrant future evaluation *in vivo* models and/or clinical studies in NEC.

ncRNAs Affecting Intestinal Microcirculation

Abnormalities in intestinal microcirculation due to maldevelopment or altered blood flow may contribute to NEC risk by causing intestinal ischemia and breach in mucosal integrity.[88] Vascular endothelial growth factor-A (VEGFA) plays a key role in intestinal vasculature development.[88,89] Association between decreased VEGF level and NEC has been established in both human as well as animal NEC models.[90] NcRNAs may regulate VEGFA genes affecting intestinal vasculature development and vasoreactivity.

The association between miRNAs modulating VEGF and NEC has been investigated. Liu et al.[91] reported downregulation of miR-429/200a/b and miR-141/200c clusters in four infants with NEC. The possible target genes for these two miRNA clusters, such as VEGFA, kinase insert domain receptor (KDR, also known as VEGFR2), FMS-related tyrosine kinase (FLT) 1, E-selectin (SELE), hepatocyte growth factor (HGF), were highly expressed in infants with NEC. Recently, these findings were confirmed by Zhao et al.[92] and also showed the interaction of miR-200c-3p and miR-22a-3p with KDR genes. The study identified three additional potential targets in apoptotic pathway, tyrosine 3-monooxygenase (TH)/tryptophan 5-monooxygenase activation protein gamma (YWHAG), YWHA protein epsilon (YWHAE), and YWHA protein beta (YWHAB).

Hypoxia is a main angiogenesis stimulus causing VEGF-mediated angiogenesis in endothelial cells. Fiedler et al.[93] identified two lncRNAs, LINC00323 and MIR503HG, in endothelial cells which are found to be highly sensitive to hypoxia and crucial for angiogenesis. Silencing of these two lncRNAs led to angiogenic defect, whereas endothelial cell treatment with VEGF increased their expression. Likewise, angiogenesis modulation by circRNAs has been explored in various pathological processes such as circ_100933, circ_100709, circ_104310 in infantile hemangioma;[94] circ_0004158, circ_0005768, circ_0008737, circ_0005324, circ_0007799, circ_0005477, circ_0000668, circ_0012698, circ_0013414 in retinopathy of prematurity;[95] circ_0005015, cZNF609, ZNF280c in diabetic retinopathy;[96] ZNF609, ZNF292, HIPK3, circ_0010729, circ_0003575, circ_0054633, antisense noncoding RNA in the INK4 locus (ANRIL), CPWWP2A, circ_0068087, circ_0008360, circ_0000109, circ_0002317 in cardiovascular diseases;[97] and SHKBP1, circ_002136 and SMARCA5 in tumorigenesis and metastasis.[98] Similar studies are needed in NEC examining the regulatory role of ncRNAs in intestinal angiogenesis.

ncRNAs Associated with Intestinal Hemorrhages in NEC

Clinical features of severe NEC commonly include coagulopathy and thrombocytopenia. There exists a knowledge gap explaining pathophysiology of coagulopathy and thrombocytopenia in NEC. The only available evidence is from a study by Giuliani et al.[99] who compared the expression of genes involved in coagulation in 11 infants with NEC with 22 controls and identified upregulation of hepatocyte growth factor (HGF), neutrophil-expressed elastase (ELANE), CD63, protein S (PROS1), and coagulation factor XII (F12) genes and downregulation of milk fat globule-EGF factor 8 (MFGE8), factor II (thrombin) receptor-like 1 (F2RL1), fibrinogen-like 2 (FGL2), plasminogen activator-tissue type (PLAT), protein C receptor (PROCR), serpin family D member 1 (SERPIND1), and hepatocyte nuclear factor-4a (HNF4A) genes. Out of these 12 genes, HNF4A is crucial for IEC maturation. Wu et al.[65] showed that overexpression of miR-432 inhibits in the Caco-2 cell model of NEC. However, the study did not highlight any effect on coagulation cascade and thrombocytopenia.

There is evidence to suggest the involvement of miRNAs in thrombocytopenia other neonatal inflammatory disorders. Cui et al.[100] identified a reduction in miR-130a expression in infants with sepsis who developed thrombocytopenia. MiR-130a targets IL-18 and/or IL-27 and was found to increase IL-18 expression without any change in IL-27 in the study. There has not been any study till date identifying specific ncRNAs associated with thrombocytopenia and coagulopathy in NEC providing an opportunity for future studies.

ncRNAs Associated with Gut Dysbiosis

Gut microbiome is a unique, complex interdependent ecosystem. With more than 3 million genes, gut microbiome can shape the gene expression in the host and determine health and diseases.[101] Dysbiosis is characterized by decreased diversity and overgrowth of pathogenic bacteria and has been linked to many inflammatory disorders, including NEC and IBD.[102] The microbiome development is a dynamic process that begins even before birth and undergoes dramatic changes during infancy due to vast contribution from various factors such as gestational age, mode of delivery, type of feeding, and antibiotic exposure.[103] Increasing information now associates genetics and the gut microbiome and *vice versa*.[104–106] Liang et al. studied conventional, germ-free, and gnotobiotic mice to characterize lncRNAs that are regulated by gut microbiota and identified six upregulated and overlapped lncRNAs, n26353, n290292, n297037, n294754, n264146, and n288632. Interestingly, most of them were highly expressed in spleen and thymus, suggesting the role of microbiome in immune modulation via lncRNAs. Dempsey et al.[107] demonstrated altered lncRNA expression in various organs such as the colon, liver, ileum, white fat tissue, jejunum, duodenum, and skeletal muscles. The mechanisms by which gut dysbiosis, lncRNA dysregulation, and intestinal inflammation may be linked need elucidation.

As mentioned earlier, miRNAs are the best-studied ncRNAs. These are more stable than other ncRNAs and are easier to measure in feces.[108–110] However, the fecal miRNA levels may be affected by the fecal microbiome.[108,111] The relationship between miRNA and gut dysbiosis has been studied in IBD and celiac disease. Mohan et al.

linked intestinal dysbiosis and altered claudin-1 expression/epithelial junctions with increased inflammation-related miRNAs, miR-203, miR-204, miR-23a, and miR-29b.[112] Studies in IBD have shown increased mir-144, mir-519, and mir-211, and downregulation of miR-577, miR-379-5p, miR-642-3p, and miR-26b-5p.[113,114] Similar studies are needed to examine the role of miRNAs in gut dysbiosis and NEC.

ncRNAs Associated with Protective Properties of Human Milk

Human breast milk, a biological "elixir," not only offers universally undisputed protection against NEC, but also reduces life-long health burden by preventing sudden infant death syndrome, bronchitis, lower respiratory tract infection, otitis media, atopy, and asthma.[115] Human breast milk contains a large spectrum of miRNAs, either as free molecules or carried in exosomes or extracellular vesicles (EVs), and is known to shape the gut microbiome.[116–120]

The influence of miRNAs in milk-derived exosomes on intestinal maturation and inflammation has been studied in the setting of IBD and NEC. The therapeutic effects of milk-derived exosomes have been studied in murine models of colitis and reported higher expression of miR-375, let-7z, miR-148, and miR-320 in milk as well as milk-derived exosome treated colon while lower expression of miR-125b in colitis. These miRNAs lower the expression of IL-1β, IL-3, IL-6, IL-12, IL-15, and TNF.[121–123] MiR-125b is known to regulate inflammation via NF-κB pathway that has a role in pathogenesis of NEC. MiR-148 also modulates immunity and has a role in metabolism and development. In intestinal cell culture models of NEC in rats and humans, milk-derived exosomes have shown a significant reduction in the incidence as well as the severity of NEC by anti-apoptotic, pro-proliferative, anti-inflammatory actions.[124,125] Future studies exploring the miRNA content of exosomes and comparing formula and breast milk content will shed some light on this innovative therapeutic option for NEC.

Many researchers have examined other ncRNAs in breast milk. Karlsson et al.[126] isolated 55 lncRNAs in EVs from human breast milk from 30 mothers within 2 months postpartum. These lncRNAs were present in more than 50% of the samples—CRNDE, DANCR, GAS5, HOTAIRM1, NCBP2-AS2, OIP5-AS1, PRKCQ-AS1, SNHG8, SRA1, TUG1, and ZFAS1. Later, Rubio et al.[127] first discovered the presence of more than 1,000 small RNAs in breast milk including piRNAs, tRNAs, snoRNAs, and snRNAs with tRNAs being the most abundant. Recently, studies have also examined lncRNAs and circRNAs in bovine as well as porcine milk-derived exosomes.[128,129] These data may be useful for future studies.

ncRNAs in IBDs in Adults

MiR-21 and miR-155 have been extensively studied in relation to IBD.[130–134] MiR-21, located on chromosome 17q23.2 in humans, regulates inflammation in the innate immune system. It directly targets the p35 subunit of Th1-promoting IL-12 and NOS in intestinal endothelial cells by modulating P13K/Akt signaling pathway encoding mRNA.[135] Increased miR-21 can alter the intestinal barrier and cause inflammation, oxidative stress, and cellular damage.[131,135] Similarly, miR-155, located on chromosome 21q21.3 in humans, induces IL-17 secreting helper T cells maturation process via IL-23/17/6 axis and has been implicated in the pathogenesis of IBD.[133,136–138] These findings are fascinating and provide future directions to confirm the role of miR-21 and miR-155 in neonates with NEC.

There is some information on the role of circRNAs and lncRNAs in IBD pathogenesis. Qiao et al.[139] profiled circRNAs and their targeted miRNAs, genes, and pathways in 13 patients with Crohn's disease (CD) and 13 controls; they found that hsa-circRNA-102685 may cause apoptosis via TLR and p53 signaling pathways via hsa-miR-146b-5p, hsa-miR-182-5p, and hsa-miR-146a-5p. Wang et al.[140] identified hsa_circRNA_0007919 disrupting mucosal integrity via miR-138 and hsa_let-7a after comparing differential expression of circRNAs between inflamed and non-inflamed intestinal mucosa from 30 patients with ulcerative colitis. Yin et al.[141] evaluated circRNAs in peripheral blood mononuclear cells obtained from IBD patients and discovered upregulation of hsa_circRNA_092520, hsa_circRNA_102610, hsa_circRNA_004662, and hsa_circRNA_103124, and correlation between circRNA_004662 and mTOR pathway via circRNA-miRNA-mRNA network prediction model. The mTOR plays a crucial role in the regulation of intestinal homeostasis and inflammation.[142] Autophagy-related 16-like 1 (ATG16L1), one of the autophagy-related genes (ATGs), is essential for maintaining immune homeostasis and may confer protection against NEC.[143,144] Genetic variation in ATG16L1 (Thr300Ala) increases risk of NEC, particularly in Caucasian infants.[145] Using animal model of IBD, Li et al.[146] showed that circRNA circPABPN1 blocked human antigen R (HuR) biding to atg16l1 mRNA and decreased ATG16L1 expression in the intestinal epithelium. Ye et al.[147] identified circRNA_103516 as a potential biomarker after showing upregulation of circRNA_103516 in 180 patients with IBD and associated downregulation of miR-19b-a-5p.

Similarly, lncRNAs can be viewed as novel potential biomarkers for diagnosis as well as promising therapeutic targets for intestinal inflammatory conditions such as IBD and NEC. LncRNAs such as lncRNA NEAT1 (nuclear paraspeckle assembly transcript 1), lncRNA H19, and lncRNA SPRy4-IT1 are essential for intestinal epithelial regeneration and repair, thus maintaining intestinal epithelial barrier function.[148] Studies have shown upregulation of lncRNA NEAT1 and lncRNA H19 in intestinal epithelium of IBD, whereas increased expression of lncRNA SPRy4-IT1 showed protective effect[149–151] by modulating barrier function. Similarly, several differentially expressed lncRNAs were potentially associated with intestinal mucosal immune homeostasis, function of pro-inflammatory cytokines, and MHC protein complex.[152] Specific inflammatory pathways affected by lncRNA dysregulation include those commonly identified in NEC pathogenesis such as NF-κB and TNF. Interleukin (IL)-1, IL-6, and IL-8 are often overexpressed in NEC and together with TNF; they stimulate NF-κB that leads to transcription of various inflammatory cytokines exacerbating the inflammation and tissue damage. Regulatory T lymphocytes (Tregs) are pivotal in keeping the excessive inflammation in check and maintenance of tolerance.[45] Qiao et al.[153] showed overexpression of lncRNA DQ786243 in 19 CD patients with active disease along with overexpression of cAMP response element binding protein (CREB) and forkhead box P3 (Foxp3), two key genes in function and development of Tregs, suggesting DQ786243, may be related to CD disease severity. Other lncRNA regulators of NF-κB have been implicated in the pathogenesis of IBD including lncRNA HIF1A-AS2, lncRNA ANRIL. Quan et al.[154] demonstrated inactivation of NF-κB/JNK pathway by lncRNA HIF1A-AS2 leading to decreased expression of cytokines IL-1β, IL-6, IL-12, and TNF-α in mice colon samples. Qiao et al.[155] demonstrated upregulation of lncRNA ANRIL in sigmoid colon mucosa obtained from 22 patients with UC and suggested that suppression of ANRIL may inhibit the development of UC by regulating miR-323-5p/TLR4/MyD88/ NF-κB pathway.

Conclusion

To expand our incomplete knowledge of complex NEC pathogenesis, we have reviewed the current literature on ncRNAs in NEC. The evidence remains imperfect due to scarcity of information on ncRNAs in NEC. Therefore, exploring the pathogenesis of other intestinal diseases such as IBD in adults and pediatric patients may provide a new direction to the future of NEC studies. Additionally, the complexity of NEC pathogenesis suggests that a single ncRNA may not explain NEC entirely. The evolution of high-throughput, in-depth next-generation sequencing techniques and bioinformatics may elucidate the interactions between different ncRNAs and their molecular mechanism in the pathogenesis of NEC.

Author Contribution

KD and JN wrote the manuscript. AM reviewed and made important revisions.

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REVIEW ARTICLE

Development and Functions of Mitochondria in Early Life

Jinghua Peng[1], Balamurugan Ramatchandirin[2], Alexia Pearah[3], Akhil Maheshwari[4], Ling He[5]

Abstract

Mitochondria are highly dynamic organelles of bacterial origin in eukaryotic cells. These play a central role in metabolism and adenosine triphosphate (ATP) synthesis and in the production and regulation of reactive oxygen species (ROS). In addition to the generation of energy, mitochondria perform numerous other functions to support key developmental events such as fertilization during reproduction, oocyte maturation, and the development of the embryo. During embryonic and neonatal development, mitochondria may have important effects on metabolic, energetic, and epigenetic regulation, which may have significant short- and long-term effects on embryonic and offspring health. Hence, the environment, epigenome, and early-life regulation are all linked by mitochondrial integrity, communication, and metabolism.

Keywords: Early life, Metabolism, Mitochondria, Mitochondrial dynamics, Neonatal development, Oocyte maturation.

Newborn (2022): 10.5005/jp-journals-11002-0013

Introduction

Mitochondria, the "powerhouses" to provide the required cellular energy, are seen to have a primary role in the oxidation of nutrients to build up proton gradients in the inner mitochondrial membrane and to make adenosine triphosphate (ATP) in eukaryotic cells.[1] Yet, the significance of mitochondria extends beyond the generation of ATP; these organelles also play central roles in the regulation of Ca^{2+} homeostasis and apoptosis, supply of intermediary metabolites, generation of heat, and the integration of various signaling pathways.[2–6] During oocyte maturation and the development of embryo prior to implantation, mitochondria go through dynamic restructuring and redistribution to support developmental processes.[7,8] After birth, many events that were previously assumed to be performed by maternal organs and placenta are actually carried out in neonatal organs such as the liver, heart, lung, and kidneys. A tremendous amount of energy is needed for these physiological processes, and hence, these are associated with a massive increase in the mitochondrial number and function.[9–12] This dynamic nature of mitochondria is essential for modulating key cellular events.

Origin of Mitochondria

The prokaryotic provenance of mitochondria is clearly distinct from the eukaryotic nuclear lineage.[13] Mitochondria were derived from a common ancestral organelle that originated from the integration of an endosymbiotic alpha-proteobacterium into a host cell related to the archaea superphylum Asgard or the Asgardarchaetoa that is capable of metabolizing oxygen.[14] It is still unclear whether it was the acquisition of mitochondria that triggered evolutionary transition of prokaryotes to eukaryotes, or if the prokaryotes had already evolved to a eukaryote-like stage (fledged eukaryotes) when mitochondrion joined in; the endosymbiotic origin of mitochondria is an important question in the study of eukaryogenesis.[14–16] The mitochondrial deoxyribonucleic acid (DNA) (mtDNA) encodes 2 ribosomal ribonucleic acids (rRNAs), 22 transfer ribonucleic acids (tRNAs), and 13 proteins that are involved in the activity of the mitochondrial respiratory chain.[17] However, there are 1,500 estimated different mitochondrial proteins,[18] and >99% of the mitochondrial proteins are likely encoded in the nucleus, synthesized in the cytosol, and imported into the mitochondria.[19]

[1]Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China

[2,3,5]Department of Pediatrics and Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America

[4]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Ling He, Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States of America, Phone: +410 502 5765, e-mail: heling@jhmi.edu

How to cite this article: Peng J, Ramatchandirin B, Pearah A, *et al*. Development and Functions of Mitochondria in Early Life. Newborn 2022;1(1):131–141.

Source of support: Nil

Conflict of interest: None

Cellular Functions of Mitochondria

Mitochondria are double-membrane-bound subcellular compartments. These are the "powerhouses" that provide eukaryotes with energy in the form of ATP. For glucose metabolism, after the split of glucose into two pyruvates in the cytosol, pyruvate enters the mitochondria and is oxidized to acetyl coenzyme A (CoA), NADH+H, and CO_2 by pyruvate dehydrogenase complex, and acetyl CoA is further oxidized to generate NADH+H, $FADH_2$, GTP, and CO_2 in the citrate acid cycle.

For the oxidation of amino acids, once the amino acids have broken down, the metabolites enter the citrate acid cycle as acetyl CoA, α-ketoglutarate (α-KG), succinyl-CoA, fumarate, and oxaloacetate. For the metabolism of fatty acids, the fatty acid is degraded to acetyl CoA with the latter entering the citrate acid cycle and generates NADH+H, $FADH_2$, GTP, and CO_2. NADH+H and $FADH_2$ will be used to generate ATP through oxidative phosphorylation in the mitochondrial respiratory chain. Under normal conditions, over 90% of ATP is made in the mitochondria.[20] The mitochondria are

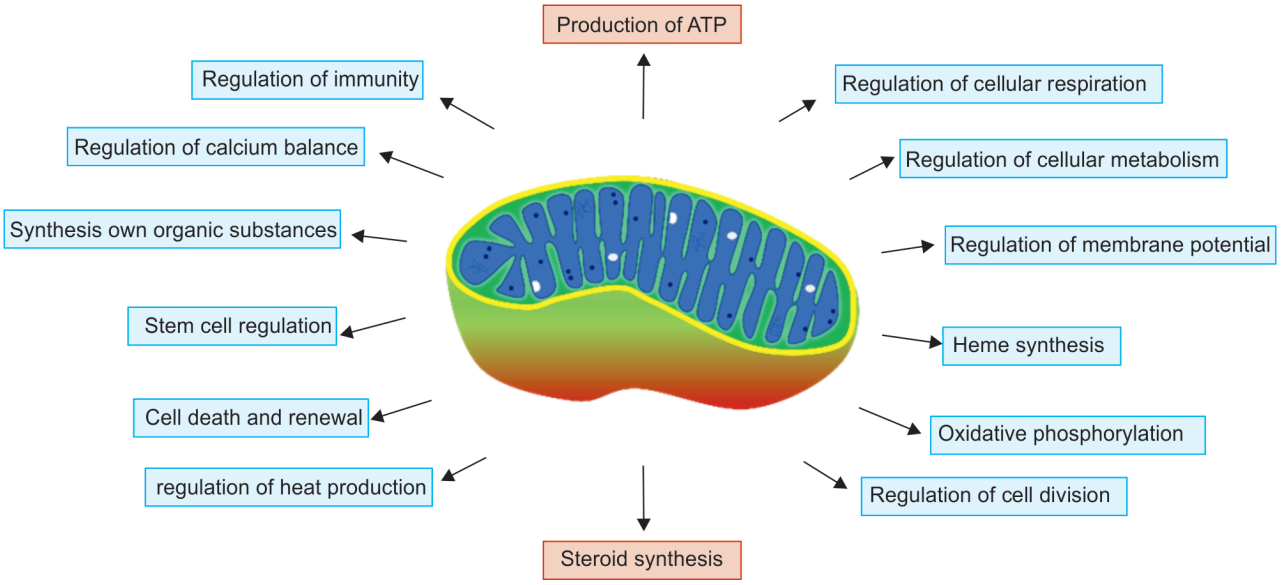

Fig. 1: Functions of mitochondria

also the subcellular compartments that regulate the biosynthesis of amino acids, lipids, and gluconeogenesis (Fig. 1).[14]

Mitochondrial Development and Functions in Embryogenesis

Mitochondria are the most abundant organelle in the oocyte and undergo significant structural and positional changes during preimplantation development.[21] These are received exclusively from the mother in uniparental inheritance; the parental mtDNA is eliminated.[22] The maternal mitochondria provide the required energy for oocyte viability until embryonic mitochondria assume their function.[23]

Mitochondria are oval or elongated in oogonia with a sparse, but even intracellular distribution. The growing oocytes show relatively dense rounded or oval mitochondria that contain a rough endoplasmic reticulum (ER). In fully grown germinal vesicle oocytes, mitochondria have a dense matrix and a few arch-like or transverse cristae and are usually not seen in the cortical part of the cytoplasm. In metaphase I and II of oocytes, mitochondria have a structure similar to that of germinal vesicle oocytes with an even distribution in the cytoplasm and aggregation around the smooth ER. At the pronuclear stage, the mitochondria are observed in a central conglomeration around the pronuclei, which persists up to syngamy. In the 8-cell cleaving embryo, the morula, and the blastocyst, mitochondria are less electron-dense and show clear areas in the matrices. In the expanding blastocysts, trophoblast, embryoblast, and endodermal cells, mitochondria look elongated with the inner mitochondrial membranes arranged into transverse cristae.[7]

Growing oocytes preferentially utilize pyruvate to make ATP via oxidative phosphorylation (OXPHOS),[24] and early embryos also use pyruvate, lactate, and amino acids to support development.[25,26] The highest mtDNA copy number and mass are found in the mature oocyte of any cell. High numbers of mitochondria in oocytes are essential for early embryonic development by providing the capacity for nutrient oxidation.[27] Fertilized oocytes have a higher mtDNA copy number than the unfertilized ones,[28] and a low mitochondrial number is correlated to fertilization failure and abnormal developments of the embryo.[22,29,30] The importance of mitochondria in embryonic development is evident in decreased fertility seen in mice with induced mtDNA mutations.[31] Inhibition of mitochondrial metabolic activity blocks maturation of oocyte and the subsequent embryonic development[32,33] as well as the growth of the fetus and the placenta in animals.[34] In humans, embryo development and implantation rates are closely correlated to ATP levels,[33] and inhibiting mitochondrial activity prevents human embryonic stem cell (ESC) differentiation.[35]

Mitochondria take up Ca^{++} under physiological conditions in a variety of cell types and are involved in Ca^{++} homeostasis.[36] Several cellular events including fertilization are regulated by the intracellular concentration of free calcium. In mammalian fertilized eggs, the Ca^{++} concentrations vary in oscillatory patterns that seem to be necessary for oocyte activation[37] and embryo development.[38] Importantly, mitochondrial ATP is needed to maintain these Ca^{++} oscillations.[39]

Development and Functions of Mitochondria in Neonates

After birth, the many functions previously assumed by maternal organs and placenta must be promptly carried out by the newborn's organs. A tremendous amount of energy is needed during the neonatal period to cope up with various energy-demanding physiological processes, and their cells show considerably increased mitochondrial number and function. To adapt to an environment with high oxygen content, the number and functions of mitochondria in the lung continue to increase during postnatal growth and development. To fulfill the function of gas exchange, postpartum mitochondria in type II alveolar epithelial cells (AECII) undergo significant morphological changes from a single and spherical shape to a complex and branched structure.[40] There are few mitochondria in the nonciliated cells of prenatal animals, but with a significant shift in mitochondrial abundance during differentiation.[41]

For a successful adaption to extrauterine life, the differentiation and proliferation of mitochondria within the neonatal liver is a key

regulatory process because the mitochondrial number and activity in hepatocytes of the fetal liver are very low.[42] The proliferation or differentiation of preexisting mitochondria to functioning mitochondria occurs rapidly after birth as well as a marked increase in mitochondrial activity concomitantly with increased ATP levels in the liver within an hour after delivery.[11] Furthermore, the expression of genes related to the mitochondrial respiratory chain activity is significantly increased in the neonatal liver.[42,43] After delivery, the neonatal kidneys must take charge of the functions of glomerular filtration, glucose reabsorption, and acid/base homeostasis previously assumed by the maternal kidneys. In the developing kidneys, mitochondrial respiration and oxygen consumption significantly increase between 21 days post coitum and 1 day postpartum, accompanied by an increase in enzymatic activity in the citrate acid cycle, fatty acid oxidation, and the levels of ATP.[12]

Importance of Mitochondria in Embryonic/Neonatal Development through the Regulation of Methylation and Acetylation of the Genome

Extensive reprogramming of the epigenetic landscape occurs to activate the embryonic genome in preimplantation embryo development. Paternal genome is also activated after fertilization through DNA demethylation.[44] Mitochondrial activity has been linked to methylation via involvement in methionine metabolism.[45] Depletion of mtDNA leads to alteration in the metabolism of amino acids including methionine, leading to increased DNA methylation.[45] *S*-adenosylmethionine (SAM) acts as a cofactor in the methylation reaction and SAM is produced from methionine by methionine adenosyl transferases (MATs).[46] In the murine embryos, knockdown of Mat2α results in 2-cell embryo arrest and reduced transcriptional activity. Furthermore, being inhibited of Mat2α or cultured in the absence of L-methionine, embryos were arrested at the morula stage and H3K4me3 levels in morula and blastocyst were much lower than those cultured under normal medium.[47] SAM supplementation promotes hatching of bovine embryo, accompanied by significant alterations in DNA methylation.[48] Consistently, in human embryonic stem cells, methionine deprivation leads to a decrease in H3K4me3 and global DNA methylation, which can be reversed by supplementation with SAM.[49]

Preimplantation embryo development requires appropriate histone demethylation mediated by the *jumonji* or the Jarid2 (JMJ) deamylase, which removes the methyl group from lysine residues.[50] JMJ demethylases catalyze the histone demethylation in an α-KG-dependent manner.[51] Thus, mitochondria regulate demethylation via α-KG through the oxidation of glucose and glutamine in the mitochondrial citric acid cycle.[52]

Chromatin remodeling also plays an essential role in embryonic epigenetic programming.[53] Histone acetylation by histone acetyltransferases (HATs) relaxes the condensed chromatin and promotes the gene transcriptions. On the contrary, deacetylation of histone condenses the chromatin and suppresses the gene transcription. Histone acetylation by HATs requires acetyl CoA, which is the product of oxidative decarboxylation of pyruvate produced by glycolysis, β-oxidation of fatty acids, and amino acid metabolism, and then shuttled out of mitochondria in the form of citrate, acetyl CoA precursor.[54] In human ESCs, increasing acetylation levels by the supplementation of precursor of acetyl CoA leads to reduced differentiation, while inhibition of acetyl CoA production from glucose results in the loss of pluripotency.[55] The availability of nicotinamide adenine dinucleotide (NAD^+) controls the activity of the conserved NAD^+-dependent histone deacetylases, the sirtuins (SIRTs).[56] SIRTs are involved in blastocyst development as the inhibition of SIRT activity leads to a significant reduction in blastocyst development.[57] NAD^+ can be synthesized *de novo* from the amino acid tryptophan or through the NAD^+ salvage pathway from nicotinamide. However, cytoplasmic NAD+ levels are very low, to maintain the NAD+ levels; NADH+H-reducing equivalents need to shuttle into mitochondria through either malate-aspartate or mitochondrial glycerol 3-phosphate dehydrogenase. This is important because blocking NADH+H into mitochondria by inhibition of malate-aspartate activity reduced blastocyst development and placental and fetal growth.[58] The significance of dynamic changes of histone acetylation and deacetylation in embryonic and neonatal development deserves further study.

Mitochondrial Dysfunction Affects Embryogenesis and Neonatal Development

Mitochondrial Dysfunction in Oocytes is Largely Responsible for Age-related Decline in Fertility

The role of mitochondria in reproduction has received increasing attention because of their importance in oocyte maturation, fertilization, and early embryo development. Less ATP and mtDNA copy number, and ultrastructural mitochondrial abnormalities are observed in mice aging oocytes.[59] Mutations of mtDNA accumulate in maternal oocytes with age.[60] In women of advanced reproductive age, oocytes have increased mtDNA deletions and mutations, which probably result in impaired mitochondrial function and subsequently lead to embryo development failure.[61]

Mitochondrial Dysfunction Causes Severe Clinical Symptoms in Neonates

Neonates require an adequate capacity of the mitochondrial energetic metabolism to support rapid growth and adaption to extrauterine life; therefore, ATP provision from the mitochondrial oxidative phosphorylation is essential. Neonates' muscles, heart, and brain are mainly dependent on aerobic metabolism that depends on mitochondrial function. Disorders of mitochondrial metabolism caused by defects in fatty acid oxidation, pyruvate metabolism, and the respiratory chain, including mitochondrial complexes I, II, III, and IV, and ATP synthase, may often present in the neonatal period. Mutations of both nuclear genes and mtDNA can cause mitochondrial dysfunction in neonates; primary and secondary mitochondrial dysfunctions are quite common in neonates.[62–64] The prognosis for newborns with mitochondrial dysfunction is often unfavorable (Table 1).[63]

Mitochondrial dysfunction affects 1 in 6,000–8,000 newborns, making mitochondrial disease almost as common as childhood cancer. Each year, about 1,000–4,000 children in the United States are born with a mitochondrial disease. Autosomal recessive inheritance, autosomal dominant inheritance, mitochondrial inheritance, and random mutations lead to mitochondrial diseases in neonates.[65] Furthermore, mitochondrial dysfunction is closely linked with obesity, diabetes, liver dysfunction, and coronary vascular disease.[66] Recent studies demonstrate that in neonates, there are 32 out of 107 patients diagnosed with mitochondrial diseases, 7 out of 73 patients are diagnosed with neonatal lactic acidosis,[64] and 11 out of

Table 1: Mitochondrial gene known to be mutated in neonatal disorder

Mutation	*Neonatal mitochondrial disorder*	*References*
Mitochondrial respiratory chain complex		
Complex I	Leigh syndrome	138, 139
	Lethal infantile mitochondrial disease	139
	Lactic acidosis	139
	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome	140
	Leber hereditary optic neuropathy	141
Complex II	Mitochondrial leukoencephalopathy	142
	Cardiomyopathy	143
	Infantile leukodystrophy	144
	Kearns–Sayre syndrome	75
Complex III	Lactic acidosis, hypoglycemia, ketosis, hyperammonemia	145, 146
	Cardiomyopathy, multisystemic dysfunction	147
	Encephalopathy	148
	Growth retardation, aminoaciduria, cholestasis, iron overload, lactacidosis, and early death (GRACILE) syndrome	146
Complex IV	Steatosis	149
	Encephalopathy, myopathy	150
	Hypertrophic cardiomyopathy, hepatomegaly, liver dysfunction and hypotonia, delayed motor development, and mental retardation	151
Complex V	Severe neonatal encephalopathy, neonatal respiratory distress, lactic acidosis, severe peripheral neuropathy, dysmorphism, cataract, arterial pulmonary hypertension, bilateral cataract, and reye-like syndrome	75, 152–156
Combined Class I, II, and III	Steatosis Fibrosis/cirrhosis	149
Fatty acid biosynthesis		
Carnitine palmitoyltransferase I (CPT I)	Hypoketotic hypoglycemia, hyperammonemia, elevated transaminases, and mild metabolic acidosis	157
Carnitine-acylcarnitine translocase (CACT)	Hypoglycemia, seizures, cardiomyopathy, cardiac arrhythmia, and apnea	158
Carnitine palmitoyltransferase II (CPT II)	Nonketotic hypoglycemia, hepatomegaly, encephalopathy, seizures, respiratory distress, and metabolic acidosis. Cardiomyopathy and arrhythmia	159
Very long-chain acyl-coenzyme A dehydrogenase (VLCAD)	Hypertrophic cardiomyopathy and fasting hypoketotic hypoglycemia	160–162
Short-chain acyl-coenzyme A dehydrogenase (SCAD)	Hypotonia, muscle weakness, and seizure	163
Long-chain 3-ketothiolase (LCKAT)	Lactic acidosis, pulmonary edema, and cardiomyopathy	164
Amino acid metabolism		
Phenylalanine hydroxylase	Phenylketonuria	165
Cystathionine synthase	Homocystinuria/homocystinuria	
Branched-chain ketoacid dehydrogenase	Maple syrup urine disease	
Solute carrier family 6 member 19 (SLC6A19)	Hartnup disease	
Pyruvate metabolism		
Pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1)	Lactic acidosis, hypotonia, seizure	166
Pyruvate carboxylase	Hypercitrullinemia and hyperlysinemia	167
Krebs cycle metabolism		
Dihydrolipoyl dehydrogenase	Severe persistent lactic acidosis, respiratory difficulties, seizures, dystonic movements, hypoglycemia, lethargy, hypotonia, vomiting, constipation, failure to thrive, and feeding difficulties	168
α-KG dehydrogenase	Choreoathetosis, opisthotonos, spasticity, hypertrophic cardiomyopathy, hepatomegaly, and sudden death	169
Fumarase	Lethargy, microcephaly, hypotonia, axial dystonia or opisthotonos, areflexia, or psychomotor retardation	170

75 patients have lethal infantile mitochondrial diseases.[67] In clinic, mutations of mitochondrial complex I lead to Leigh syndrome; lactic acidosis; and renal, cardiac, and hepatic disorders in newborns.[68,69] The mutations of succinate dehydrogenase complex flavoprotein subunit A (SDHA), succinate dehydrogenase complex iron sulfur subunit B (SDHB), and succinate dehydrogenase complex assembly factor 1 (SDHAF1) genes in complex II can cause mitochondrial defects that are associated with neonatal cardiomyopathy and infantile leukodystrophy.[70–72] Mutations in complex III has been reported in one neonate who has severe lactic acidosis associated with hypotonia, irritability, and muscle wasting. Complex III deficiency is mainly caused by mutations in homolog of the S. cerevisiae bcs1 protein, ubiquinol-cytochrome c reductase complex chaperone (BCS1L), ubiquinol-cytochrome C reductase binding protein (UQCRB), ubiquinol-cytochrome C reductase complex III subunit VII (UQCRQ), and mitochondrially encoded cytochrome B (MTCYB) genes, which is passed down maternally. Mutations in complex IV cause neonatal hypertrophic cardiomyopathy, hepatomegaly, liver dysfunction and hypotonia, delayed motor development, mental retardation, encephalopathy, and myopathy.[73] The biogenesis and assembly of cytochrome c oxidase (COX) in complex IV depend on numerous ancillary factors, including copper chaperones, all nuclear encoded. Specifically, disease-causing mutations were found in genes encoding surfeit locus protein 1 (SURF1), essential for the formation of early assembly intermediates; mutation in complex IV is associated with severe neonatal encephalopathy, neonatal respiratory distress, lactic acidosis, severe peripheral neuropathy, dysmorphism, cataract, and arterial pulmonary hypertension.[74] The defects in ATP synthase can cause fatal neonatal mitochondrial encephalopathy.[75]

Pyruvate dehydrogenase complex (PDHc) catalyzes oxidative decarboxylation of pyruvate to produce acetyl CoA and initiates the tricarboxylic acid (TCA) cycle. PDHc deficiency is most often due to mutations in the first component of the enzyme complex, pyruvate dehydrogenase E1α (responsible for 70% of the PDHc deficiencies). There is a spectrum of clinical presentations in E1α mutations. In the most severe form of PDHc mutations, lactic acidosis develops within hours of birth. This is often associated with an altered level of consciousness, profound hypotonia, lethargy, feeding and respiratory difficulties, and coma.[76]

Mitochondrial Transfer from One Cell to Another

Mitochondria and mtDNA can be transferred between cells. It was reported that transient focal cerebral ischemia induced mitochondria release from astrocytes and enter into the adjacent neurons mediated by a calcium-dependent mechanism involving CD38 and cyclic adenosine diphosphate (ADP) ribose signaling.[77] In this way, the survival signal was amplified in cells. In cancer models, mtDNA of host cells in the tumor microenvironment can be horizontally transferred to tumor cells that have a defective respiratory function, leading to the re-establishment of respiration and tumor growth.[78] Mechanisms of horizontal transfer of mitochondria that have been discovered include forming tunneling nanotubes between cancer cells and cells in the tumor microenvironment,[79] packaging mtDNA into extracellular vesicles (EVs)[80] and connexin 43 gap junctions.[81]

A recent study demonstrated that cells from obese mothers may carry fewer mitochondria, which relates to higher levels of triglycerides, free fatty acids, and more lipids. The fewer numbers of mitochondria alter the placental lipid metabolism and transfer of the lipids to the fetus, causing lipid-related diseases such as newborn adiposity.[82] Likewise, reduced mitochondrial function has been found to induce brain injury in newborns. The alteration of cellular oxygen dependency by reduced mitochondrial function that decreased oxygen delivery into the brain causes brain injury and severe encephalopathy.[83] Metabolic shift of fetal/neonatal is important for the function of cardiomyocytes. This metabolic shift is controlled by the mitochondria biogenic surge that involves cardiomyocyte maturation in neonates. Mutation mtDNA and mitochondrial dysfunction leads to dilated cardiomyopathy via mitochondrial transcription factor A (TFAM).[84,85]

Negative Impacts of Inflammation on Mitochondrial Function

Inflammasomes Promote mtDNA Release

Pathogen-associated molecular patterns and damage-associated molecular patterns stimulate the formation of inflammasomes, which bind to apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) to form a platform for caspase-1 activation and ultimately process pro-interleukin (IL)-1beta and pro-IL-18 to mature IL-1beta and IL-18. Caspase-1 activated by inflammasomes trigger mitochondrial damage including dissipation of mitochondrial membrane potential, mitochondrial permeabilization, and fragmentation of the mitochondrial network. Simultaneously, Caspase-1 inhibits mitophagy leading to the accumulation of defective mitochondria partially mediated by Parkin.[86]

Neutrophils are the first line of defense when external microorganisms attack the human body. It was reported that stimulation with IL-8, or lipopolysaccharide, leads to neutrophils releasing granule proteins and chromatin to form neutrophil extracellular traps (NETs).[87] NETs degrade virulence factors and killing bacteria, in which mtDNA is identified.[88] In systemic lupus erythematosus (SLE), mtDNA in NETs is demonstrated as the interferogenic DNA. Oxidized mtDNA retention in neutrophils and autoantibodies against oxidized mtDNA are observed in SLE patients.[89] In healthy people, neutrophils remove damaged mitochondria by extruding the mitochondrial components including mtDNA devoid of oxidized residues. Once being oxidized, mtDNA is degraded in lysosomes, which requires PKA phosphorylation of the TFAM. In SLE, neutrophils have reduced PKA activation that blocks TFAM phosphorylation.[90] As a result, oxidized mtDNA is accumulated and extruded as interferogenic complexes.

Inflammation Affects Mitochondrial OXPHOS

Mitochondria are the site of OXPHOS in eukaryotes. During OXPHOS, NADH, provided by the TCA cycle, is oxidized and provides electrons to the electron transport chain (ETC) , which consists of complexes I–IV, and ATP synthase. Decreased expression of the mitochondrial respiratory complexes I–V genes was found in patients of Alzheimer's disease that is characterized by progressive neuronal loss and neuroinflammation.[91] In acute inflammation, tumor necrosis factor (TNF) reduces the activity of complexes I, III, and IV in hepatocytes by triggering intracellular signaling cascade, which partially accounts for a shift of energy production from aerobic metabolism to glycolysis.[92] TNF, a proinflammatory cytokine, is elevated in the blood, cerebrospinal fluid (CSF), and striatum region of the brain in patients with Parkinson's disease. TNF upregulates the expression of miRNA targeting mitochondrial complex I, decreasing ATP levels, and increasing ROS production and induces dopaminergic cells death in Parkinson's disease.[93] TNF is also

reported to inhibit OXPHOS via peroxisome proliferator-activated receptor (PPAR)-γ co-activator 1α (PGC-1α), a transcriptional cofactor regulating transcription of ETC genes.[94,95]

Inflammation Affects Mitochondrial Dynamics

Mitochondria are dynamic organelles whose structure, localization, and balance between biogenesis and degradation are under tight control referred to as mitochondrial dynamics. It includes fission and fusion as well as mitochondrial trafficking and mitophagy.[96] In brain slices, proinflammatory stimuli promote mitochondrial fragmentation in astrocytes by triggering phosphorylation of the pro-fission protein dynamin-related protein-1 (Drp-1) and ultimately result in reduced respiratory capacity.[97] TNFα induces mitochondrial fragmentation accompanied with increased mitochondrial fission protein fission-1 and decreased mitochondrial fusion protein optic atrophy protein 1 (Opa1) in adipocytes.[98,99] In a model of dopaminergic neuron degeneration akin to Parkinson's disease, long term and low dose of TNFα exposure induce mitophagy by visualization of the colocalization of the autophagosome marker microtubule-associated protein 1A/1B-light chain 3 (LC3) with mitochondria.[93]

Inflammation Affects Mitochondrial Cell Death Pathways

The proinflammatory cytokine TNFα initiates necroptosis by tumor necrosis factor receptor 1 (TNFR1) signaling under caspase-8-deficient conditions. Receptor-interacting serine/threonine-protein kinase (RIPK)-1 and its downstream RIPK-3 are necessary for necrosome formation. It has been demonstrated that the mitochondrial proteins, PGAM family member 5 (PGAM5), and Drp-1 are downstream of RIPK-3 in the necroptosis pathway.[100] RIPK-3 phosphorylates mixed lineage kinase domain-like (MLKL) and long form of PGAM family member 5 (PGAM5) to activate Drp-1 leading to mitochondrial fission and necroptosis. In the cortical lesions of human samples of multiple sclerosis, TNFα activates necroptosis that is indicated by defective caspase-8 activation, as well as activation of RIPK1, RIPK3, and MLKL.[101]

A consequence of OXPHOS is the production of ROS, which acts as signaling molecules to modulate numerous processes at physiological levels.[102] ROS regulate the activity of phosphatases, which oppose the activity of protein kinases. It has been demonstrated that phosphatases including protein tyrosine phosphatase 1B,[103,104] phosphatase and tensin homolog (PTEN, lipid phosphatase), and mitogen-activated protein kinase ((MAPK)[105–107] are inhibited by ROS under physiological conditions. Mitochondrial ROS regulate TNF-mediated cell death.[108] TNF-induced signaling activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[109] and pro-apoptotic signaling via c-Jun N-terminal kinase (JNK).[110] ROS promote TNF-induced death and sustained JNK activation by inhibiting MAPK phosphatases.[107] The mitochondrial antioxidant protein superoxide dismutase 2 regulates NF-κB-mediated expression of anti-apoptotic genes.[107]

ROS are necessary signals for self-renewal and cellular differentiation of stem cells. Quiescent stem cells have lower levels of ROS, and mitochondrial ROS promote epidermal differentiation and hair follicle development.[111–113] High expression of pluripotent genes including Nanog, octamer-binding transcription factor 4 (OCT4), and Sox2 reflects the self-renewing ability.[112] In mouse ESCs, knockdown of the mtDNA polymerase DNA polymerase gamma (POLG)[114] or the mitochondrial protein growth factor erv1-like[115] decreases the expression of pluripotent markers including the OCT4, Nanog Homeobox (NANOG), and the putative thiosulfate sulfurtransferase (SSEA). In physiological conditions, mitochondrial H_2O_2 stabilizes the hypoxia-inducible factor proteins,[116] which is indispensable for self-renewal and the pluripotency of mouse and human ESCs.[117]

Mitochondrial Abnormalities and Mitochondrial Diseases in Neonates

Mitochondrial diseases can be described as heterogeneous, progressive, and multisystemic and these characteristics are thought to be due to the early neonatal period when high energy is suddenly demanded from the newborn's organs that can cause the development of significant illnesses such as Leigh syndrome, Alpers syndrome, anemia, seizures (epilepsy), stroke, heart failure, diabetes, and enteropathy.[118,119] Mitochondrial dysfunction in newborns is the result of the mtDNA and nuclear DNA (nDNA).[120] Under dual genetic control, there are approximately 80 subunits of the respiratory chain with 13 of these subunits being encoded by the mtDNA and the remaining 67 subunits are thought to be encoded by the nDNA. There are two groups of mutations classified as originating from the mtDNA that is inherited maternally or from the nDNA.[121] A recent study in pediatrics stated about 36% of cases reveals that the neonatal period produces onset of oxidative phosphorylation disorders.[122,123] In infants and children, other than OXPHOS disorders as affected by mitochondrial dysfunction, mutations and defects in protein importation, mitochondrial dynamics, and mtDNA expression and regulation have been found.[124] However, mutations in the mtDNA have been found to not be primarily responsible for neonatal disease symptoms but rather that mtDNA mutations are consistent with early spontaneous abortions and/or neonatal deaths as revealed by anecdotal experience.[125]

The most common mitochondrial disease in neonates as explained by various larger cohort studies is Leigh syndrome that contains an ATPase6 gene mutation in complex V, m.8993 T >G. Leigh syndrome is the result of at least 26 distinct mtDNA mutations and it is within the first 2 years of life that the child acquires this disease, however, this can present much sooner with most pediatric patients showing symptoms within the first month of life as well as clinical phenotypes such as dystonia, abnormal eye movements, and respiratory abnormalities.[126,127] Another syndrome occurring with neonatal onset is Pearson's syndrome that is characterized by refractory sideroblastic anemia along with vacuolization of bone marrow precursor cells in addition to pancreatic dysfunction. The exocrine pancreas, liver, and kidney also experience dysfunction in this syndrome oftentimes leading to premature death; however, the pediatric patients that do survive acquire Kearns-Sayre syndrome (KSS) in late childhood. Both of these disorders explained (Leigh and Pearson) undergo a large deletion in their mtDNA.[128] Additionally, reversible COX-deficient infantile myopathy is a third syndrome that presents within the neonatal period affecting newborns with hypotonia and severe muscle weakness beginning as early as the life of the first few days to the first few weeks. The underlying mutations of this disease are found in m.14674 T>C of tRNAGlu and often require mechanical ventilation as well as containing severe lactic acidosis. The muscle biopsy material demonstrates absent muscle complex IV or COX activity but patients with these results experience spontaneous improvement between 5 and

20 months of age with a complete reversal to normal COX activity in addition to being phenotypically healthy without any other severe symptoms.[129,130]

These are not the only significant reports of diseases with neonatal onset but rather there are other cases displaying a homoplasmic mutation of m.1624C >T in the $tRNA^{Val}$ gene in a family with six children who fatally died with diagnosed severe lactic acidosis, whereas the seventh had Leigh syndrome.[125] Two of the children in this family with the homoplasmic mutation, m.3303C >T in the $tRNA^{Leu}$ gene died of infantile cardiomyopathy.[131] To further examine infantile and pediatric mitochondrial diseases, a recent study of 262 patients was conducted revealing that approximately 17% of diseases were a result of mtDNA mutations. The majority of mtDNA diseases addressed in this study were "classical" mitochondrial syndromes, including mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), leber hereditary optic neuropathy (LHON), KSS, and myoclonic epilepsy with ragged red fibers (MERRF).[132] Mutations causing MELAS such as the ND1 and ND5 gene mutations are derived from mtDNA-encoded subunits within complex I.[133] Additionally, protein-encoding gene mutations occurring in complex IV and COXIII have been found to induce MELAS.[134] Historically, the first mtDNA point mutation described to give rise to a mitochondrial disease was the m.11778 G >A mutation inducing LHON with more than 95% of patients with LHON containing one of the three following mutations: m.11778 G >A, m.3460 G >A, or m.144484 T >C.[135] One of the progressive myoclonic epilepsies is MERRF and is classified clinically by a set of four constant features: myoclonus, generalized epilepsy, ataxia, and ragged red fibers within muscle tissues. Greater than 80% of patients with MERRF have the mutation of the gene encoding tRNALys at m.8344A >G.[136] Another severe disease that occurs in premature infants is necrotizing enterocolitis (NEC) known as one of the most common gastrointestinal emergencies during the neonatal period due to mitochondrial dysfunction. NEC occurrence is significantly high among infants with very low birth weight (VLBW) with approximately 14% weighing less than 1000 g.[137]

Perspective

This dynamic nature of mitochondria is essential for providing energy and modulating key cellular events. Besides providing energy, mitochondria serve as the other essential cellular functions to support key developmental events of the reproductive process, fertilization, oocyte maturation, and preimplantation embryo development. The highest mtDNA copy number and mass are found in the mature oocyte of any cell. High numbers of mitochondria in oocytes are essential for early embryonic development by providing the capacity for nutrient oxidation.[27] Inhibition on mitochondrial metabolic activity blocks maturation of oocyte and the subsequent embryo development[32,33] as well as fetal and placental growth in animals.[34] After birth, the many functions previously assumed by maternal organs and placenta must be promptly carried by the newborn's organs. A tremendous amount of energy is needed for the newborn to cope with increased energy-demanding physiological processes; thus, the neonates experience a considerable increase in mitochondrial number and function. Neonates' muscles, heart, and brain are mainly dependent on aerobic metabolism that depends on mitochondria function. Disorders of mitochondrial metabolism caused by defects in fatty acid oxidation, pyruvate metabolism, and the respiratory chain, including mitochondrial complexes I, II, III, and IV and ATP synthase, may often present in the neonatal period. Mutations of both nuclear genes and mtDNA can cause mitochondrial dysfunction in neonates since primary and secondary mitochondrial dysfunctions are quite common in neonates.[62–64]

With promising expectations, mitochondrial research is expanding constantly. Finding out more about mitochondrial functions and the underlying mechanisms in the embryonic/neonatal stage in mammals will give us insight on how to develop novel clinical interventions to address mitochondrial dysfunction during this stage. There are still many unknown factors, such as the long-term effects of developmental conditions on mitochondrial function in the early stage, how these observed effects affect health and disease susceptibility during the life cycle, long-term effects of involved molecular mechanisms, and identification of biomarkers that provide information about the mitochondrial function across tissues. A framework for future research is provided by these questions and issues. Additionally, studies using newborn cells offer great promise in terms of understanding mitochondrial function using *ex vivo* experimental challenge paradigms.

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REVIEW ARTICLE

Rotavirus Infection in Neonates and Young Infants

Preeti Shakya[1], Biplov Adhikari[2], Amit S Nepal[3], Pragyik Pandey[4], Akhil Maheshwari[5]

Abstract

Rotavirus is the primary cause of acute, frequently severe gastroenteritis among growing premature neonates, young infants, and children under the age of five globally. It contains a double-stranded ribonucleic acid genome is a member of the Reoviridae family. In this review, we have discussed the structure and characteristics of the virus, the pathogenesis of rotaviral diarrhea, clinical features, methods of diagnosis, clinical management, and available vaccines. This article combines peer-reviewed evidence from our own clinical studies with results of an extensive literature search in the databases PubMed, EMBASE, and Scopus.

Keywords: Diarrhea, Double-stranded ribonucleic acid, Gastroenteritis, Nonenveloped virus, Reovirus, Vaccine, World Health Organization.

Newborn (2022): 10.5005/jp-journals-11002-0014

Introduction

Rotaviruses are one of the leading causes of life-threatening acute gastroenteritis among infants and young children worldwide. It is associated with substantial morbidity and mortality, mostly in developing countries. World Health Organization estimates that about 453,000 children aged under 5 years die each year from rotavirus infections worldwide.[1] The infections begin occurring during the neonatal period, and the number increases significantly beyond 3 months of age.[2] This article reviews the rotavirus disease burden, its pathogenesis, clinical presentation, management, complication, and the latest developments in its treatment including vaccination.

Virology

Rotaviruses are members of the rotavirus genus of the Reoviridae family. Rotaviruses are large particles (1000 Å) comprised of a viral genome surrounded by three concentric protein layers. In electronic micrographs, rotaviruses appear "wheel-shaped" with a central axle composed of a few dots and concentric linear shadows in the periphery.[3] The genome is composed of 11 segments of double-stranded ribonucleic acid (dsRNA) that encodes six viral proteins (VPs) and six nonstructural proteins (NSPs). Two of the VPs, VP1, and VP3 have an enzymatic role, whereas VP2, VP4, VP6, and VP7 contribute to the structure (Fig. 1). Each genome segment codes for one protein with the exception of segment 11, which codes for two proteins. The role of these proteins is summarized in Table 1.

Rotavirus species are classified on the basis of antigenic differences of VP6 and genetic sequence. Ten different species (A-J) have been identified.[4] Serogroups A, B, and C are most commonly implicated in humans. Group A rotaviruses are the single most common serogroup of human rotaviruses.[5]

Rotaviruses exhibit unusual structural and replication properties that allow them to establish clinically significant disease. The capsid, comprising three layers, is extremely stable and ensures feco-oral transmission as well as delivery of viral particles to the intestine. The capsid contains 60 spikes that project from its surface which are the initial viral attachment proteins—these complex molecules bind to the receptors on the enterocytes. These spikes undergo conformational change following proteolytic cleavage by trypsin. The virus, which does not get fully uncoated, gets its outer capsid removed to deliver the dsRNAs that then produce capped messenger RNAs (mRNAs) which are then translated into proteins and new genomic RNA. The molecular mechanisms involved in rotaviral disease are shown in Figure 2.[3]

The rotavirus NSPs are produced in infected cells and help in viral replication. These NSPs act on the host cells and play a major role in the pathogenesis of rotaviral disease. The VP7, a glycoprotein (G-type antigen), forms the outer capsid shell, and the VP4, which forms spikes that protrude through the protein capsid shell, form the basis for the classification system (binary) of viral serotypes. The VP7 protein is an important neutralization target (Table 1). Based on genotype sequencing and neutralization assays, severe serotypes of VP7 have been recognized and classified as the G serotypes (named as the G1, G2, G3, and so on). On the other hand, VP4 serotypes are not so consistent in neutralization assays and genotyping sequencing. These proteins have been classified using a dual system, including a (a) P subtyping, where P genotypes are denoted using numerals in brackets (such as P[8], P[4], and so forth); and (b) P serotypes, which are classified in a numerical order (such as P1, P2, and so on). Currently, 32 G and 48[P] subtypes are known.[6] Fortunately, both sets of proteins are recognized by the host immune system; these induce antibody responses and therefore can be used to augment the host's defense responses.[3] Both are protease-sensitive proteins (P-type antigen) and can be targeted.[3]

[1]Janaki Medical College, Tribhuvan University, Janakpur, Nepal

[2]MedStar Union Memorial Hospital, Baltimore, Maryland, United States of America

[3]Institute of Medicine, Tribhuvan University, Kathmandu, Nepal

[4]Lumbini Medical College, Kathmandu University, Tansen, Nepal

[5]Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Preeti Shakya, Janaki Medical College, Tribhuvan University, Janakpur, Nepal, Phone: +977 9849669131, e-mail: drshakyapreeti@gmail.com

How to cite this article: Shakya P, Adhikari B, Nepal AS, *et al.* Rotavirus Infection in Neonates and Young Infants. Newborn 2022;1(1):142–150.

Sources of support: Nil

Conflict of interest: None

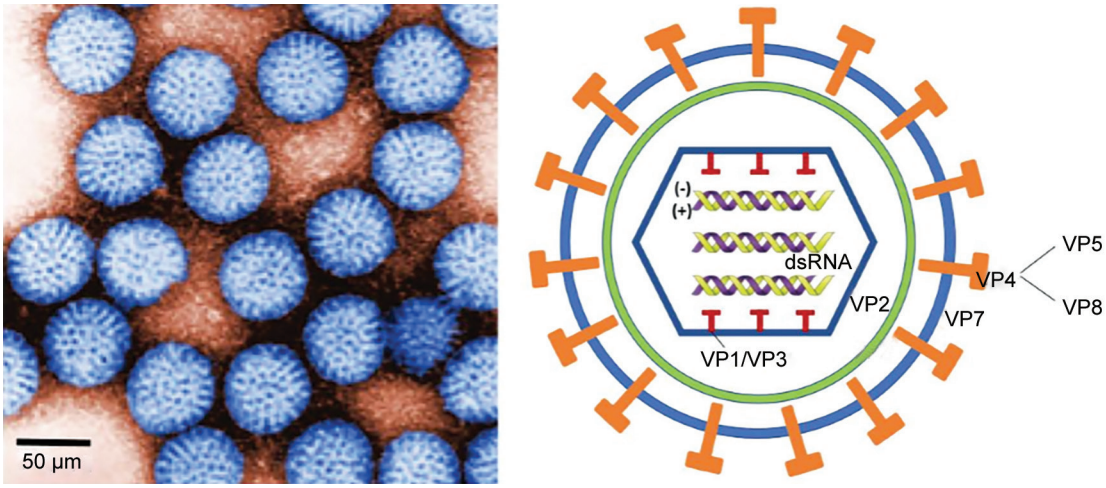


Fig. 1: Rotavirus. (A) Electron microscopy shows rotaviruses as spherical particles with short spikes; (B) Schematic diagram of the virus particle. The virion core is composed of dsRNA and the VP1/VP3 that have an enzymatic role, and these are enclosed in the inner capsid composed of VP2. The core is covered by a middle capsid layer of VP6, and an outer capsid of VP7. The outer layer has spikes composed of VP4, which can be proteolyzed into VP5 and VP8. VP6 in the middle capsid layer determines species, group, and subgroup specificities. VP4 and VP7 in the outer capsid are antigenic and elicit immune responses with specific antibodies

Table 1: Rotavirus viral proteins (VPs) and non-structural proteins (NSPs)

VP1	Polymerase
VP2	Inner capsid (protein shell)
VP3	Capping enzyme
VP4	Neutralization target; breaks into VP5 and VP8
VP6	Middle shell
VP7	Neutralization target
NSP1	Interferon antagonist
NSP2	Octamers regulate viroplasm formation and, possibly, function in reassortment restriction between rotavirus groups
NSP3	Promotes systemic spread; promotes viral mRNA translation by directing circularization of viral polysomes. In addition, may inhibit translation of and relocalization of host polyadenylated mRNAs from cytoplasm to nucleus
NSP4	Enterotoxin; mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-trisphosphate production
NSP5 and NSP6	Viroplasm

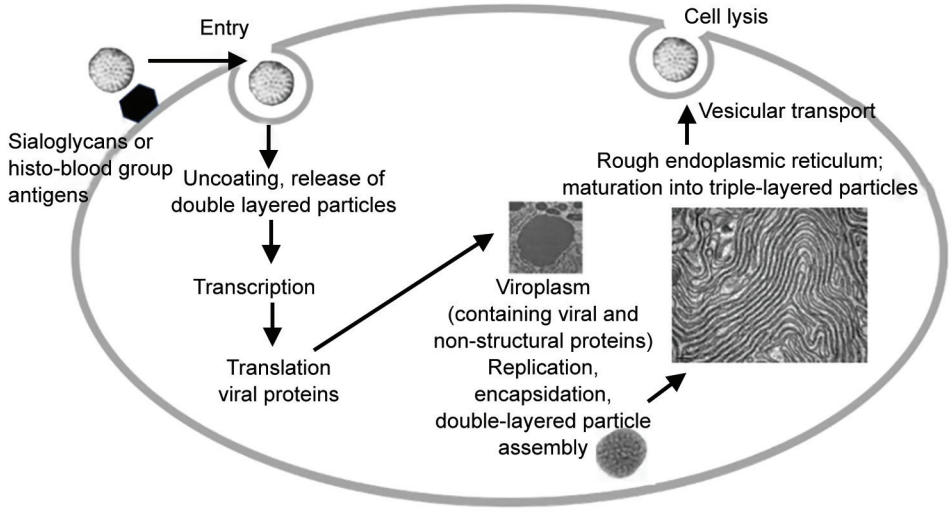


Fig. 2: Intracellular replication of rotaviruses

Epidemiology

Occurrence

Human rotaviruses were discovered 52 years ago, as an important cause of diarrhea in infants and children across the world. These viruses are one of the most common cause of diarrhea in children younger than 5 years of age, particularly in children between the age of 6 months and 2 years.[7] The incidence of primary rotavirus infection may vary between low- and high-income countries; in developing countries, the rotavirus infections occur frequently in infants, and 80% of all children get infected during the first year after birth. Some studies show the median age of acquisition of rotavirus infections to be between 6 and 9 months, whereas others show the infections to start much earlier. In the developed world, the infections may start later during infancy and may affect young children at 2–5 years of age.[8]

Rotavirus infections have been documented in neonates, particularly in the developing world. Newborn nurseries in many parts of the world have reported outbreaks of rotavirus diarrhea.[9] These endemics can last for extended periods; an outbreak lasted for over 2 years in Sweden.[10] Newborn infants can develop rotavirus diarrhea soon after birth and can begin shedding the virus in feces starting as early as the first week following birth (both above). Studies have found that 3.5–15% neonates in hospital nurseries excrete rotavirus in the feces, and this prevalence can reach up to 50% during outbreaks. Up to 28% of neonates excreting rotavirus particles exhibited diarrhea and other clinical symptoms.[11–13]

During the early 1980s, rotaviruses were implicated in 870,000 deaths per year in children below the age of 5 years.[3] Nearly one in five children may visit a clinic for rotavirus diarrhea by the age of 5 years. One in 50 children may be hospitalized, and 1 in 205 may die.[3,14] In 2013, 215,000 children below 5 years of age succumbed to rotavirus gastroenteritis.[15] It has been estimated that each year rotavirus is responsible for 114 million diarrheal episodes, 24 million hospital visits and 2.4 million hospitalizations.[16]

Rotavirus infections are more severe and rampant in low-and-middle income countries. More than 85% of these deaths occurring in low-income countries of Africa and Asia factors like malnutrition, frequent concurrent infections, overcrowding, and poor living conditions including lack of clean water, proper sanitation and unhygienic practices perpetuate and make children living in such reasons more vulnerable to the disease.[7] Almost half of all rotavirus deaths were estimated to occur in Nigeria, India, and Democratic Republic of Congo (DRC); almost three-fourth (73%) of all global mortality occurred in 10 countries.[17]

Reservoir

The gastrointestinal tract and stool of infected humans act as the reservoir of rotaviruses. Even though rotavirus infection occurs in many mammals other than humans, transmission from animals to humans is found to be uncommon. Human infections have not been reported from animal strains. A true carrier state has not been identified but immunocompromised people may shed the virus for a prolonged period.[18]

Transmission and Communicability

Rotavirus is highly contagious and is considered to be a universal infection. Nearly all children aged 3–5 years were exposed to the infection prior to the pre-vaccine era. The virus is shed in high concentrations in the stool of infected infants beginning 2 days before the onset of diarrhea and for several days after the onset of symptoms. In immunodeficient patients, rotavirus may be detected in stool for more than 30 days after infection.

At room temperature, the viral particles can survive for months in stool samples. Transmission is primarily by the fecal–oral route and can occur via both person-to-person contact or by fomites. It is common for the virus to spread within families, institutions, hospitals, and child care settings.[18]

Seasonal Variation

In temperate climates, rotaviral epidemics usually occur during the fall and winter. The seasonal variations are less distinct in tropical climates although the transmission may be more common during the relatively drier and cooler months. A biennial pattern of disease activity has been noticed with less notable differences in timing by geographic region post vaccine introduction.[18]

Pathophysiology

Rotavirus diarrhea has different mechanisms, including malabsorption secondary to enterocyte destruction, virus-encoded toxin, stimulation of enteric nervous system (ENS), and villus ischemia.[19] Rotavirus accesses the intestines through the mouth and replicates in mature, nondividing enterocytes in the middle and the tip (upper two-third) of villi[5] and enteroendocrine cells in the small intestine.[4]

Rotavirus attaches to host cells by outer capsid protein VP4 via its VP8 domain, binding partners on the host cell surface, include sialoglycans like gangliosides GM1 and GD1a and HBGAs (histoblood group antigens).[4] The virus then starts replicating in the upper small intestine, leading to the destruction of absorptive enterocytes and thus decreases surface area leading to malabsorption. Rotavirus stimulates intestinal secretion via the NSP4 and activation of the enteric nervous system.[4] Osmotically active infectious particles are also released into the intestinal lumen. These osmotically active infectious particles impair water reabsorption in the large intestine, thus causing watery diarrhea.

Along with enterocyte destruction, absorption of sodium, water, and mucosal disaccharides are decreased but mucosal cyclic AMP is unaltered.[19] Malabsorption results in the transit of undigested mono- and disaccharides, carbohydrates, fats, and protein into the colon, further perpetuating osmotic diarrhea.[19] Studies also show epithelial damage caused by villus ischemia can cause diarrhea.[20] The pathogenesis of rotaviral diarrhea is still an area for intense study (Flowchart 1).

Animal studies have shown no visible lesions or slight lesions like enterocyte vacuolization/loss, or larger changes like villus blunting and crypt hyperplasia, suggesting that there is no absolute correlation between histological lesions and disease symptoms.[20]

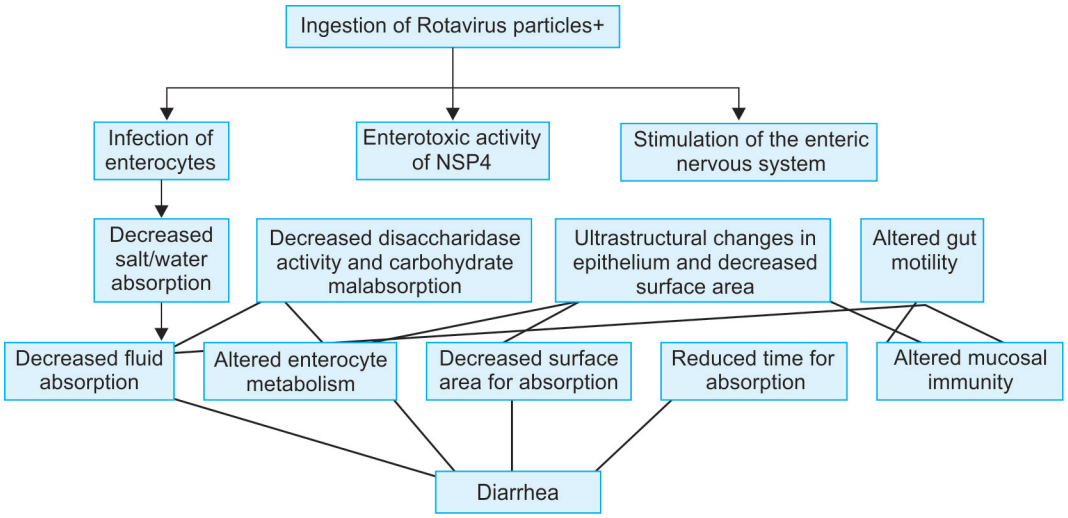
Clinical Features

The incubation period of rotavirus is usually short and varies between 1 and 3 days.[21] Infections may be asymptomatic—43–78% of rotavirus infections in neonates may be asymptomatic.[2] Although clinically silent, studies show that natural infections in neonates may confer protection against severe disease in the future.[22]

The most common presenting features are fever, diarrhea, and vomiting. Vomiting is usually the first to occur, followed by mild watery diarrhea of short duration to severe diarrhea with vomiting and fever. Fever is found in approximately 33% of infected patients.[23]

In one study done in Korea, the clinical symptoms among premature and full-term babies were found to vary. Premature

Flowchart 1: Pathogenesis of rotaviral diarrhea



neonates manifested lethargy, feeding difficulties, and abdominal distension, whereas those born at full-term babies were more likely to have fever, diarrhea, and vomiting.[24]

The clinical manifestations of rotaviral infections vary and depend on whether it is the first infection or reinfection. The symptoms are more severe in patients if the first infection is after 3 months of age. Younger infants usually present with relatively less intense symptoms. In few cases, infants have presented with necrotizing enterocolitis.[21]

The symptoms of rotavirus infection are usually identical with other acute gastrointestinal infections, but these tend to be more severe in presentation. In addition, it is also difficult to differentiate rotavirus infection from other pathogens causing gastrointestinal infections with only the physical examination. Some common physical examination findings include fever, and signs of dehydration such as dry mucous membranes, decreased skin turgor, tachycardia, diminished urine output, and prolonged capillary refill.[25] Most cases take 5–7 days for recovery.

Effect of Age on Clinical Severity of Rotaviral Disease

Age is an important determinant of disease severity. There is some variability in the severity of rotaviral disease in young infants. With low antibody levels, premature neonates can develop severe disease during the first month and during early infancy. Many young infants born at full term can also develop severe symptoms, but clear predictive factors are not clearly known.[26] Many full-term infants can tolerate the infection with minimal symptoms because of protective levels of the antibodies received from their mother during the third trimester. However, these infants could become reservoirs of disease. Neonates can also become symptomatic with infections with unusual viral strains that are not frequently seen in the community. During follow-up, many full-term infants can again become susceptible to rotaviral disease at the age of 6 months to 2 years, when immunity from passively-received maternal antibodies begins to wane.

Studies conducted in temperate as well as tropical regions show formation of anti-rotaviral antibodies in children only by the age of 3 years.[27] In infants who recover from rotaviral infections, develop IgA, IgG, and neutralizing antibodies that may protect them against subsequent infections.[28] Various studies have shown a significant correlation between the formation of neutralizing antibodies, both in the bloodstream (IgA, IgG) and in stool (IgA), and the protection from severe disease.

Investigation

In most cases, rotavirus infections manifest with a mild fever accompanied by vomiting and watery diarrhea. Fortunately, intestinal hemorrhage occurs in a smaller subset. The detection of acid-reducing substances in the stool, and reduced serum bicarbonate levels are also more likely to be found in rotavirus induced gastroenteritis.[4] Currently, lab testing offers the only way to confirm the diagnosis, and can assist in designing appropriate treatment and shortening the hospital stay.[29]

A confirmatory lab test can help in appropriate diagnosis and treatment of severe, intractable infections.[30] Specific diagnosis of rotavirus infection is made by the identification of virus in the stool samples using enzyme-linked immunosorbent assay (ELISA) or immunochromatography. ELISA offers 70–80% sensitivity and 71–100% specificity.[31,32]

Reverse transcription polymerase chain reaction (RT-PCR) assays are sensitive and allow genotyping of virus isolates. These tests may also facilitate epidemiological studies.[4] Additional methods of detection include electron microscopy, polyacrylamide gel electrophoresis, antigen detection assays, nucleic acid hybridization, sequence analysis, and virus isolation.[30]

Many patients, particularly those born premature, show a mild increase in blood levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), suggesting that they may have rotavirus-induced mild hepatitis.[4] These infants with severe disease may need additional evaluation with abdominal x-ray, blood gas and electrolytes, blood urea nitrogen, serum creatinine levels, and complete blood counts.

Differential Diagnosis

The differential diagnosis for rotavirus infection can be broad and include a variety of viral, bacterial, and parasitic causes including acute abdominal pathologies.[33]

Viruses that mimic acute gastroenteritis similar to that of rotavirus include norovirus, adenovirus, and astroviruses. As more than 5% of stool cultures test positive for nonviral etiologies, viruses are sought to be the most common cause of acute infectious diarrhea.

The most common bacterial causes of gastroenteritis are *Shigella*, *Salmonella*, *Campylobacter*, *Escherichia coli*, *Yersinia*, *Vibrio*, *Listeria*, and *Clostridium difficile*. Infants with bacterial infections need careful observation because of the risk of sepsis.[34]

Infants in tropical countries also need to be evaluated for infection with parasites such as *Giardia*, *Cryptosporidium*, *Cyclospora*, *Isospora*, and *Mycobacterium* species. Abdominal pathologies and noninfectious extraintestinal causes may also need to be considered in any case of acute diarrhea.[34]

Treatment

No specific treatment is yet available for rotavirus infections. Although the disease is self-limiting in many cases, it can cause considerable morbidity in infants, particularly those born premature or are in the age range of 3–24 months. At present, the treatment of rotavirus infection is mainly supportive.

In tropical, remote areas, careful observation for dehydration and oral rehydration therapy is indicated in infants with mild or moderate dehydration. The World Health Organization (WHO)-recommended formula for oral rehydration solutions (ORS) consists of an isotonic salt solution supplemented by glucose (Table 2).[35] Since 2005, a reduced-osmolarity ORS preparation has shown therapeutic effectiveness (Table 1).[35]

In low-resource settings, alternative methods for maintaining hydration have been tried. One example is the resolution prepared by mixing 50 g of rice powder in 1 L of water and boiling the solution (rice water therapy). However, such therapies are frequently difficult to administer and may not be as effective as ORS formulations.[37,38]

Patients with severe dehydration or in cases where oral rehydration is not possible, need intravenous fluids which can consist of Ringer's lactate solution, normal saline, or a similar solution.[39,40] In some situations where intravenous administration of fluids is not available, there may be a need for alternative, relatively extreme solutions such as subcutaneous hypodermoclysis.[41,42] The indications for hospitalization are summarized in Table 3.

The severity of diarrhea has been linked with the nutritional status of the patient. There is a need for adequate caloric intake for recovery, which can be difficult in infants with severe diarrhea.[43] Severe zinc deficiency is also a risk factor for adverse outcomes in developing countries,[44] and although not specific, there may be some additive benefit with zinc supplementation.[45–47] In low-income countries, prophylactic vitamin A supplementation may also reduce rotavirus-related mortality in children >6 months of age.[48–50] Vitamin A could possibly strengthen the innate immune defense system and gut integrity.[50]

Immunocompromised children may need rotavirus specific immunoglobulin preparation. In a study by Guarino et al., children receiving a single dose of 300 mg/kg of orally administered human immunoglobulin showed statistically significant clinical improvement compared to placebo arm. Rotaviral diarrhea in children who received immunoglobulin lasted for 76 hours; the diarrhea lasted an average 131 hours in the group receiving placebo.[51]

Multiple antiviral drug therapies have been studied for the treatment of rotavirus diarrhea. Nitazoxanide, an antiviral agent that interferes with viral morphogenesis, has been found to reduce the total duration of hospitalization and diarrhea among children. Other potential drugs against rotavirus diarrhea are racecadotril, clioquinol, and smectite. These therapies have not been extensively studied in neonates and combinations of more than one drug have not been tried. Most standard drug regimens for viral diarrhea do not include drugs. The safety of these agents in young infants still needs to be proven. Antiemetic agents like metoclopramide, ondansetron, and dimenhydrinate are "possibly recommended" in rotavirus diarrhea—they can reduce the need for hospitalization and intravenous fluid therapy.[54]

Prevention

Administration of the rotavirus vaccine is the most effective way to prevent rotavirus gastroenteritis in children. The use of rotavirus vaccines should be part of a comprehensive strategy to control rotavirus infections with preventative (promotion of early and exclusive breastfeeding for 6 months, vitamin A and zinc supplementation, hand-washing, improved water supply, and sanitation) and treatment measures.[55]

Breastfeeding has been found to have an equivocal relationship with the prevention of diarrhea. Some studies have found that exclusive breastfeeding among infants can prevent rotavirus diarrhea which may be attributed to rotavirus IgA antibodies and trypsin inhibitors in breast milk.[56,57] Studies have also suggested that breastfeeding can augment the effects of the rotavirus vaccine. But a study conducted by Misra et al. in a country with a heavy disease burden did not find any significant impact of breast feeding on prevention of rotavirus infection.[58] A meta-analysis conducted to assess the correlation between breastfeeding and

Table 3: Indications for hospitalization of children include[52,53]

- Shock
- Severe volume depletion
- Moderate volume depletion with refusal of oral fluids
- Clinical deterioration
- Intractable or bilious vomiting
- Failure of oral rehydration
- Neurologic abnormalities (e.g., lethargy, seizures)
- Possibility of severe illness or condition other than acute gastroenteritis that requires specific therapy (e.g., bowel obstruction)

Table 2: ORS solutions developed by WHO[36]

ORS solution	*Glucose (mmol/L)*	*Na (mmol/L)*	*K (mol/L)*	*Cl (mmol/L)*	*Base (mmol/L)*	*Osmolarity (mosm/L)*
WHO 2005	75	75	20	65	10	245
WHO 2002	75	75	20	65	30	245
WHO 1975	111	90	20	80	30	311

rotavirus infection, too, did not find the role of breastfeeding to be effective in prevention.[59]

The role of supplemental zinc in the prevention of acute diarrhea has proven to be quite effective. The analysis of randomized, controlled trials of zinc supplementation performed in nine low-income countries from Latin America and the Caribbean, South and Southeast Asia, and the Western Pacific suggested an 18% reduction in the incidence of diarrhea and a 25% reduction in the prevalence of diarrhea.[60]

Improved hand hygiene has been found to reduce the incidence of acute gastrointestinal diseases by 31% (95% confidence interval, 19–42). Despite reducing the incidence of gastroenteritis, another study found that hand hygiene had little effect on disease transmission.[61,62]

The major complication of rotavirus infection in infants and young children is dehydration, which can lead to electrolyte imbalance, metabolic acidosis and eventually circulatory collapse and death in the most severely-afflicted cases.

Vaccination

Endeavors to develop effective vaccines against human rotaviruses began as early as the 1980s.[3] Rotavirus vaccines are attributed to prevent 15–34% of severe diarrhea in third world countries and 37–96% of severe diarrhea in developed countries. It is estimated that vaccines have saved more than 28,000 lives (95% CI 14,600–46,700) in sub-Saharan Africa.[65]

There are four vaccines available for protection against rotaviruses (Table 4). The first vaccine against rotavirus—RotaShield of RRV-TV—was found to be highly effective (80–100%) in preventing severe diarrheal illness. After administration in 600,000 infants in the United States, RRV-TV was stopped in July 1999 when it was found to be associated with an 25-fold increased relative risk of intussusception within the first 10 days of administration.[3] In the 7 years of vaccine hiatus, as newer alternatives were explored, rotavirus-related diarrheal illness continued to cause significant mortality and mortality.

In 2006, two new vaccines—Rotateq (a reassorted bovine-human rotavirus) and Rotarix (derived from a single common strain of human rotavirus). These vaccines were found to be safe and not associated with intussusception, in fact, the recipients of these vaccines had lower incidence of intussusception.[3]

- Infants who are born preterm should be immunized according to their chronological age. A study showed no increase in adverse events following vaccination in premature infants.[69]
- Ninety-two countries had incorporated rotavirus vaccine into their national immunization programs by the year 2018; six other countries introduced rotavirus on a phased or regional basis.[66]

The rotavirus vaccination drive met with a challenge in 2018 and 2019 due to its short supply which led to the efforts of development of newer vaccines—Rotavac (naturally occurring bovine-human reassortant neonatal G9P, also called 116E); and RotaSiil (bovine-human reassortant with human G1, G2, G3, and G4 bovine UK G6P5 backbone). These new vaccines are currently used in Palestine (Rotavac) and India (both vaccines).

WHO recommends the incorporation of rotavirus vaccination in all national immunization programs, particularly in countries with high risk of disease severity and fatality. It is estimated that in Asia alone vaccination could potentially save the lives of 109,000 children, prevent 1.4 million hospital admissions, 7.7 million OPD visits; it can decrease the healthcare cost by US$ 191 million.[67]

Despite four WHO-prequalified oral rotavirus vaccines available, and several newer vaccines under development, the global disease burden of rotavirus continues to impose a challenge.[68] Many countries are yet to introduce rotavirus vaccines in their national immunization schedule. With a favorable risk-benefit profile and proven clinical efficacy demonstrated in most parts of the world, vaccination appears to be the most effective way to curb this illness.

Prognosis

Rotavirus infection has a considerable public health burden. In the prevaccine era, rotavirus was the nearly universal infection of children by age 5 years and was responsible for up to 500,000 deaths worldwide.[63] After the introduction of the vaccine, globally, it is estimated that the number of rotavirus deaths in children <5 years of age declined from 528,000 (range, 465,000–591,000) in 2000–215,000 (range, 197,000–233,000) in 2013.[64] The substantial decline in diarrhea mortality over the past decade can also be

Table 4: Rotavirus vaccines

	Rotateq	*Rotarix*	*Rotavac*	*Rotasiil*
Composition and strains	Attenuated human strain R1X4414 of G1P[8] strain	Pentavalent rotavirus reassortant with human G1, G2, G3, G4 and P[8]	Monovalent vaccine containing live attenuated Rotavirus 116E	Live attenuated bovine—human Rotavirus Reassortant (G1, G2, G3, G4 and G9 grown on vero cells) 10 5.6 FFU/serotype
Administration	Oral	Oral	Oral	Oral
Dose regimen	3—at 2, 4, and 6 months of age	2—at 2 and 4 months of age	3—at 6, 10, and 14 weeks	3—at 6, 10, and 14 weeks
Protection against severe disease	85% (72–92)	95% (91–97)	53.6%	33.6–66.7%
Virus shedding	9%	≥50%		
Intussusception	No	No	No	No
% Reduction in hospitalization	63	42		
First licensure date	Licensed in the USA and Europe in 2006	First approved in Mexico in 2004	Licensed in India in 2014	Licensed in India in 2006
WHO prequalification date	October 7, 2008	March 12, 2009	January 5, 2018	September 21, 2018
Presentation	Liquid	Liquid	Liquid	Lyophilised active component

largely attributed towards general improvements in sanitation and hygiene.

Complications

Rotavirus infection in infants has been found to be associated with pneumonia, necrotizing enterocolitis (NEC), sudden infant death syndrome (SIDS), encephalitis, seizures, bradycardia-apnoea, and diffuse intravascular coagulopathy (DIC).[70–75] Rotavirus infection, especially in premature babies, may be associated with necrotizing enterocolitis and secondary bacteremia.[76–78] Immunocompromised children owing to congenital immunodeficiency or bone marrow or solid organ transplantation may experience persistent infection lasting weeks or months. They may develop abnormalities in multiple organ systems, particularly the kidney and liver.

Approximately, 2–3% of children suffer from neurological complications, most common manifestation being febrile or afebrile seizures.[79,80] In rare cases, rotavirus have also been implicated in the causation of meningoencephalitis, cerebellitis, and encephalopathy.[81] Rotavirus infection has also been associated with aseptic meningitis, sudden infant death syndrome (SIDS), and Kawasaki syndrome.[82–86]

Epilogue

Rotavirus gastroenteritis causes substantial child mortality and morbidity worldwide, with almost 80% of deaths occurring in developing countries. Rotavirus in neonates although less prevalent than in infants is a topic that's under studied and requires further studies to understand the differences in the disease incidence, prevalence, and pathogenesis. While infants were prone to get community-acquired infections, neonates, on the other hand, were more likely to acquire nosocomial infections.

The tremendous global burden of this disease emphasizes the urgent need for interventions, such as vaccines, particularly to prevent childhood deaths in developing nations. Administration of the rotavirus vaccine is the most effective way to prevent rotavirus infection along with sanitation and hygiene measures. Rapid progress towards the development of rotavirus vaccines has prompted a reassessment of the disease burden of rotavirus diarrhea in developing countries.

Author Contribution

Preeti Shakya and Biplov Adhikari contributed equally to this work.

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REVIEW ARTICLE

Neonatal Hypoglycemia

Raghavendra Bangrakulur Rao

Abstract

Hypoglycemia is the most common metabolic problem in the neonatal period with a potential to cause brain injury. However, there are controversies in diagnosis, significance, and treatment of neonatal hypoglycemia. Several large-scale prospective and retrospective studies have reported the impact of neonatal hypoglycemia on neurodevelopment in high-risk infants. Significance of short-term hypoglycemia on neurodevelopment in healthy infants remains unresolved. There are also concerns that rapid correction of hypoglycemia may worsen brain injury. Conflicting recommendations from professional societies have further muddied the field. This review examines the current knowledge on the epidemiology of neonatal hypoglycemia, its impact on neurodevelopment, current screening and treatment recommendations, and the emerging role of dextrose gel for management of neonatal hypoglycemia.

Keywords: Hypoglycemia, Neonatology, Neurodevelopment, Newborn, Newborn infant, Preterm infants.

Newborn (2022): 10.5005/jp-journals-11002-0011

Introduction

Hypoglycemia is a common metabolic problem in the neonatal period. Severe, prolonged, and recurrent hypoglycemia in the neonatal period is associated with brain injury. Several endocrine disorders and inborn errors of metabolism also present as neonatal hypoglycemia. A thorough understanding of neonatal hypoglycemia and its effects is necessary to prevent brain injury. However, there are controversies in the definition, significance, and treatment of neonatal hypoglycemia. Conflicting recommendations from professional societies have led to additional confusion. In the following sections I will review populations at risk for neonatal hypoglycemia, current knowledge on neurodevelopmental outcome after neonatal hypoglycemia, and commonly practiced screening and treatment strategies. Hypoglycemia due to congenital hyperinsulinism, endocrine disorders, and inborn errors of metabolism is not discussed.

Perinatal Glucose Metabolism

Prior to birth, the fetus is dependent on a continuous supply of glucose from the mother. Following the abrupt cessation of maternal glucose supply at birth, blood glucose levels decrease, reaching a nadir at around 2 hours of age.[1] Glucose levels normalize over the next several hours[2] due to a combination of feeding, glycogenolysis, and gluconeogenesis. The transient decrease in blood glucose occurs in all mammals and is probably essential for postnatal metabolic programming. In most infants, the transient decrease in blood glucose does not cause problems. However, under certain conditions it could lead to complications, including brain injury. The common causes of neonatal hypoglycemia are presented in Table 1. Hypoglycemia soon after birth is typically due to failure of metabolic adaptation or inadequate energy stores. Hyperinsulinism is the most common cause of persistent hypoglycemia beyond 24–48 hours of age.

Definition and Incidence

Definition of neonatal hypoglycemia has changed over time. A blood glucose concentration of <47 mg/dL (2.6 mmol/L) is commonly used at present. This value represents the 10th percentile blood glucose concentration in healthy full-term infants during the first 48 hours after birth.[2] A blood glucose concentration of <35 mg/dL (<2.0 mmol/L) is considered severe hypoglycemia. Using a blood glucose concentration <45 mg/dL (2.5 mmol/L) as definition, a study of approximately 2000 infants of 23 to 42 weeks gestation reported an 19% incidence of hypoglycemia in the first three hours after birth.[3] Severe hypoglycemia (blood glucose <35 mg/dL) was seen in 6%. Continuous glucose monitoring (CGM) detects more cases of hypoglycemia. Using a combination of CGM and intermittent plasma glucose measurements, Harris and colleagues found that 39% of healthy full-term infants have one or more episodes of blood glucose <47 mg/dL (<2.6 mmol/L) during the first five days after birth.[2] Incidence is higher in preterm infants, infants of diabetic mothers, small-for-gestation and large-for-gestation infants.[4,5] Standardized glucose monitoring of over 500 preterm, small-for-gestation and large-for-gestation infants, and infants of diabetic mothers during the first week after birth found a 51% incidence of hypoglycemia (<47 mg/dL [<2.6 mmol/L]) with no difference in incidence among the different risk groups.[4] The mean number of hypoglycemia episodes was one per infant (range: 1–7), and the mean duration was 1.4 hours (range: 0.2–4.5 hours). Most (81%) episodes occurred on the first day after birth. Nineteen percent had severe hypoglycemia (blood glucose <36 mg/dL [<2.0 mmol/L]) with 90% of cases occurring within 12 hours after birth. Recurrent hypoglycemia occurred in 19% with the majority (70%) occurring on day one.[4] Maternal obesity and cesarean section without labor have emerged as additional risk factors for neonatal hypoglycemia.[6]

Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, United States of America

Corresponding Author: Raghavendra Bangrakulur Rao, Department of Pediatrics, University of Minnesota, Minneapolis, Minnesota, United States of America, Phone: + 6514977324, e-mail: raghurao@umn.edu

How to cite this article: Rao RB. Neonatal Hypoglycemia. Newborn 2022;1(1):151–157.

Source of support: Nil

Conflict of interest: None

Table 1: Infants at risk for neonatal hypoglycemia

Failure of metabolic adaptation
Maternal drugs (β blockers and β agonists)
Prenatal/perinatal hypoxia-ischemia
Poor energy reserves
Prematurity (<37 weeks)
Intrauterine growth restriction
Birth weight <10th percentile (small-for-gestation)
Increased energy demand
Cold stress
Seizures
Sepsis
Heart failure
Endocrine causes
Transient and persistent hyperinsulinism (birth weight >90th percentile, maternal diabetes, maternal obesity)
Hypopituitarism
Congenital adrenal hyperplasia
Inborn errors of metabolism
Disorders of amino acid metabolism (e.g., maple syrup urine disease)
Disorders of carbohydrate metabolism (e.g., galactosemia, glycogen storage disease)
Disorders of fatty acid oxidation (e.g., CPT-1 deficiency, medium- and very long-chain acyl-CoA dehydrogenase deficiency)

Inadequate time for metabolic transition and fewer opportunities for skin-to-skin care and early feeding are likely responsible for hypoglycemia in infants delivered by cesarean section.[6]

Brain Injury in Neonatal Hypoglycemia

Animal Data

Animal models demonstrate that the newborn brain is resistant to injury than the mature brain during acute hypoglycemia, likely because of its ability to maintain energy metabolism using alternative substrates.[7–9] Scattered neuronal injury is seen in brain regions important for attention, learning, and emotion (anterior cingulate and orbital cortex) and cognitive function (temporal cortex).[7] Newborn nonhuman primates exposed to prolonged acute hypoglycemia (10 hours) have impaired motivation and adaptability in infancy and require additional training and procedural modification for learning the task.[10] Recurrent hypoglycemia in the neonatal period negatively impacts neurodevelopment, leading to increased anxiety, affective dysregulation, poor socialization, and altered stress response.[11,12] Animal studies also show that excess dextrose administration during treatment of hypoglycemia worsens brain injury.[9]

Human Data

Since first reported in 1959, over 60 studies have reported neurodevelopmental outcome after neonatal hypoglycemia. A review of 16 studies (89 infants) reported that more than 95% of infants with neurological sequelae had a plasma glucose <25 mg/dL (<1.4 mmol/L).[13] Most of the studies included in the review were retrospective, of small sample size, lacked a control group, or did not controlled for comorbidities. Boluyt and colleagues in a 2006 systematic review identified 18 eligible studies on neurodevelopmental outcome after neonatal hypoglycemia.[14] Only two were found to be of high methodologic quality. None of the studies provided a valid estimate of the effect of neonatal hypoglycemia on neurodevelopment. Since then, six studies of large sample size have been published (Table 2). They are briefly reviewed below:

In a cohort of 832 preterm infants of 32–35 weeks gestation, neurodevelopment was assessed at 43–49 months using ages and stage questionnaire (ASQ).[15] Children who had hypoglycemia (defined as blood glucose <30 mg/dL [<1.7 mmol/L]) in the first 72 hours after birth (*N*=67) had >2 folds higher odds of developmental delay (20% vs 9%), compared with those who did not have neonatal hypoglycemia (*N*=765).[15] Odds for abnormal ASQ total-problem scores increased with decreasing glucose levels with the glucose value <20 mg/dL (1.1 mmol/L) being associated with an odds ratio of 3.04 (95% CI: 1.03–9.00).[15] Glucose monitoring was not standardized in the study, which could have led to a selection bias. Treatment details were also not provided.

In a population-based study of approximately 2000 infants born at 23–42 weeks gestation, 1395 infants who had at least one recorded glucose measurement in the first three hours after birth were matched with their academic performance at 10 years.[3] Transient hypoglycemia (defined as a single blood glucose value below threshold) was associated with decreased probability of proficiency on literacy (adjusted odds ratio [ORs], 0.49, 0.43, and 0.62) and mathematics achievement tests (adjusted ORs, 0.49, 0.51, and 0.78) for the three hypoglycemia cutoffs (glucose level <35 mg/dL [<2.0 mmol/L], <40 mg/dL [<2.2 mmol/L], and <45 mg/dL [<2.5 mmol/L]), respectively.[3] These data are consistent with the learning difficulties demonstrated in nonhuman primates with prolonged neonatal hypoglycemia.[10] A limitation of the study is that glucose concentrations were determined only for the first two values in the first three hours of birth; the potential effects of persistent or late-onset hypoglycemia were not tested. Moreover, effects of treatment were not examined.[3]

A secondary analysis of data from a national, multisite, randomized controlled longitudinal intervention study of long-term health and developmental outcomes in preterm infants ≤37 week gestation and ≤2500 g birth weight did not find differences in cognitive, academic, and behavioral outcomes at 3, 8, or 18 years between infants who had neonatal hypoglycemia (blood glucose ≤45 mg/dL [≤2.5 mmol/L]; *N*=461) and those that remained normoglycemic (*N*=284) after adjusting for demographics and confounding variables.[5] Interestingly, children with a history of severe neonatal hypoglycemia (blood glucose ≤35 mg/dL [≤2.0 mmol/L]) had lower problematic behaviors than the other groups.[5] This study has limitations. In addition to being a secondary analysis, screening and treatment criteria were not standardized. Methods of glucose measurement were not uniform, and duration of hypoglycemia was not reported.

In a prospective study involving 614 at-risk infants, blood glucose concentration was determined up to one week after birth (more intensely in the first 48 hours).[16] Additionally, CGM was performed in a subset. Hypoglycemia, defined as blood glucose <47 mg/dL (<2.6 mmol/L) was treated using a combination of feeding, buccal dextrose gel application, or intravenous dextrose. Neurosensory impairment (NSI) and processing difficulty were evaluated in 404 infants at 2 years of age using bayley scales of Infant Development III, vision screening, global motion perception and executive function. The risk of NSI or processing difficulty was

Table 2: Recent studies on neurodevelopmental outcome after neonatal hypoglycemia

First author (year)	*Study type*	*Population and sample size*	*Hypoglycemia definition*	*Glucose monitoring and method*	*Treatment and goal*	*Age at follow-up*	*Neuro-developmental tests/outcomes*	*Main study results*
Kerstjens (2012)[15]	Retrospective cohort	32–35 weeks preterm infants. HG, 67; no-HG, 765	Plasma Glc, <30 mg/dL in first 72 hr	Several times during the first 24 hr and longer as necessary. Method not described.	Not mentioned	43–49 months	Ages and stages questionnaire/ developmental delay	>2 folds higher odds of developmental delay in those with HG. Higher odds with lower glucose values
Kaiser (2015)[3]	Retrospective cohort	All infants between 23 and 42 wk. HG, 89; no-HG, 1306	Glc <35 mg/dL (primary). <40 mg/dL and <45 mg/dL (secondary)	1–3 hr after birth, repeated in 1 hr in those with HG. Method not described.	No standardized treatment. IV dextrose or early feeding when glucose ≤35 mg/dL	10 years	Benchmark examination in fourth grade/ Proficiency on literacy and mathematics achievement tests.	Decreased probability of proficiency on literacy tests (adjusted ORs, 0.49, 0.43, and 0.62) and on mathematics tests (adjusted ORs, 0.49, 0.51, and 0.78) with glucose <35 mg/dL, <40 mg/dL, and <45 mg/dL
McKinlay (2015)[16]	Prospective cohort	Preterm and term at-risk infants. HG, 216; no-HG, 188	Glc <47 mg/dL	Regular measurement for 24–48 hours or until no concerns. Masked CGM in a subset.	Feeding, dextrose gel, and IV dextrose to maintain blood glucose at least 47 mg/dL	2 years	BSID-III, vision screening, global motion perception and executive function	No increase in NSI or processing with HG. Unstable glycemia and steep rise in blood glucose in those with NSI
Goode (2016)[5]	Retrospective cohort	≤37 weeks gestation and ≤2500 g birth weight. HG, 461; no-HG, 284	Glc ≤45 mg/dL	No standardized protocol. Highest and lowest Glc levels by Dextrostix and/or plasma sample.	No standardized treatment	3, 8, and 18 years	Cognitive, academic, and behavioral assessments*	No difference in cognitive, academic, and behavioral outcomes. Lower problematic behaviors in those with severe HG.
McKinlay et al. (2017)[17]	Prospective cohort	Preterm and term at-risk infants. HG, 280; no-HG, 197	Glc <47 mg/dL	Blood Glc and masked CGM up to 7 days	Treated to maintain blood glucose at least 47 mg/dL	4.5 years	BSID-III, vision screening, global motion perception, and executive function	No increase in neurosensory impairment with HG. Low executive function and visual motor dysfunction with HG. Highest risk with severe, recurrent or clinical undetected HG. Steeper rise in interstitial Glc in those who developed NSI between 2 and 4.5 years

(Contd...)

Table 2: (*Contd...*)

First author (year)	*Study type*	*Population and sample size*	*Hypoglycemia definition*	*Glucose monitoring and method*	*Treatment and goal*	*Age at follow-up*	*Neuro-developmental tests/outcomes*	*Main study results*
van Kempen (2020)[18]	Prospective, randomized, controlled trial	Late preterm (35–37 wk) and at-risk term infants. Lower-threshold group, 348; traditional-threshold group, 341	Plasma Glc <47 mg/dL between 3 and 24 hr	Before feeding at 3, 6, 9, 12, 18, and 24 hr after birth	Randomized 1:1 to lower-threshold (Glc <36 mg/dL) and traditional-threshold (Glc <47 mg/dL) groups. Goal: ≥36 mg/dL in lower threshold and ≥47 mg/dL in traditional-threshold group	18 months	BSID-III-NL	No differences in Bayley-III-NL scores in the two groups. More and severe HG in the low-threshold group; more diagnostic and treatment interventions in the traditional-threshold group

*Cognitive: stanford-binet intelligence scales, peabody picture vocabulary test-revised (PPVT-R) and PPVT-III, wechsler intelligence scale for children and wechsler abbreviated scale of intelligence; academic achievement: woodcock-johnson tests of achievement-revised; Behavior: Child Behavior checklist and the youth report behavior surveillance system: *BSID-III*, bayley scales of Infant and toddler development, third edition; *BSID-III-NL*, bayley scales of infant and toddler development, third edition, dutch version; *CGM*, continuous glucose monitoring; *Glc*, glucose; *HG*, hypoglycemia; *NSI*,neurosensory impairment

not higher in children with neonatal hypoglycemia, irrespective of its frequency and severity. On the contrary, there was an indication for NSI with higher and unstable glucose concentrations in the first 48 hours after birth. Children with NSI had slightly higher (approximately 3 mg/dL) interstitial glucose concentrations than those with normal neurosensory function. Greater time outside of a blood glucose range 54 mg/dL (3 mmol/L) to 72 mg/dL (4 mmol/L) was associated with a 40% higher risk of NSI, particularly, cognitive delay. A steeper rise in interstitial glucose following treatment of first hypoglycemia episode increased the risk of NSI.[16]

Four hundred seventy-seven of the 604 eligible children were followed at 4.5 years.[17] Cognitive, executive, visual, and motor functions were assessed. Similar to the assessment at 2 years, neonatal hypoglycemia was not associated with an increased risk of NSI at 4.5 years. However, the risk of impaired executive and visual motor functions was increased, especially in children with severe (blood glucose <35 mg/dL [<2.0 mmol/L]), recurrent (>1 episode), or hidden (detected only on CGM) hypoglycemia in the neonatal period. Unlike the effect at 2 years, there was no association between NSI and time outside the central blood glucose (54–72 mg/dL [4.0–5.0 mmol/L]) range. However, children who developed NSI between 2 and 4.5 years had a steeper rise in interstitial glucose concentration after hypoglycemia.[17] Collectively, these data suggest that (1) severe and recurrent hypoglycemia in the neonatal period is associated with neurosensory, visual motor, and executive function impairments in early childhood; (2) these deficits may not be apparent in early infancy; and (3) glycemic fluctuations may worsen neurological outcomes. A limitation of the study is that the cohort included only newborn infants at risk for hypoglycemia. Infants with these conditions are known to be at risk for abnormal neurodevelopment even in the absence of hypoglycemia. Relevance of the data to healthy newborn infants with transient hypoglycemia is not known.

A recent multicenter trial (Hypoglycemia–Expectant Monitoring vs Intensive Treatment trial; the HypoEXIT trial) randomized late preterm and full-term infants at risk for hypoglycemia to treatment at a blood glucose <36 mg/dL (<2 mmol/L; lower-threshold group; *N*=348) or <47 mg/dL (<2.6 mmol/L; traditional-threshold group; *N*=341). The goal was to maintain blood glucose ≥36 mg/dL in the lower threshold group, and ≥47 mg/dL in the traditional-threshold group.[18] Neurodevelopment was assessed at 18 months of age. Cognitive and motor outcome scores were similar in the two groups. Infants in the lower-threshold group had more frequent and severe hypoglycemia than the traditional-threshold group. Conversely, there were more invasive diagnostic and treatment interventions in the traditional-threshold group.[18] The results cannot be extrapolated to all infants as the trial excluded neonates with severe hypoglycemia. Further, as mentioned above,[16,17] impairments may not become apparent until later in childhood. Long-term follow-up is necessary before treatment at lower glucose threshold can be recommended.

A meta-analysis involving 11 studies and 1657 infants demonstrated no association between neonatal hypoglycemia and NSI, risk of epilepsy, cognitive impairment, emotional and behavioral difficulty, visual and hearing impairment, motor deficits, and cerebral palsy in early childhood (2–5 years).[19] However, children with a history of neonatal hypoglycemia had a 3.5-fold higher risk of visual–motor impairment and a 2.5-fold higher risk of executive dysfunction. There was a statistically nonsignificant association between hypoglycemia and low language and literacy.

Assessment at mid-childhood (6–11 years) showed that neonatal hypoglycemia increased the risk of NSI by 3.6 folds and the risk of low language/literacy and numeracy by 2 folds. A statistically nonsignificant risk of emotional–behavioral difficulty was present. There was no impact on risk of epilepsy, motor, cognitive, visual, and hearing impairments.[19]

There are no outcome data at adolescence and beyond.

Management Considerations

The primary goal of screening and treatment of neonatal hypoglycemia is prevention of brain injury. Current diagnosis and treatment strategy is based on blood glucose levels and is focused on raising blood glucose concentrations above a predetermined threshold. While practical, this strategy may not ensure neuroprotection for the following reasons: (1) blood glucose levels do not reflect the dynamic metabolic changes in the developing brain during hypoglycemia;[8] (2) the risk of brain injury cannot be predicted by a single blood glucose without considering the severity and duration of hypoglycemia, availability of alternative substrates and associated comorbidities; and (3) there is no evidence that normalizing blood glucose above a certain level (typically, >45 mg/dL, >2.5 mmol/L) ensures neuroprotection.[3,20,21] Nevertheless, in the absence of an alternative evidence-based strategy, a blood glucose-based management strategy remains the recommendation of professional societies (Table 3).[22–26]

Screening

Universal screening will pick-up all cases of hypoglycemia. The disadvantages of this strategy are pain associated with blood collection, parental anxiety, over diagnosis and unnecessary treatment of a transient and potentially benign condition, and increased healthcare cost. Up to 39% of healthy full-term infants have at least one blood glucose concentration <47 mg/dL in the first five days after birth.[2] Currently, all professional societies recommend screening only infants at-risk for hypoglycemia.[22–26] This strategy is cost-effective but could miss asymptomatic hypoglycemia in infants without known risk factors. The concerns on unnecessary intervention remain and it is not clear whether such a screening and treatment strategy ensures normal neurodevelopment for the reasons mentioned above.[3,20,21]

Typical recommendation is to screen at-risk infants for hypoglycemia within 1–4 hours of birth, typically 30–60 minutes after a feeding, and then every 3–4 hours until two to three consecutive pre-prandial blood glucose levels in the normal range are confirmed. The duration of monitoring depends on the underlying risk factor.[22,24] However, a uniform duration of screening may be appropriate and easier to implement as there is no difference in the incidence and severity of hypoglycemia among the various risk groups.[4] The fact that hypoglycemia could occur after several normal blood glucose values in up to one third of infants and that 6% have the first episode of hypoglycemia on the second day [4] also supports a uniform length (e.g., 48 hours) of screening.

Screening is commonly performed using point-of-care nonenzymatic methods. Although convenient, point-of-care techniques are not sensitive at lower glucose levels and require confirmation using an enzymatic method in the laboratory. Hand-held point-of-care enzymatic methods are available. While they are more expensive per test, they are overall cost-effective because of the reduced need for laboratory confirmation.[27] Continuous monitoring of interstitial glucose using indwelling catheters is an alternative method. The method is reliable, detects "hidden" hypoglycemia[16,17] and reduces the need for intermittent glucose monitoring, but may result in over diagnosis and treatment.

Treatment

The primary goal of treatment is prevention of brain injury. Treatment depends on blood glucose concentration, presence or absence of symptoms and signs, infant's ability to feed, and response to intervention. Symptomatic infants, particularly those exhibiting neurological signs, require prompt measures to raise their blood glucose. Typically, an intravenous bolus of 10% dextrose at a dose of 200 mg/dL (2 ml/kg), followed by a continuous dextrose infusion at a glucose infusion rate (GIR) of 5–8 mg/kg per minute is provided.[22,28] The goal is to achieve a blood glucose concentration 40–50 mg/dL (2.2–2.8 mmol/L).[22] Some professional societies recommend a target range of 47–80 mg/dL (2.6–5.0 mmol/L) for infants <72 hours of age and 60–80 mg/dL (3.3–5.0 mmol/L) for those 72 hours or older.[24] The Pediatric Endocrine Society recommends maintaining plasma glucose >50 mg/dL (>2.8 mmol/L) during the first 48 hours, and >60 mg/dL (>3.3 mmol/L) after 48 hours in high-risk infants without suspected congenital hyperinsulinism.[23] A higher target (>70 mg/dL) is recommended for those with suspected or confirmed hyperinsulinism.[23] The target glucose concentration is maintained by frequent blood glucose checks and adjustment to infusion rate. IV dextrose is weaned when blood glucose remains stable for 12 hours.[24] Persistent hypoglycemia, requirement of high GIR (≥8 mg/kg per min), or inability to wean dextrose infusion after 3 days indicates the possibility of hyperinsulinism and the need for additional work-up.[24,26]

Asymptomatic infants who are able to feed are offered breastfeeding or formula with follow-up blood glucose checks 1 hour later.[22,24] Some professional societies use different blood glucose thresholds depending on postnatal age (e.g., <25 mg/dL [<1.4 mmol/L] in the first 4 hours and <35 mg/dL [<1.9 mmol/L]

Table 3: Operational thresholds for management of neonatal hypoglycemia

	Postnatal age (hours)				
Professional Society	*0–4*	*4–24*	*24–48*	*48–72*	*>72*
American Academy of Pediatrics[22,a]	<25	<35	–	–	–
Pediatric Endocrine Society[23,b]	Maintain plasma glucose >50			Maintain Glc. >60	
World Health Organization[35]			<47		
Canadian Pediatric Society[24]			<47		<60
British Association of Perinatal Medicine[26]	<18 any time; a single value <40 in a symptomatic infant; two values <36 in asymptomatic at-risk infant				

Values are mg/dL; to get mmol/L, multiply by 0.0555. *Glc*, glucose: [a]Symptomatic infants with blood glucose <40 mg/dL require IV glucose: [b]Maintain plasma glucose >70 mg/dL in suspected/confirmed congenital hyperinsulinism

between 4 and 24 hours).[22] One study showed that formula feeding led to higher blood glucose than breastfeeding or feeding of expressed breastmilk.[29] Intravenous dextrose with or without a mini bolus as described above is used if blood glucose remains low. Approximately 5% of infants with hypoglycemia require parenteral dextrose.[6]

Dextrose Gel for Prevention and Treatment of Hypoglycemia

Application of 40% dextrose gel to the buccal mucosa at a dose of 0.5 mL/kg (200 mg/kg) has emerged as an alternative to intravenous dextrose infusion.[24,29–31] Dextrose gel application is followed with breastfeeding or bottle feeding of expressed mother's milk, donor breastmilk, or formula. Type of feeding determines success with dextrose gel. In one study, donor milk and formula achieved higher blood glucose levels than breastfeeding.[32] The primary benefit of dextrose gel is improved success with breastfeeding, likely because of the mother and infant can remain together.[30,31] There was no effect on NSI at 2 years of age.[20,33] Preventive application of dextrose gel reduces the risk of hypoglycemia in at-risk infants.[34]

Conclusions

Despite being a common metabolic problem with the potential to cause brain injury, diagnosis and management of neonatal hypoglycemia remains controversial. Severe and recurrent hypoglycemia is associated with impaired executive and visual motor functions in infants at high-risk for hypoglycemia. Detection of these impairments at preschool age suggests the need for long-term follow-up in children exposed to neonatal hypoglycemia. The higher risk of NSI with glycemic instability suggests the importance of avoiding glycemic fluctuations during treatment. Current recommendations from professional societies are expert opinions and not evidence based. Well-designed, prospective, randomized, controlled trials with long-term neurodevelopmental assessment are needed to optimize management.

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REVIEW ARTICLE

New Therapeutic Targets in Neonatal Pulmonary Hypertension

Julie A Dillard[1], Claire Murray[2], Amit A Mathur[3]

Abstract

Persistent pulmonary hypertension of the newborn (PPHN) is a significant cause of morbidity and mortality in neonates. Despite advances in medical care, mortality remains high. In the United States, inhaled nitric oxide is the gold standard treatment in patients with PPHN. However, while it decreases the need for extracorporeal membrane oxygenation, many patients do not respond to inhaled nitric oxide, and it does not improve overall mortality in those with PPHN. Furthermore, its use is cost-prohibitive in many parts of the world. Thus, there is a critical need to research alternative therapies to improve neonatal outcomes. In this review, we present the animal and human data of some emerging therapeutic targets for pulmonary hypertension, prioritizing pediatric and neonatal data when available. Specifically, we discuss the role of soluble guanylate cyclase stimulators and activators, prostacyclin and analogues, phosphodiesterase 3, 4, and 5 inhibitors, rho-kinase inhibitors, endothelin receptor blockers, PPARγ agonists, and antioxidants in the treatment of neonates with PPHN.

Keywords: Extracorporeal membrane oxygenation, Neonate, Persistent pulmonary hypertension of the newborn, Pulmonary hypertension.

Newborn (2022): 10.5005/jp-journals-11002-0015

Introduction

In utero, the lungs are fluid-filled and pulmonary vascular resistance (PVR) is high, with only 10–20% of cardiac output reaching the lungs (1). At birth, with the establishment of ventilation and removal of the low resistance placental circuit, PVR falls dramatically, resulting in the establishment of pulmonary blood flow (PBF).[1] Persistent pulmonary hypertension of the newborn (PPHN) results when the normal circulatory transition at birth fails to occur and pulmonary vascular pressures remain elevated. This leads to right-to-left shunting of blood across the foramen ovale and ductus arteriosus (DA), with resulting hypoxemia. The need for extracorporeal membrane oxygenation (ECMO) in these infants is high, and despite advances in medical care, mortality remains up to 10% in the United States.[2] Inhaled nitric oxide (iNO), the only FDA-approved treatment for PPHN, continues to be the mainstay of therapy, and has been shown to decrease the need for ECMO.[3,4] However, up to 40% of neonates with PPHN have a suboptimal response to iNO, and there is a lack of evidence-based alternative therapies for this population.[3–5] Furthermore, iNO does not clearly reduce mortality or improve neurodevelopmental outcomes among survivors.[6–8] Thus, there is a critical need to identify novel therapeutic targets to improve patient outcomes. In this paper, we will review the preclinical and clinical research of some emerging therapeutic targets for neonatal pulmonary hypertension.

Soluble Guanylate Cyclase Stimulators and Activators

Nitric oxide (NO) exerts its vasodilatory effects through activation of the enzyme, soluble guanylate cyclase (sGC), resulting in greater cyclic guanosine monophosphate (cGMP) concentrations. The native form of sGC contains a prosthetic heme group that serves as the location of the NO-binding site and is required for NO–sGC activation. In the heme-free state, sGC is dysfunctional and ultimately degraded, which occurs under conditions of oxidative stress.[9,10] In disease states characterized by reduced NO bioavailability or the development of NO tolerance, therapeutics that are able to modulate sGC in the absence of NO may prove beneficial in clinical practice. Two such classes of drugs, sGC stimulators and sGC activators, bind directly to sGC to activate the enzyme via an NO-independent mechanism.[9,11]

Soluble guanylate cyclase stimulators directly bind and stimulate the heme-containing form of sGC, increasing cGMP concentrations. Additionally, these compounds stabilize the binding of NO to sGC, and thus, exhibit synergism with NO. Experimental animal models demonstrate sustained pulmonary vasodilation in the presence of the sGC stimulators, BAY 41-2272 and BAY 41-8543.[12–16] In fetal lambs, intravenous infusion of BAY 41-2272 resulted in a 75% reduction in PVR with a threefold increase in PBF.[14] Furthermore, the pulmonary vasodilator effects were not attenuated by the addition of an NO synthase inhibitor, suggesting that the effects of BAY 41-2272 were independent of NO.[14] Of concern in this study, systemic effects were observed at higher doses and during prolonged infusion, although the study drug was infused directly into the LPA.[14] In another study of severe PPHN generated by prenatal ligation of the DA, BAY 41-2272 infusion resulted in a 75% reduction in PVR by day 5, greater than that of sildenafil-treated fetal lambs.[15] Moreover, in neonatal sheep, while BAY 41-2272 infusion resulted in greater pulmonary vasodilation than iNO, the combined treatment with both agents resulted in enhanced pulmonary vasodilation and improved oxygenation compared to either treatment alone.[15] In fact, multiple animal studies provide evidence of synergy between NO and sGC

[1–3]Department of Pediatrics, Saint Louis University, St Louis, Missouri, United States of America

Corresponding Author: Julie A Dillard, Department of Pediatrics, Saint Louis University, St Louis, Missouri, United States of America, Phone: +17249864412, e-mail: julie.dillard@health.slu.edu

How to cite this article: Dillard JA, Murray C, Mathur AA. New Therapeutic Targets in Neonatal Pulmonary Hypertension. Newborn 2022;1(1):158–169.

Source of support: Nil

Conflict of interest: None

stimulators.[12,15–17] In adult rats, pulmonary vasodilator response to BAY 41-8543 was attenuated if endogenous NO production was inhibited and could be restored by additional treatment with an NO donor.[16] Unfortunately, given as an IV infusion in this study, BAY 41-8543 resulted in similar dose-dependent decreases in systemic arterial pressure, which may limit its clinical applicability as an IV infusion in neonates.[16]

The oral sGC stimulator, BAY 63-2561, or riociguat, is FDA-approved for the treatment of adults with pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension. In phase 3 double-blind study of 443 adults with symptomatic PAH, riociguat improved several clinical outcomes including 6-minute walk distance, PVR, and time to clinical worsening.[18] In the open-label follow-up study, improvements in exercise and functional capacity were maintained for up to 1 year, with a survival rate of 97%.[19] While serious adverse events were rare, hypotension was documented in 9% and syncope in 7% of patients receiving treatment.[19] Interestingly, a clinical trial demonstrated that riociguat was effective in the majority of adult patients who had inadequate response to treatment with PDE5 inhibition.[20] Riociguat is currently being evaluated in children aged 6–18 years with PAH in an open-label, dose adjustment study, PATENT–CHILD, and results are pending (https://clinicaltrials.gov/, NCT 02562235). A single case report of a 3.5-year-old boy with therapy-resistant PAH who was treated with riociguat for 6 months demonstrated improved PVR/systemic vascular resistance (SVR) ratio, right ventricular hypertrophy (RVH), and pediatric functional class without adverse effects of systemic hypotension.[21] Currently, there are no reports of studies utilizing sGC stimulators in infants and neonates, and more research is needed to elucidate the safety and efficacy in this population.

In contrast to sGC stimulators, sGC activators bind to the dysfunctional, heme-free form of sGC that is unresponsive to NO.[9,22] Considering that pathophysiological conditions of oxidative stress can oxidize the heme moiety of sGC, rendering it unresponsive to NO, the use of sGC activators may have wide-reaching clinical implications, including in neonatal pulmonary hypertension (PH). Experimental animal studies demonstrate that the sGC activator, BAY 58-2667 (cinaciguat), elicits potent and sustained pulmonary vasodilation.[23–26] In a fetal ovine model, infusion of cinaciguat resulted in dose-dependent and long-lasting increase in PBF and reduced PVR by 80%.[23] Importantly, sGC oxidation by ODQ enhanced the pulmonary vasodilatory effects of cinaciguat *in vitro* and resulted in a 14-fold increase in cGMP levels in pulmonary artery smooth muscle cells (PASMC) *in vivo* compared to non-ODQ treated cells.[23] Likewise, in PASMC isolated from a lamb model of PPHN, cinaciguat increased cGMP generation by >60-fold following oxidation with ODQ and approximately 20-fold after exposure to moderate hyperoxia.[24] In this PPHN lamb model induced by prenatal DA ligation, newborn sheep treated with cinaciguat had increased PBF and decreased PVR, and the vasodilatory effects were greater than treatment with oxygen or iNO.[24] In heme-deficient sGC mice, the ability of NO to relax precontracted aortas was abolished, whereas the ability of cinaciguat to relax the vessels was enhanced.[27] Not surprisingly, rat studies utilizing intravenous injection of sGC activator, BAY 60-2770 reported potent and long-lived reduction in systemic arterial pressures as well (Fig. 1).[25]

In the COMPOSE studies, a series of three randomized, double-blind, placebo-controlled trials among adults with acute decompensated heart failure, researchers determined that treatment with intravenous cinaciguat did not meaningfully improve cardiac index or dyspnea and was associated with significant reductions in systemic blood pressure.[28] The study was terminated early due to an increased occurrence of hypotension and poor recruitment.[28] At present, no studies have examined the use of sGC activators in the pediatric population. The findings in animal models and adults of substantial systemic hypotension are concerning and may be prohibitive in researching this therapy in neonates. Interestingly, inhalation of microparticles containing sGC stimulators (BAY 41-2272 and BAY 41-8543) or sGC activator (BAY 58-2667) produced pulmonary vasodilation and transpulmonary cGMP production without impacting systemic hemodynamics in an experimental lamb model of pulmonary hypertension,[26] suggesting that inhalational therapy may show efficacy while avoiding adverse effects on the systemic circulation.

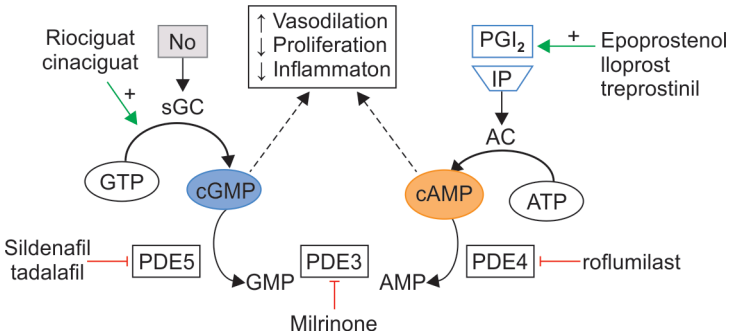

Fig. 1: Pulmonary vascular tone is regulated by cAMP and cGMP, which are hydrolyzed by the phosphodiesterases. NO activates sGC to increase cGMP concentrations. Additionally, sGC stimulators (riociguat) and sGC activators (cinaciguat) directly bind and stimulate sGC independent of NO. PDE5 hydrolyzes cGMP, thus PDE5 inhibition with sildenafil or tadalafil results in increased cGMP levels. Prostacyclin and its analogues bind to a prostacyclin receptor which activates adenylate cyclase to increase cAMP concentrations. Cyclic adenosine monophosphate is hydrolyzed by PDE4, which can be inhibited with roflumilast. PDE3 hydrolyzes both cyclic nucleotides and is inhibited by milrinone. Increase in cAMP and cGMP concentrations results in pulmonary vasodilation and decreased smooth muscle cell proliferation. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; NO, nitric oxide; sGC, soluble guanylate cyclase; PDE, phosphodiesterase; PGI_2, prostacyclin; IP, prostacyclin receptor

Prostacyclins

Prostanoids and their analogues have pulmonary vasodilatory and immunomodulatory effects. Upon binding to its receptor, prostacyclin activates the enzyme, adenylate cyclase, thereby increasing intracellular cyclic adenosine monophosphate (cAMP) concentrations. This activates protein kinase A which relaxes smooth muscle, leading to vasodilation of the pulmonary arteries.[29] The use of prostacyclins in the treatment of PPHN is an active area of research and their use in adults and pediatric patients with PH is well-established.[29,30] However, their use has been somewhat limited by their short half-life. The prostacyclins are available in IV, subcutaneous, and inhaled formulations.

Animal models of PH utilizing prostacyclin and analogues have shown promising results. In piglets with acute respiratory failure and PH, investigators compared iNO, IV, and inhaled prostacyclin. Inhaled NO and inhaled prostacyclin significantly increased PaO_2/FiO_2 and decreased mean airway pressure and mean pulmonary artery pressure (PAP) without impacting systemic blood pressure.[31] There was no difference in efficacy between iNO and inhaled prostacyclin; however, IV prostacyclin improved oxygenation parameters to a lesser extent at all doses.[31] In isolated lungs from a fetal lamb model of PPHN induced by antenatal ligation of the DA, protein expression of both prostacyclin synthase and prostacyclin receptor were decreased.[32] Furthermore, precontracted pulmonary arteries showed impaired relaxation to prostacyclin, iloprost, and milrinone. Interestingly, pretreatment with milrinone significantly enhanced the vasorelaxation to both prostacyclin and iloprost, suggesting that dual therapy with these agents may be of benefit in neonates with PPHN.[32]

One challenge with the currently available prostanoids and analogues is their short half-life, limiting delivery strategies. Epoprostenol is one such type of synthetic prostanoid that requires continuous intravenous administration and is unstable at room temperature.[33] Case studies of infants given epoprostenol reported that pulmonary arterial pressure was decreased by an average of 19.4 mmHg[34] and oxygenation index improved by a mean of 32[35] without the side effect of systemic hypotension. In a recent retrospective review of iNO-unresponsive PPHN, IV epoprostenol resulted in a rapid and sustained reduction in oxygenation index (OI) in a small subset of patients who were considered responders.[36] There was an increased need for volume resuscitation after initiation of treprostinil in the subgroup of infants deemed unresponsive to the drug. Additionally, per institutional unit protocol, treatment with IV milrinone preceded initiation of epoprostenol in 95% of the study patients.[36]

The current prostanoid analogues (treprostinil and iloprost) are more chemically stable than epoprostenol.[37] Treprostinil has subcutaneous, intravenous, and inhalation delivery options with a slightly longer half-life than its sister drug, epoprostenol. In a retrospective cohort study, 17 patients with congenital diaphragmatic hernia (CDH)-associated PH who were treated with IV treprostinil for a median of 54.5 days had a significant reduction in B-type natriuretic peptide and improvement in echocardiographic parameters of PH at one month.[38] Despite this, patients treated with treprostinil were more likely to require ECMO, had a longer length of mechanical ventilation and hospital stay, and had an overall mortality of 35%.[38] There is currently an ongoing placebo-controlled clinical trial enrolling babies with PPHN to receive IV remodulin (treprostinil) vs placebo in addition to standard of care (https://clinicaltrials.gov/, NCT02261883).

Iloprost is available for use as an inhaled medication and has shown pulmonary selectivity.[37] In an early randomized controlled trial (RCT) evaluating the efficacy of inhaled iloprost in 203 adults with severe PH, 40% of the iloprost group increased their 6-minute walk distance by greater than 10%, and approximately 25% had improvement in functional class over a 12-week period.[39] In an open-label extension study of 71 adults with PH, long-term treatment with inhaled iloprost improved functional class after 1 and 3 years and had survival rates of 83, 78, and 58% at 1, 3, and 5 years, respectively.[29] Large, prospective studies examining the safety and efficacy of inhaled iloprost in neonates are lacking. In a small, prospective study of neonates with PPHN, inhaled iloprost 4–8 times per day resulted in improved echocardiographic parameters of PH and respiratory severity score in 8 of 9 patients.[40] In another prospective study, 47 neonates with PPHN were given either oral sildenafil or inhaled iloprost as first-line therapy. The group who received inhaled iloprost showed decreased time to clinical response, ventilatory parameters, and length of mechanical ventilation.[41] Furthermore, while there was a significant decrease in systemic blood pressure in the sildenafil group, this was not noted with inhaled iloprost. Inhaled NO was not used in these low resource settings and both authors concluded that inhaled iloprost may be a beneficial first-line agent for this purpose.[40,41] The original delivery of nebulized iloprost that was studied in ambulatory adults with PH could not be used in a closed ventilator circuit. Recent advances have identified reliable methods of drug delivery in mechanically ventilated adults, but studies are lacking in infants. A recent *in vitro* study of iloprost delivery utilizing a neonatal test lung model found that delivery of iloprost was optimized when using a vibrating mesh nebulizer proximal to the patient airway and was more efficient during high-frequency ventilation than conventional ventilation.[42] Definitive *in vivo* studies need to be completed to confirm these results.

The Phosphodiesterases

The cyclic nucleotide phosphodiesterases (PDEs) are composed of a superfamily of 11 enzymes with various tissue and cellular distribution, and cell-specific function and regulation.[43] PDEs degrade the ubiquitous second messengers, cAMP and/or cGMP, and thus, are effectors in many cellular processes including vascular tone and remodeling, and inflammation.[43,44] While nearly all of the PDE families have been identified in the pulmonary vasculature,[43] this review will focus on the most well-studied PDEs in neonatal PH: PDE3, PDE4, and PDE5.

PDE3

PDE3 hydrolyzes both cAMP and cGMP with high affinity. It is known as the cGMP-inhibited PDE, as its rate of hydrolysis for cAMP is greater than that of cGMP. Increased PDE3 activity has been reported in pulmonary arteries from a rat model of PH[45] as well as in isolated PASMC from patients with PH.[46] Moreover, the PDE3 inhibitor, milrinone has beneficial effects in animal models of PH[32,47–49] and in case reports of neonates with PH.[50–52]

Recent data suggest that treatment with NO leads to an increase in PDE3 expression and activity. In experimental animal models, PDE3 expression and/or activity is increased in the pulmonary vasculature following treatment with NO.[53–55] Pulmonary arteries of newborn sheep treated with iNO and 100% oxygen had the highest PDE3 activity and the greatest relaxation response to milrinone.[54] Interestingly, in this study, the second-highest PDE3 activity

was seen in one-day-old spontaneously breathing lambs, which suggests a role for PDE3 in the transitional circulation. We recently demonstrated that treatment of neonatal human PASMC with an NO donor resulted in increased PDE3 activity and decreased cAMP concentrations.[56] Moreover, there is evidence of synergy between iNO and milrinone. In an experimental model of PH induced by a thromboxane mimetic, rabbits who received the combination of iNO + IV milrinone had a greater drop in the PAP and PVR compared to either treatment alone.[55] These data strongly support a role for PDE3 in neonatal PH. Moreover, as NO treatment appears to increase PDE3 activity, we speculate there is a role for PDE3 inhibition in neonates with PPHN that is unresponsive to iNO.

Milrinone has inotropic, lusitropic, and vasodilatory properties and is researched for use in multiple disease conditions. Currently, milrinone is FDA-approved for short-term use in adults with acute decompensated heart failure. Additionally, in children and infants, milrinone is used for the treatment and prevention of low cardiac output syndrome.[57–59] Case reports of the use of milrinone in neonates with iNO-unresponsive PPHN have found promising results, with evidence of improved oxygenation.[50,51,60,61] Some reports have found no impact on systemic hemodynamics,[50,51] while others have found a decrease in systemic arterial pressures.[52,61,62] In a prospective study of 11 neonates with PPHN resistant to iNO, the addition of milrinone led to improved oxygenation, decreased iNO dose, and decreased PAP.[52] While there was a statistically significant reduction in systolic arterial pressure following the milrinone bolus, there was an overall improvement in cardiac output and markers of systemic perfusion, including lactate and base deficit.[52] In a recent RCT evaluating the use of milrinone + sildenafil (a PDE5 inhibitor) in neonates with PPHN in a resource-limited setting, the investigators reported that combination therapy with milrinone and sildenafil resulted in a greater decrease in the PAP and OI than either monotherapy, further evidence of synergy between the two treatments.[63] Importantly, there was no statistically significant difference in either the systolic or diastolic blood pressures before and after treatment in any of the three groups.[63] Despite the promising animal and human data supporting the use of milrinone in neonates with PPHN, large RCTs in this population have not been performed. This is likely secondary to low enrollment of a rare disease process, as well as difficulty in adherence to study arms given the critically ill-nature of the patients.[64]

PDE4

PDE4 consists of four isoforms that are ubiquitously expressed and hydrolyze cAMP with high affinity.[43,65] PDE4 isoforms are highly expressed in inflammatory cells, and therefore have been implicated for a role in respiratory disorders characterized by chronic inflammation, including chronic obstructive pulmonary disease (COPD) and asthma.[66,67] The PDE4 inhibitor, GPD-1116, attenuated the increase in RV systolic pressure and RVH (as measured by Fulton's Index) in rats with monocrotaline-induced PH and resulted in a 57% increase in pulmonary cAMP concentrations in non-diseased rats.[68] Furthermore, the effects of GPD-1116 on the above measures were greater than that seen with the PDE5 inhibitor, tadalafil.[68] However, it was discovered that GPD-1116 also potently inhibits PDE1, so the effects seen from this molecule may not be attributed solely to PDE4. Several experimental animal studies of bronchopulmonary dysplasia (BPD) evaluating the effects of PDE4 inhibition on neonatal lung injury have shown promising results.[69–72] In a hyperoxia model of acute lung injury in newborn rats, treatment with the PDE4 inhibitor, rolipram, improved survival and decreased lung inflammatory cell count and cytokine expression compared to controls.[70] Similar findings were reported in a preterm rat model of hyperoxic lung injury in which PDE4 inhibitors prolonged survival, reduced capillary alveolar protein leakage, alveolar fibrin deposition, and influx of neutrophils and macrophages into the lung. PDE4 inhibition also resulted in reduced expression of inflammatory genes.[71] In another experimental BPD study in neonatal rats, researchers investigated the effects of prophylactic and rescue PDE4 inhibition on hyperoxia-induced lung injury. Prophylactic treatment with the PDE4 inhibitor, piclamilast improved mortality and prevented the development of PH assessed by increased pulmonary vessel density, reduced arteriolar medial wall thickness, and attenuation of RVH.[72] While rescue treatment with piclamilast on day 6 reduced arteriolar wall thickness and attenuated RVH, it did not restore lung angiogenesis or alveolar development.[72] In the majority of studies, PDE4 inhibition did not significantly improve alveolarization, and we speculate that this may be related to the growth retardation seen in animals treated with PDE4 inhibitors, which may have important clinical implications for the neonatal population.[70–72]

The PDE4 inhibitor, roflumilast is FDA-approved for use in adults with COPD. In patients with moderate-to-severe COPD, roflumilast improves lung function and lowers the risk of exacerbation in some patients.[73,74] However, in these studies, rates of gastrointestinal adverse events were high, including diarrhea and weight loss. Thus, this represents a major barrier to utilizing PDE4 inhibitors in practice, especially in the pediatric and neonatal populations. One approach to improve tolerability and mitigate adverse effects is to develop inhaled formulations, which should avoid systemic effects, and is currently being evaluated in animal models and adult COPD patients.[75] Furthermore, PDE4 is comprised of 4 isoforms: A-D. Studies have shown that PDE4B inhibition is predominantly responsible for the anti-inflammatory effects of the PDE4 inhibitors, while PDE4D may be the primary cause of emesis and poor weight gain,[75,76] and thus, it may be beneficial to study isoform-specific PDE4 inhibition.

PDE5

PDE5 hydrolyzes cGMP. Thus, agents that inhibit PDE5 lead to increased cGMP concentrations, resulting in vasorelaxation and inhibition of cellular proliferation. PDE5 is the most abundant PDE in the pulmonary vasculature, and therefore is the most well-studied for its role in PH.[77,78] PDE5 expression is increased in the lungs of patients with PH[79] in animal models of PH,[80] and following exposure to hyperoxia.[81,82] Multiple experimental animal models of PPHN demonstrate that PDE5 inhibition decreases PVR and improves oxygenation.[83–85] In chronic hypoxia-induced PH in newborn piglets, a single dose of oral sildenafil decreased PAP and PVR compared to control and did not impact systemic hemodynamics.[86] Notably, prophylactic sildenafil did not prevent the development of PH in hypoxia-exposed piglets, which contrasts with the findings in adult animal models of hypoxia-induced PH.[87,88]

Sildenafil is a well-established therapeutic option in adults with symptomatic PH, with notable improvement in pulmonary hemodynamics and enhanced exercise capacity.[89,90] Despite the compelling adult and animal data, and although sildenafil is commonly utilized as a second-line agent in neonates with PPHN,[91] no large RCTs evaluating the use of sildenafil in this population have

been published. In an open-label dose-escalation trial of 36 neonates with PPHN, IV sildenafil was associated with a sustained reduction in OI.[92] Hypotension occurred in five patients, necessitating discontinuation of the drug in three cases, one patient went on ECMO, and one died. Most patients received iNO + sildenafil which was demonstrated to be safe, while six neonates never required iNO after initiation of sildenafil.[92] In resource-limited settings where iNO is cost-prohibitive and ECMO is not readily available, oral sildenafil appears to be a reasonable alternative based on the results of three RCTs in neonates with PPHN.[93–95] In the largest of the three studies, an RCT of 51 term infants with PPHN, oral sildenafil given every 6 hours resulted in improved oxygenation compared to placebo.[95] Moreover, oral sildenafil improved mortality, which was 40% in the placebo group, compared to only 6% in the sildenafil group.[95] A 2017 meta-analysis evaluating the use of sildenafil for PPHN, utilizing data from 166 enrolled patients in five eligible trials, concluded that sildenafil may be of benefit in improving oxygenation and reducing mortality, specifically in resource-poor environments in which iNO is not readily available.[96] However, they did not find a survival benefit when sildenafil was compared to an active control (magnesium sulfate)[97] or when combined with iNO.[98] Two recent RCTs evaluating the use of sildenafil alone compared to sildenafil + milrinone[63] or sildenafil + bosentan[99] in neonates with PPHN both concluded that combination therapy was more effective than either monotherapy. A few retrospective chart reviews evaluating the use of sildenafil in BPD-associated PH have reported reduction in PAP,[100,101] but no RCT has been reported in this population despite its widespread use.[102]

The use of sildenafil in neonates with PH is still being actively investigated. A large, international, multicenter trial evaluating the use of IV sildenafil or iNO in infants with CDH-associated PH is currently enrolling.[103] Finally, a large, multicenter RCT evaluating the use of IV sildenafil in PPHN completed enrollment with 59 patients, and results are pending (https://clinicaltrials.gov/, NCT01720524).

Tadalafil, another PDE5 inhibitor is FDA-approved for use in adults with PH and has a longer half-life than sildenafil, allowing for the convenience of once-daily dosing. In a prospective, open-label study of 25 patients aged 2 months to 5 years who were started on daily tadalafil either as initial therapy or transitioned from sildenafil, tadalafil improved mean PAP and was well tolerated.[104] In two recent RCTs, comparing the efficacy of tadalafil and sildenafil in infants with PH, investigators found no difference between the two treatments.[105,106]

Rho-kinase Inhibitors

Emerging evidence implicates rho-kinase (ROCKs) for a role in vascular tone and remodeling, and thus, an important contributor to neonatal pulmonary hypertension. The small GTP-binding protein Ras homolog gene family member A (RhoA) acts on the serine-threonine kinase, ROCKs, which inhibits myosin light chain phosphatase, resulting in sustained smooth muscle cell contraction.[107] The RhoA/ROCK pathway activity has been found to be increased in experimental animal models of PH, including in pulmonary arteries of neonatal rats.[108] In fetal lambs, brief intrapulmonary infusions of the ROCK inhibitors, Y-27632 and HA-1077, resulted in potent pulmonary vasodilation.[109] Furthermore, treatment with Y-27632 prevented vasoconstriction induced by inhibition of endogenous NO production.[110] These data are compelling in that they elucidate a potential role for RhoA kinase in the maintenance of high PVR in utero, and the rapid drop in PVR in the transitional circulation, although mechanistic pathways remain unknown. Furthermore, several studies have demonstrated complex reciprocal interactions between NO and the RhoA/ROCK pathway. In a rat model of bleomycin and hypoxia-induced PH, the elevated PVR did not respond acutely to inhaled or systemic NO yet normalized completely after giving a ROCK inhibitor.[108] These data suggest that the RhoA/ROCK pathway may be responsible for the suboptimal response to iNO seen in some neonates with PPHN. Furthermore, RhoA/ROCK pathway is involved in both the serotonin and PPARγ pathways, which are also undergoing interrogation for their role in the pathophysiology of PH (Fig. 2).

The ROCK inhibitor, fasudil, is currently being investigated in clinical trials in adult and pediatric PH patients in China and Japan. Overall, studies in adults utilizing fasudil for PH have shown promising results.[111–114] In prospective studies in adults with PAH, IV fasudil decreased PVR by 17%[112] and long-acting oral fasudil improved cardiac index from baseline.[113] A randomized clinical trial in 209 hospitalized adults with PH and right heart failure demonstrated markedly improved in-hospital mortality and 30-day rehospitalization rates.[114] In these studies, adults were receiving maximal therapeutic treatment of PH, including multiple other pulmonary vasodilators. Interestingly, fasudil was well tolerated and had no significant impact on systemic hemodynamics, suggesting that it is at least somewhat selective for the pulmonary vasculature.[112] In a prospective study of 12 pediatric patients with a mean of 12.3 years diagnosed with congenital heart disease (left-to-right shunt) and mild-to-moderate PH, treatment with IV fasudil led to a significant decrease in PAP and PVR, and an increase in cardiac output, PBF, and mixed venous oxygen saturation.[115] While the investigators did note a small drop in systemic arterial blood pressures and SVR, there was an overall decrease in the PVR/SVR ratio.[115] No studies have been done in neonates with PH.

Endothelin Receptor Blcokers

In PH pathophysiology, there is a decrease of vasodilator mediators and an increase of vasoconstrictor mediators. One such vasoconstrictive mediator is endothelin (ET-1) which acts on

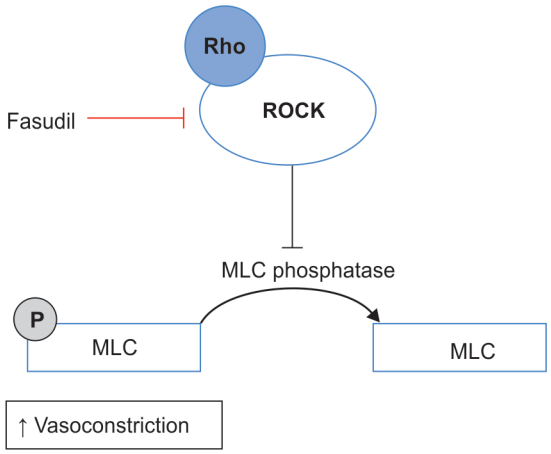

Fig. 2: Rho-kinase is an effector of the small GTPase RhoA. Activation of the RhoA-ROCK pathway results in inhibition of MLC phosphatase, resulting in greater phosphorylated MLC and sustained smooth muscle contraction. Fasudil is a potent ROCK inhibitor. ROCK, rho-kinase; MLC, myosin light chain

endothelin receptors in the smooth muscle cell and increases ionic calcium concentration, resulting in vasoconstriction. ET-1 has been found to be elevated in infants with PPHN.[116] Early on, medications classified as endothelin receptor antagonists were discovered in lamb models to block the binding of endothelin to its receptors, thereby negating endothelin's deleterious effects on the pulmonary vasculature (Fig. 3).[30,117]

Bosentan, an endothelin receptor antagonist, was FDA approved in 2017 for the treatment of pulmonary hypertension for patients ages 3 years and older. In an open-label prospective study of 19 pediatric patients between 10 and 40 kg, pharmacokinetics were found to be similar to that of adults.[118] Improvements in PAP and PVR were significant. Bosentan was well tolerated, and while there were small decreases in systemic blood pressure, no symptomatic hypotension was observed.[118] Fifty-eight percent of the study patients were receiving IV epoprostenol and the use of the two drugs together appeared to be safe.[118] A database search in 2009 included 21 studies, both retrospective and prospective, examining the evidence on the effectiveness and safety of bosentan in the treatment of pediatric arterial hypertension.[119] The authors state that bosentan appears to improve long-term functional status and hemodynamics in children with PAH without safety concerns. Adverse events include liver enzyme elevations were seen less frequently than in studies utilizing bosentan in the adult population[119] Several retrospective studies examining long-term outcomes of pediatric patients with PAH treated with bosentan report stabilization or improvement in WHO functional class, with no major safety concerns.[120,121]

In a 2018 retrospective chart review of infants with PPHN, the combination of iNO + bosentan or bosentan alone both improved oxygenation.[122] Additionally, several RCT utilizing treatment with bosentan have been performed in neonates with PH. In a randomized, double-blind, placebo-controlled trial of 24 infants given bosentan for PPHN, OI decreased by an average of 10 and mechanical ventilation days decreased by 7.2 days.[123] In the FUTURE-4 trial, an exploratory trial designed to assess feasibility of enteral bosentan as adjuvant to iNO in neonates with PPHN, the investigators assessed the safety and pharmacokinetics of bosentan in 21 neonates who received the study drug.[124] Blood concentrations of bosentan were variable in the first 12 hours after administration and did not reach steady state until day 5. Overall, bosentan was well tolerated, and no adverse effects on systemic hemodynamics or liver transaminases were noted.[124] While the study was not powered to evaluate efficacy, the investigators reported that oxygenation and time on iNO and mechanical ventilation were not improved in the bosentan group.[124] Overall, while several studies have demonstrated the safety and feasibility of bosentan use in neonates with PPHN, the efficacy remains largely unknown. Mixed results could be due, in part, to trials with small numbers of participants and the use of multidrug therapy. Notably, bosentan is only available orally, and thus in critically ill neonates, there may be delayed or variable absorption of the medication.

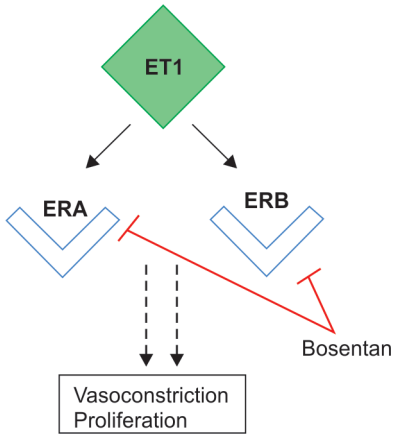

Fig. 3: Endothelin acts on endothelin receptors in the smooth muscle cell and increases ionic calcium concentration resulting in vasoconstriction. Bosentan is an endothelin receptor antagonist. ET-1, endothelin; ER, endothelin receptor

PPARγ

Peroxisome proliferator-activated receptors (PPARs) are members of the superfamily of nuclear receptors. They are ligand-activated transcription factors that exert their effects by binding to DNA and altering gene expression. PPARs play a major role in energy homeostasis, specifically lipid metabolism. The most well-studied of the three subtypes, PPARγ, has also been studied for its role in inflammation and cellular proliferation[125] and is highly expressed in both pulmonary vascular endothelial cells and smooth muscle cells, as well as adipocytes.[126,127] Importantly, metabolic derangements are common in PH and significantly worsens the disease course. Thus, treatment of metabolic dysfunction in PH patients may be of value to prevent disease progression.[126] Epithelial cell PPARγ-deficient mice develop airspace enlargement with decreased tissue resistance and increased lung volumes, suggesting a role for PPARγ in alveolar development.[128] Many experimental animal models have implicated a role for PPARγ in the development of PH. In a rat model of PPHN and in adults with PAH, PPARγ expression was decreased.[129,130] Mice with targeted deletion of PPARγ in smooth muscle cells spontaneously developed PAH, characterized by RVH, elevated RV systolic pressure, and distal pulmonary artery muscularization.[131] In a rat model of chronic hypoxia-induced PH, the PPARγ agonist, rosiglitazone, attenuated PA remodeling and prevented muscularization of the distal arterioles. Moreover, it reversed the vascular remodeling and arteriole muscularization in mice previously exposed to chronic hypoxia.[132] However, while this agent attenuated RVH, PA pressures remained significantly elevated in this model, suggesting PH.[132] In an experimental BPD model in which newborn rats were exposed to chronic hyperoxia, both antenatal and neonatal administration of rosiglitazone enhanced lung maturation and ameliorated lung injury in pups compared to controls (Fig. 4).[133,134]

The pathogenic mechanisms of PPHN are complex and likely involve the interaction of multiple key pathways. In a fetal lamb model of PPHN, inhibition of the RhoA/ROCK pathway results in restoration of PPARγ activity, whereas PPARγ inhibition increased ROCK activity and proliferation in PASMC.[135] Additionally, it has been shown that ET-1 decreases PPARγ activity, leading to pulmonary artery endothelial cell (PAEC) dysfunction and impaired angiogenesis. In a fetal lamb model of PPHN, ET-1 decreased PPARγ activity and reduced endothelial cell tube formation of isolated PAEC.[129] The addition of a PPARγ agonist restored endothelial cell tube formation and increased endothelial nitric oxide synthase (eNOS) activity and NO production.[129] Thus, PPARγ agonists may be most beneficial in PPHN when used in combination with a bosentan, endothelial receptor antagonist or fasudil, a ROCK inhibitor.

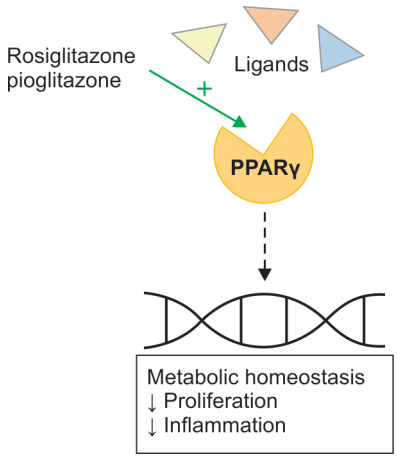

Fig. 4: PPARγ are ligand-activated transcription factors that exert their effects by binding to DNA and regulating gene expression. PPARγ is important in metabolic health and energy homeostasis and its activation has been shown to be beneficial in models of PH, including decreased proliferation and inflammation. Rosiglitazone and pioglitazone are two PPARγ ligands. PPARγ, peroxisome proliferator-activated receptor gamma

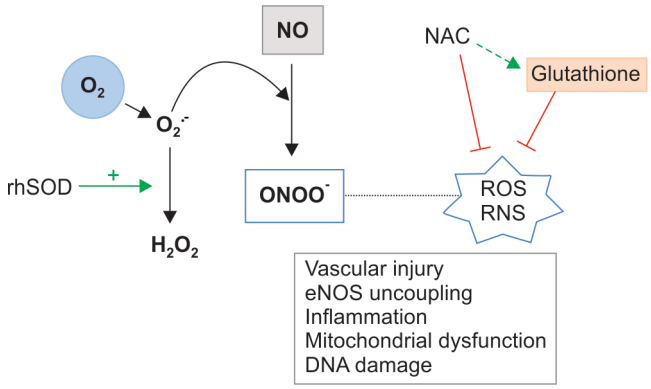

Fig. 5: In diseased states, NO can combine with $O_2^{\cdot-}$ to form the damaging oxidant, $ONOO^-$. $ONOO^-$ along with other ROS and RNS can have severe adverse effects in pulmonary hypertension including vascular dysfunction, eNOS uncoupling, inflammation, and alterations in metabolism. The antioxidant, rhSOD can eliminate $O_2^{\cdot-}$, thus decreasing the formation of $ONOO^-$. NAC has both direct and indirect antioxidant effects and is a precursor to glutathione, which is a potent cellular antioxidant. O_2, oxygen; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; $ONOO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; rhSOD, recombinant human superoxide dismutase; NAC, N-acetylcysteine

Two PPARγ agonists, rosiglitazone and pioglitazone, were approved by the FDA in 1999 for the treatment of adults with Type 2 diabetes mellitus. However, their use was later restricted after it was discovered that they can cause or exacerbate congestive heart failure. While preclinical PH models utilizing PPARγ agonists show promising results, human studies are lacking for this clinical indication. Moreover, no studies have been done on children or neonates.

Antioxidants

Pathologic changes in PH can be mediated by free radical damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS).[136,137] Oxidative stress results when there is an overproduction of ROS and RNS that overwhelms the antioxidant defenses and can result in alterations in energy metabolism, inflammation, cellular proliferation, DNA injury, and vascular dysfunction.[137] NO and hyperoxia, two common treatments for PPHN, may alter these homeostatic conditions. Under normal conditions, NO combines with oxyhemoglobin to form nitrate. However, in diseased states, NO can combine with superoxide to form the damaging oxidant, peroxynitrite.[138] Peroxynitrite leads to vascular endothelial dysfunction by multiple methods, including oxidation of the NOS cofactor, tetrahydrobiopterin (BH4), resulting in eNOS uncoupling.[139] Additionally, it can lead to inactivation of prostacyclin synthase, decreasing levels of the vasodilator, prostacyclin, and increasing levels of vasoconstrictors.[138] Oxidant stress and hydrogen peroxide and superoxide generation have been implicated in animal models of PPHN,[140–143] as has eNOS uncoupling.[138,144,145] Overall, these studies suggest that there is an increased burden of oxidant stress and a deficiency in antioxidant activity in neonates with PH (Fig. 5).

N-acetylcysteine (NAC) is a precursor to glutathione, important in antioxidant defense, and acts as a direct ROS scavenger.[146] It is FDA-approved for the use of acetaminophen overdose resulting in hepatotoxicity and is trialed in adults with COPD. In a model of acute lung injury created by intratracheal administration of meconium, adult rabbits who received NAC had a reduction in lung inflammation and peroxidation and improvement in oxygenation compared to controls.[147] In fetal sheep PASMC, the administration of NAC prevented the hyperoxia-induced increase in PDE5 activity and restored cGMP concentrations.[80]

Despite the promising results in animal data, studies in neonates have been less positive.[148,149] An RCT of 391 extremely low birth weight infants examining the impact of NAC on death or BPD showed that there was no difference between the two groups, with 51% of infants in the NAC group meeting criteria for BPD or death compared to 49% of infants in the control group.[149] However, a recent RCT has garnered significant attention and placed an emphasis on the need for further research of NAC for BPD prevention in extremely preterm infants. In the study, antenatal administration of NAC to pregnant women with impending preterm birth resulted in less resuscitation at birth and was protective against the development of BPD, which was only 3% in the NAC-exposed group vs 32% in the control group.[150]

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. SOD, a predominant antioxidant enzyme in the pulmonary vasculature, is decreased in pulmonary arteries from neonates with PH,[151] which results in reduced availability of NO secondary to NO-superoxide interactions.[152] In a lamb model of PPHN, mechanical ventilation with 100% oxygen increased ROS burden, blunted the expected rise in eNOS expression, and decreased BH4 levels. While treatment with either iNO or recombinant human SOD (rhSOD) decreased ROS production and increased eNOS expression compared to PPHN lambs ventilated with 100% oxygen alone, only rhSOD restored eNOS function.[153] In a few studies utilizing lamb models of PPHN, investigators demonstrated that intratracheal rhSOD improves oxygenation and causes pulmonary vasorelaxation. Importantly, this was enhanced by the combination use of rhSOD and iNO.[154,155] Interestingly, two placebo-controlled RCT published in 1996 and 1997, enrolled a total of 59 preterm infants and evaluated the use of single and multiple intratracheal doses of rhSOD given 30 minutes after surfactant.[156,157] Both studies demonstrated enhanced SOD activity in the serum, urine,

and tracheal aspirates and decreased neutrophil chemotactic activity and albumin concentration in tracheal aspirates of treated infants.[156,157] A large RCT involving 302 premature infants randomized to receive rhSOD vs placebo every 48 hours for up to one month found no difference in the incidence of death or BPD at 28 days or 36 weeks corrected gestational age.[158] However, follow-up data on 80% of the enrolled infants at one year of age showed a 36% reduction in wheezing that necessitated treatment with asthma medications. Furthermore, in infants <27 weeks, there was a 55% decrease in emergency room visits and a 44% decrease in hospitalizations.[158]

Conclusion

In conclusion, despite advances in medical care for the neonate with PPHN, overall morbidity and mortality remain high. Our understanding of the pathobiology of PH in neonates continues to evolve, and with it, the emergence of new therapeutic compounds. However, definitive trials in this population are lacking. Sildenafil, the most well-studied of the adjuvant therapies, appears to be a safe and effective alternative when iNO is not available, although it is less clear if it provides benefit when used in combination with iNO or as a rescue therapy after failed iNO. Unfortunately, for many of the other therapies discussed in this review, while the animal data are compelling, studies in adult and pediatric patients have not clearly demonstrated efficacy, as in the case of endothelin receptor blockers and antioxidants, or safety, as in the case of PDE4 inhibitors and PPARγ agonists. Researchers are attempting to overcome many of these obstacles, in part, by studying various delivery mechanisms, including inhalational. Additionally, due to the complex interactions of the multiple pathways involved in PPHN, it may be beneficial to use combination therapies to target two or more underlying mechanisms, which is also being investigated. Finally, while the majority of therapies are being interrogated for their use as rescue therapy in the iNO-unresponsive infant, it is also prudent to target therapies that work synergistically with iNO and enhance iNO-responsiveness in neonates with PPHN.

Orcid

Julie A Dillard https://orcid.org/0000-0003-3391-5154

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REVIEW ARTICLE

Necrotizing Enterocolitis Associated with Congenital Heart Disease—A Review Article

Sriya Roychaudhuri[1], Gurpreet Grewal[2], Sakethram Saravu Vijayashankar[3], Pascal Lavoie[4], Akhil Maheshwari[5]

Abstract

Necrotizing enterocolitis (NEC) is a relatively rare but devastating entity associated classically with the preterm cohort in the neonatal intensive care unit. Preterm and term babies with congenital heart disease are at risk of a number of comorbidities because of the hemodynamic derangements due to a structurally abnormal heart and the corrective procedures adopted. Necrotizing enterocolitis is one of the dreaded complications associated with this cohort and impacts the course of these babies in the hospital in a major way. A large majority of term babies with NEC are in the backdrop of a significant congenital cardiac lesion. This review article summarizes the literature and elaborates this entity including its specific features, risk factors associated with its causality, histopathology and related aspects of hemodynamics, and feeding in this vulnerable population. It also provides insight into modifiable risk factors and early markers of detection of gut necrosis to facilitate prevention and early detection. It highlights the subtle but definite difference in outcome variables to help physicians enable the parents of babies with heart disease to develop a better understanding of the entity and its expected course while counseling.

Keywords: Congenital heart disease, Necrotizing enterocolitis, Neonatal mortality, Preterm newborns.

Newborn (2022): 10.5005/jp-journals-11002-0016

Introduction

Necrotizing enterocolitis (NEC) is one of the most severe gastrointestinal emergency conditions in the neonatal period, with high morbidity and mortality rates. Nearly 85–97% of all cases of NEC occur in premature infants.[1] The other 3–15% occur in full-term infants with predisposing factors such as maternal cocaine exposure, intrauterine growth retardation, birth asphyxia, and congenital heart disease (CHD).[2–5]

Any cardiac lesion causing hemodynamic alterations in the gut could cause ischemia and NEC.[6] Poor outflow and poorly oxygenated systemic circulation, due to left-sided cardiac lesions, heart failure, or congenital cyanotic heart disease could all result in gut necrosis.[4,5] Consequently, the severity and complexity of cardiac lesions seem to have a major impact on the causality of NEC. The rate of NEC in term newborn infants with symptomatic CHD has been estimated to be 10–100 times higher than in those with structurally normal hearts,[4] with an estimated incidence ranging from 1.6 to 9%.[4,7–10] The mortality rates in infants who are born with CHD and go on to develop surgical NEC can be as high as 20–30%.[11] Infants with single ventricular physiology are at the greatest risk, and NEC can be fatal in up to 97% of these infants.[12–14] These infants also have considerable morbidity related to severe intestinal injury, and many develop intestinal strictures, short bowel syndrome, and neurodevelopmental delay.[12,14]

In a systemic review, Siano et al.[1] described cardiogenic NEC to be an entity that was distinct from the classical, idiopathic NEC seen in the preterm gut. The demographics, pathogenic mechanisms, and outcomes differ in the two cohorts. They showed that the presence of a CHD increased the incidence of NEC in very low birth weight (VLBW) infants only slightly from 6.3 to 8.9%. Fewer infants with CHD and NEC required surgery (31 vs 66% in classical NEC), but the overall mortality in these infants was higher.[15] Most cardiogenic NEC (50–70%) occurred in the postoperative period.[8,15]

Prematurity continues to be an independent risk factor for NEC, where the likelihood of developing NEC remains higher in preterm in infants recovering after surgery for CHD, even mild medical NEC impeded feeding and weight gain resulting in prolonged hospital stay.[19–21] The pathophysiology of NEC in CHD patients is still unknown, but is thought to be multifactorial with lower bowel perfusion pressures due to poor diastolic pressures along with lower systemic oxygenated blood flow. This may have caused bowel ischemia, loss of gut wall integrity, and bacterial overgrowth.[9,12]

[1]Department of Pediatrics, Division of Neonatology, BC Women's Hospital, Vancouver, Canada

[2,4]Department of Neonatal-Perinatal Medicine BC Women's Hospital and Health Centre, British Columbia, Vancouver, Canada

[3]Department of Pediatric Cardiology, BC Children's Hospital, British Columbia, Vancouver, Canada

[5]Department of Neonatal-Perinatal Medicine, Global Newborn Society, Baltimore, Maryland, United States of America

Corresponding Author: Sriya Roychaudhuri, Department of Pediatrics, Division of Neonatology, BC Women's Hospital, Vancouver, Canada, Phone: + 17789842320, e-mail: dr.sriyarc@gmail.com

How to cite this article: Roychaudhuri S, Grewal G, Vijayashankar SS, *et al*. Necrotizing Enterocolitis Associated with Congenital Heart Disease—A Review Article. Newborn 2022;1(1):170–176.

Source of support: Nil

Conflict of interest: None

Histopathological Characteristics of NEC in Infants with CHD

The pathophysiology of NEC in infants with CHD which is likely to develop primarily due to gut ischemia may differ from that of NEC in preterm infants, which is thought to result from a mixture of inflammatory and vascular injury.[22] The incidence of NEC in premature infants with CHD is actually higher than those with

idiopathic NEC, presumably because these infants not only have the classic risk factors of preterm babies but also the hemodynamic changes observed in CHD.[4,20,23–25]

Existing studies on cardiogenic NEC emphasize the importance of vascular phenomena involving mesenteric hypoperfusion from diastolic steal and flow reversal in the abdominal aorta.[26,27] A recent retrospective case–control study revealed that postnatal age at onset was lower in CHD NEC patients than in preterm infants (4 [2–24] vs 11 [4–41] days, p <0.001). The pH nadirs were lower in the CHD cohort (7.21 [7.01–7.47] vs 7.27 [6.68–7.39], $p = 0.02$) in the one with structurally normal heart.[23]

Interestingly, the contribution of inflammation and bacterial colonization may not be different between cardiogenic and idiopathic NEC, although there may be more neutrophils in the lesions of NEC associated with CHD.[23,28] In one study, the highest C-reactive protein levels were higher in cardiogenic NEC (112.5 mg/L [5.0–425.0] vs 66.0 [5.2–189.0], $p = 0.05$).[23] The anatomic localization of the disease may be different, with some studies showing more lesions in the colon,[23] but not others.[29] The colon can be involved more often than would be predicted based on the gestational age (86 vs 33%, $p = 0.03$). Mortality caused by NEC was not different (22 vs 11%, $p = 0.47$).[23]

The histopathological characteristics of NEC in infants who have CHD vs those who have a structurally normal heart are not markedly different. Diez et al.[12] described that infants with cardiogenic NEC seemed to have more prominent macroscopic necrosis, but also had a higher bacterial load noted on histopathology. One example of CHD-related NEC is shown in Figure 1.

Specific Cardiac Abnormalities Associated with NEC

Many cardiac anomalies are associated with increased risk of NEC (Table 1). Infants with single ventricular physiology are at the greatest risk of intestinal injury and NEC, which can be fatal in up to 97% of these infants.[12] Hypoplastic left heart syndrome (HLHS), which constitutes 7% of all CHD, is the single most common heart lesion associated with NEC.[18,30,31] Infants with HLHS and other single ventricle defects sustain dramatic hemodynamic challenges in the first few days after stage 1 palliative shunt surgery which aims to improve the systemic perfusion by shunting it from the pulmonary circulation.[18] In this process, the gut undergoes periods of hypoperfusion and is at risk of ischemic injury.[30] Lopez et al.[32] examined the National Pediatric Cardiology-Quality Improvement Collaborative (NPC-QIC) database to compare mid-term that is interstage outcomes for NEC in infants with HLHS. NEC was seen in 5.8% (68 of 1163) with preterm infants with HLHS. The incidence was higher in those born at <37 weeks (18.3%, 11 of 60) compared to 5.2% (57 of 1103) in gestational age >37 weeks. ElHassan et al.[18] examined a larger retrospective cohort of HLHS babies who went on to develop NEC in 41 hospitals over 10 years. Of the 5720 infants with HLHS, 349 patients (6.1%) were diagnosed with medical or surgical NEC. Fifty-two patients (0.9%) required laparotomy or percutaneous abdominal drainage.

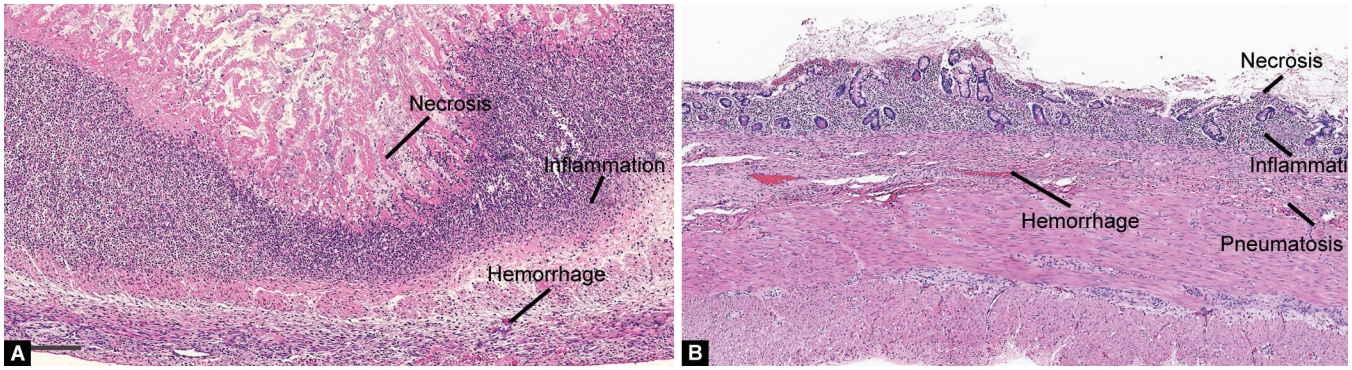
Table 1: Outcomes in mild and severe CHD vs controls

	Mild CHD, n = 130	*Severe CHD, n = 40*	*Controls, n = 4508*	*p value*
NEC (%)	5 (4)	4 (10)[a]	109 (2)	0.014
Mortality (%)	22 (17)[a]	16 (40)[a]	234 (5)	<0.001

p value corresponds to χ^2 analysis (exact test) or analysis of variance including all groups. [a]Pairwise comparison vs controls, p <0.025 (Fischer's exact test with Bonferroni correction). (*From:* Motta et al. Journal of Perinatology 2015;35:949–953. CHD, congenital heart disease; NEC, necrotizing enterocolitis)

Bain et al.[33] looked at the incidence of NEC in VLBW infants with atrial and ventricular septal defects (ASDs and VSDs), or both, and found that the incidence of NEC was 6.2% in infants without septal defects, 9.3% in those with an ASD, 7.8% in those with a VSD, and 10.3% in infants with both an ASD and a VSD. Compared with infants without septal defects, the adjusted odds ratios for developing NEC for each group, ASD alone, VSD alone, and those with both an ASD and a VSD were 1.26 (95% confidence interval (CI) 1.07–1.49), 1.27 (1.07–1.51), and 1.79 (1.03–3.12), respectively. They concluded that septal defects did increase the risk of NEC. The association could be due to direct factors causing hemodynamic alterations like cardiac surgery and cardiopulmonary bypass or due to other factors like an elevated circulating endotoxin and proinflammatory cytokines in the background of altered mesenteric blood flow leading to intestinal ischemia thus encouraging bacterial overgrowth with intestinal breakdown.[33]

Diez et al. looked into the characteristics of preterm newborns with NEC with or without a patent ductus arteriosus (PDA) or a CHD. Lesions causing a diastolic steal commonly seen in hemodynamically significant PDAs are often implicated in NEC.[12,34] In another study, Harkin et al. noted increased risk of NEC following ligation of PDA.[35]



Figs 1A and B: Hematoxylin and eosin-stained microphotographs of resected (A) Distal jejunum; and (B) Colon from a 28-week gestation infant with HLHS show typical features typical of NEC, including necrosis, inflammation, and interstitial hemorrhages. Scale bars: 100 µm

Becker et al.[14] examined a large multicenter cohort with CHD, and noted the risk of NEC to be higher in infants with various forms of duct-dependent CHD and were on prostaglandin E infusion. This association was the strongest in those with single ventricle heart defects such as HLHS, tricuspid atresia, and hypoplastic right ventricle syndrome. They also examined this cohort for the association of NEC with feeding, and noted that the risk was highest in infants with HLHS. The incidence of NEC was higher in any single ventricle heart disease, even after adjusting for gestational age. In their own cohort, infants born at an earlier gestational age and with lower birth weights had a higher incidence of NEC.[12,21] Enteral feeding seemed to increase the odds of NEC but the difference was not statistically significant. The median time of starting feeds was seen to be later in patients who went on to develop NEC as compared to those who did not (5 days vs 2 days postoperative). NEC occurred mostly in the postoperative period after feedings were started but did not seem to be related to feeding regimens.[5]

HLHS, which causes major hemodynamic compromise and may need multiple surgeries, had a major impact on the quality of life with a 9% incidence of NEC.[15] In other duct-dependant lesions, NEC was seen in 5% infants. When the complexity of the cardiac anomaly was quantified using the Risk Adjustment for Congenital Heart Surgery criteria, a score >2 was more likely to develop NEC.[36] Motta et al.[37] examined a cohort of preterm infants to determine whether *severe* CHD had an independent association with NEC. They defined *severe lesions* as cyanotic or left-sided obstructive lesions, or those associated with congestive heart failure. Mild lesions included septal defects and various other lesions not included in the list of severe ones. Among 4678 infants, 170 (3.6%) had CHD and 118 (2.5%) developed NEC. The risk for NEC increased with severe CHD (adjusted relative risk (RR) = 3.72; 95% CI = 1.37–10.10 but not with mild CHD (RR = 0.65; CI = 0.27–1.55).

Hemodynamic Changes and Gut Perfusion in CHD

The risk of NEC in infants with CHD suggests that altered intestinal perfusion and consequent gut wall ischemic–reperfusion injury may trigger the cascades of mucosal breakdown and bacterial translocation typically associated with NEC.[38,39] The histopathological changes of NEC do not differ from those who developed NEC related to prematurity and did not have CHD.

The specific hemodynamic changes that increase the incidence of NEC in infants with HLHS still remain unclear. Diez et al.[12] described how reduced perfusion is documented in a large number of infants with CHD or PDA who developed NEC. The location of gut necrosis in CHD vs patients with normal cardiac anatomy was noted to be similar, in the small intestine. In other studies, Bubberman et al.,[23] Diez et al.,[12] and Giannone et al.[3] found most lesions in the colon. The risk of ileal and colonic injury might be related to inferior collateral blood supply and consequent tissue hypoxia.

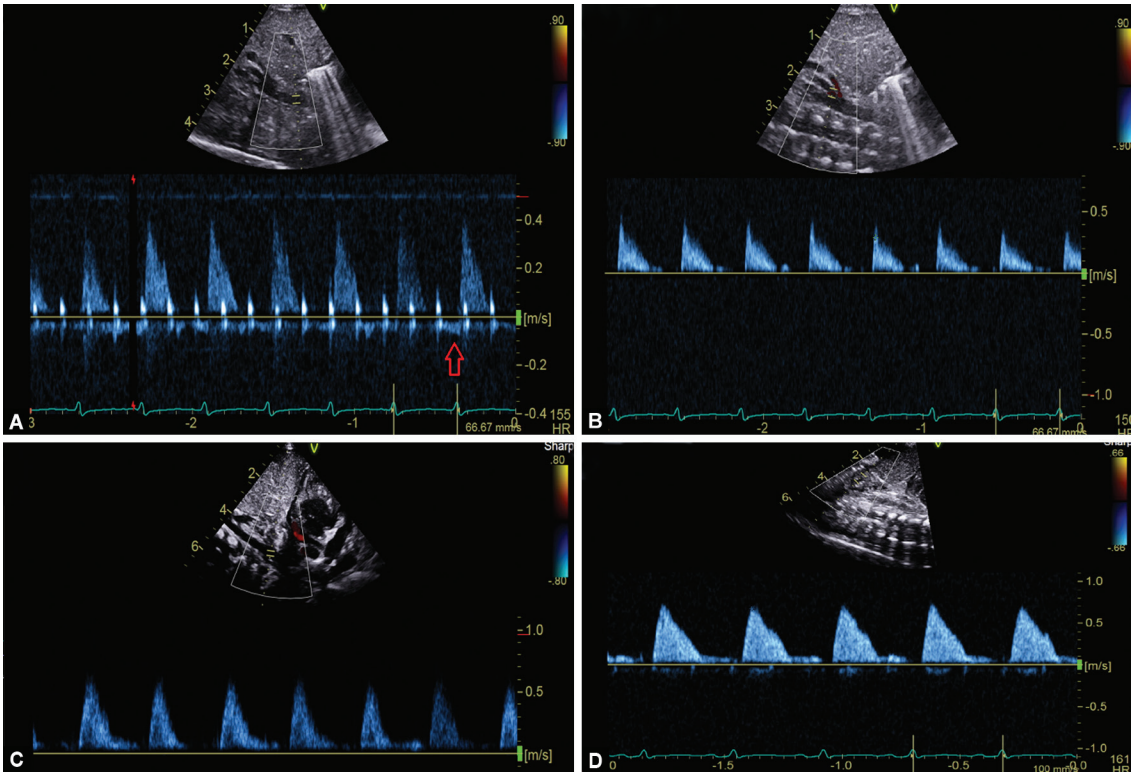
In patients who develop NEC, the diastolic velocities of DA were significantly lower, and were even reversed in some. The ratio of diastolic reverse to systolic forward flows was significantly increased in the NEC group. In addition, the resistive index of DA was an independent risk factor for the development of NEC.[12] In another study, Papneja et al.[40] noted that lower descending artery flows in ultrasound Doppler studies in infants with small left-sided heart structures were associated with feeding intolerance and NEC. Figure 2 shows typical Doppler changes related to a large PDA with systolic steal, compared to those from a structurally normal heart with no PDA.

Two separate studies by Cheung et al.[41] and Castillo et al.[34] examined the postprandial mesenteric blood flow velocities in cohorts with palliative shunts for single ventricle physiology. Cheung compared the basal and postprandial mesenteric blood flow velocities and vascular resistance in infants after shunt palliation with those in non-shunted infants with underlying heart disease. They selectively looked at the systemic to pulmonary or the modified-blalock taussig shunt (m-BTS) which is known to reverse diastolic flow in the mesenteric vasculature. The correlation between these factors was complex. Disturbances of splanchnic perfusion were noted in the shunted cohort with their superior mesenteric artery (SMA) blood flow being either absent or reversed during diastole both prior to, and after feeding. However, there was also some compensatory lowering of resistance of the splanchnic circulation in the shunted population. Johnson et al.[42] compared mesenteric blood flow in m-BTS cohort to the right ventricle-to-pulmonary artery conduit or the Sano shunt population, postprandially. The Sano shunt was expected to improve systemic including mesentery perfusion due to better diastolic blood flow in the descending aorta. However, they saw similar changes in the SMA and celiac artery blood flow, and an unexpected absence of increase in forward flow in the postprandial period in the Sano cohort. Thus, the risk of NEC was higher in infants with palliative shunts in both studies.

Miller et al.[43] noted that low cardiac output and diastolic run-off in HLHS might be only inconsistently associated with NEC. They suggested these defects may alter the development of systemic vascular beds, and that those with low abdominal aorta pulsatility index have significantly higher chances of developing NEC. Univentricular physiologies presenting with heterotaxy syndromes pose the complex scenario of unstable hemodynamics with intestinal malposition. Sharma et al.[44] showed that the timing of Ladd's procedure in such babies needs careful consideration as the chances of NEC increases if it was done prior to establishing a more balanced circulation, ideally after cavopulmonary connection. In another study, Van der Heide et al.[27] looked at two decades of retrospective data of near-term infants with CHD. They noted that infants who had birth asphyxia with lower Apgar scores were at higher risk of NEC.

Cardiac Surgery, Perioperative Risk Factors, and NEC

Other than critical cardiac lesions, NEC can also result due to other associations of CHD like prostaglandin therapy, inotrope use,[14] cardiopulmonary bypass (CPB), extracorporeal membrane use,[45] red cell transfusions, especially if multiple.[19] The postoperative period is particularly vulnerable for gut necrosis resulting in significant morbidity and mortality.[9,38,46] The surgical procedure for the cardiac lesion itself compromises the gut flow with patients needing longer times of cardiopulmonary bypass having increased chances of NEC in the postoperative period.[47] Lopez et al. found that the incidence of extracorporeal membrane use was higher and HLHS patients who developed NEC were more likely to have moderate to severe atrioventricular valve regurgitation at times of discharge from the NPC-QIC database analysis.[45] In another study, Weiss et al.[48] noted the rates of intra-abdominal complications to be more frequent in infants



Figs 2A to D: (A, B) Pulse wave Doppler signals through the abdominal aorta showing diastolic reversal of flow in (A) and normal forward flow in (B); (C, D) Pulsed Doppler signals through splanchnic circulation showing blunted peak systolic velocity in (A) and normal systolic velocity in (B); (A), (C) are from a patient with a large patent ductus arteriosus (PDA) with systolic steal while (B), (D) are from a structurally normal heart with no PDA

who underwent the hybrid procedure than those with the Sano or m-BTS procedure.

During the postoperative period, the diagnosis of NEC can be challenging. Clinical signs may be difficult to elicit and characteristic radiologic signs may note be notable. Delayed diagnosis and the presence of unusual pathogens may increase morbidity and mortality.[29] The gastrointestinal (GI) system may be particularly prone to complications after cardiac surgery because of the interactions between the GI and cardiovascular systems. Complications may result from perfusion abnormalities of the splanchnic circulation, which may cause ischemia–reperfusion injury. Potential abdominal complications include NEC, GI bleeding, colitis, enteric ischemia, intestinal perforation, and pancreatitis. The gut is particularly at risk of ischemia during the postoperative low cardiac output state because of the sensitivity of the splanchnic circulation to endogenous and exogenous catecholamines, and selective vasoconstriction effects of angiotensin. Splanchnic ischemia may result in inflammation and development of endotoxemia leading to multiorgan dysfunction, and potentially death.[38]

Giannone et al.[3] reviewed the occurrence of NEC in newborns with CHD. They noted considerable systemic inflammation with activation of the nuclear factor-κβ in these infants during both the pre- and postoperative periods. There were inflammatory changes in cardiac myocytes and higher levels of the tumor necrosis factor and endotoxin released during and after cardiac surgery, which were associated with heart failure. Contact of blood with foreign surfaces and ischemia–reperfusion injury also results in systemic inflammatory response.

The association between feeding and necrotizing enterocolitis results in frequent feed withdrawal which further contributes to malnutrition in the postsurgical cardiac neonate. Neonates with CHD manifest with early and gradual falls in their growth trajectory compared to healthy infants, increasing their length of hospital stay and risk of death postsurgery.[49]

Impact of Feeding in Infants with Cardiogenic CHD

Golbus et al.[50] did a systematic review of all literature between 1950 and 2010 for feeding issues in babies with HLHS. The rationale that it is modifiable morbidity and specific strategies if implemented could improve outcomes in this cohort. The feeding issues reported in literature range from dysphagia due to recurrent laryngeal nerve dysfunction, gastroesophageal reflux (GERD), and increased glottic gap due to weakness to complications like NEC and poor growth and development all resulting in prolonged hospital stay and increased morbidity and mortality. Insertion of a gastrostomy tube has shown to improve survival. The other two measures to show significant benefit are implementation of a standardized feeding protocol and a home-monitoring system consisting of daily weight and systemic oxygen saturation measurements. The thresholds for parents to seek medical advice included a resting SpO_2 <70%, failure to gain 20 g during a 3-day period, and weight loss of >30 g during 24 hours.

A single-center retrospective study at Texas Children's Hospital from 2010 to 2016 found that an exclusive unfortified human milk

diet was associated with a significantly lower risk of preoperative NEC (OR 0.17, 95% CI 0.04–0.84, $p = 0.03$) in a multivariable regression model controlling for cardiac lesion, race, feeding volume, birth weight, SGA, inotrope use, and prematurity.[51] Therefore, exclusive breastfeeding and an exclusive unfortified human milk diet, whether the milk is maternal or donated, is the most significant enteral feeding strategy to decrease the incidence of NEC in infants with CHD.

Standardized feeding protocols have been shown to reduce the incidence of postoperative NEC, shorten the duration of total parenteral nutrition intake days, and reach RDA in a significantly shorter time. Gephart et al.[30] found that having a standardized feeding protocol in a unit does reduce the chances of NEC in the vulnerable population including babies with CHD. But they also agree that there is no defined way to standardize an ideal feeding regime for babies with heart disease. A unit-specific feeding policy with well-defined indications and timings to stop, hold, or progress feeds are often used to reduce the chances of NEC.[9] Delayed feeding might also be required in low cardiac output states such as in neonates with ductal-dependent cardiac lesions or during treatment with extracorporeal membrane oxygenation or when on inotropic support.[30]

Investigations to Detect Early Gut Compromise in the Postoperative Period

Abdominal radiography is used as a diagnostic tool for NEC; however, its utility is limited in the early stages when pneumatosis intestinalis is absent. In recent years, there has been accumulating data regarding the benefits of using bowel ultrasound (BUS) in the diagnosis and management of NEC. Bowel ultrasonography provides a more detailed and dynamic understanding of the state of the bowel in patients with NEC and may thus make management decisions easier and potentially change the outcome. BUS can detect early signs of NEC (such as bowel wall thickening, decreases in bowel perfusion, and peristalsis), which can then translate to earlier treatment before more advanced NEC develops.[52] In atypical presentations, abdominal CT scan is a sensitive modality to diagnose NEC.[53]

Dewitt et al. studied the splanchnic NIRS in single ventricle physiology babies intra and postoperatively and as they were being fed in the postoperative period. They saw lower average regional oximetry (rSO_2) values for prolonged durations in patients who went on to develop proven NEC compared to those who did not. These rSO_2 values were lower when they reached one-fourth feed volumes.[47]

There has been a fair bit of work to look at specific biochemical markers in postoperative babies to predict the occurrence of NEC. O'Connor et al.[49] found that fecal calprotectin levels were significantly increased in postop babies with NEC compared to without increased levels of intestinal-fatty acid-binding protein in the immediate postoperative period after a cardiothoracic surgery is an indicator of the degree of enterocyte injury, and is associated with subsequent development of NEC.[54] Other biochemical markers identified in relation to NEC are FOXP3+ regulatory T-cell levels or increased levels of platelet-activating factor and increased expression of its receptor in the ileum. These factors are also more specific to small gut injuries.[12]

Conclusions

Neonates with CHD who go on to develop NEC are a particularly vulnerable cohort and are distinct from the typical population of preterm babies with classical NEC. As advancement in technology allows more newborns to have interim and corrective surgeries for CHD at earlier gestational ages, the likelihood for NEC increases in this vulnerable population. There is a requirement for large randomized prospective studies to understand this entity better but this is difficult as the overall incidence is low. The "high risk" in this group are the ones with complex cardiac lesions like HLHS which significantly reduce gut perfusion. Majority of the cases of NEC are seen in the postoperative period probably due to the hypoperfusion–reperfusion injuries to the intestinal cells. This mandates holding of enteral feeds, complicates recovery, delays growth, and prolongs hospital stay. This review article highlights some key areas of knowledge and gives insight into specific populations at risk, demographics, anticipated outcomes, and management strategies of cardiogenic NEC. It will be helpful to discuss course and expected trajectory of such babies while counseling parents instead of citing data more applicable to preterms with classical NEC.

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REVIEW ARTICLE

Role of Near-infrared Spectroscopy in the Diagnosis and Assessment of Necrotizing Enterocolitis

Vinayak Mishra[1], Amit A Mathur[2], Shakir Mohamed[3], Akhil Maheshwari[4]

Abstract

Near-infrared spectroscopy (NIRS) is a noninvasive, bedside diagnostic tool that could assist in the early diagnosis of necrotizing enterocolitis (NEC) in preterm neonates. NIRS is a safe and effective clinical tool in the neonatal intensive care unit to detect abnormal alterations in tissue perfusion and oxygenation. In addition, NIRS could also detect the complications of NEC, such as bowel necrosis and perforation. NEC is the most common gastrointestinal complication associated with preterm birth and critically ill infants. It is observed in 6–10% of preterm neonates, weighing below 1500 g, leading to considerable morbidity, mortality, and healthcare cost burden. The mortality rate ranges from 20 to 30%, highest in NEC infants undergoing surgery. NIRS is a promising diagnostic modality that could facilitate the early diagnosis of NEC and early detection of complications alone or with the imaging modalities.

Keywords: Near-infrared spectroscopy, Necrotizing enterocolitis, Neonatology, Newborn, Preterm infant.

Newborn (2022): 10.5005/jp-journals-11002-0001

Introduction

Near-infrared spectroscopy (NIRS) is a clinical tool that provides a bedside method of noninvasively measuring continuous oxygen consumption and assessing for potential ischemia of tissues such as in the brain, kidneys, and intestinal tract. NIRS utilizes transparency of biological tissue to near-infrared radiation (700–1000 nm wavelength) to differentiate among various forms of chromophores, such as hemoglobin, myoglobin, and cytochromes.[1–5] In health care, it is used to detect tissue oxygenation levels, measure hemoglobin and myoglobin levels,[6,7] and assess hemoglobin oxygenation and tissue oxygenation noninvasively in real time.[1] One of its first medical applications was to monitor cerebral oxygenation and perfusion after traumatic brain injury and during cardiac and neurosurgical operations.[8] In pediatrics, it was first used to examine cerebral oxygenation in hospitalized preterm neonates.[9] The clinical application of NIRS in neonates has expanded in the last two decades, one of the domains being the early diagnosis of necrotizing enterocolitis (NEC).[8,10]

NEC is one of the most prevalent devastating diseases in neonates, affecting around 7% of preterm neonates with a birth weight below 1500 g in the United States and Canada.[11,12] The mortality rate is estimated to be around 20–30%, highest in those undergoing surgical intervention.[13,14] Bowel ischemia and eventual necrosis are pivotal aspects of the pathogenesis of NEC.[15] Plain abdominal radiography is the imaging modality of choice in NEC diagnosis; however, it often fails to detect the early stages of NEC.[16,17] Early detection of ischemic bowel may help in the earlier institution of necessary interventions and prevent bowel necrosis and perforation, thus reducing morbidity and mortality rates.[16]

In NEC, NIRS has been used to evaluate the effects of bowel perfusion deterioration on bowel ischemia and injury.[18] Besides the early diagnosis of NEC, NIRS can also differentiate between complicated and uncomplicated diseases in the first 48 hours after the onset of symptoms.[19,20] This review elaborates on the role and future implications of NIRS in the diagnosis and management of NEC.

[1]Department of Pediatrics, Grant Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India

[2]Department of Pediatrics, Saint Louis University, St Louis, Missouri, United States of America

[3]Department of Neonatal-Perinatal Medicine, Saint Louis University School of Medicine, St Louis, Missouri, United States of America

[4]Global Newborn Society, Baltimore, Maryland, United States of America

Corresponding Author: Vinayak Mishra, Department of Pediatrics, Grant Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India, Phone: +91 8828079692, e-mail: vinayakmishra3009@gmail.com

How to cite this article: Mishra V, Mathur AA, Mohamed S, *et al*. Role of Near-infrared Spectroscopy in the Diagnosis and Assessment of Necrotizing Enterocolitis. Newborn 2022;1(1):177–181.

Source of support: Nil

Conflict of interest: None

Technical Aspects of NIRS

The NIRS device contains a light-emitting diode (LED) that emits light rays of wavelengths 730 and 810 nm. These light photons pass through superficial and deep layers of tissue and are absorbed by oxygenated and deoxygenated hemoglobin differently.[21] The nonabsorbed fraction is reflected from the superficial to the proximal arc detector and the deep to the distal arc detector as illustrated in Figure 1. These signals are analyzed, and data from the superficial tissue are subtracted to estimate the tissue oxygen levels at a depth of 1–2 cm, called the regional saturation (rSO_2) of the underlying tissue.[22] The value of the rSO_2 reflects the tissue blood flow and tissue oxygenation. The device also calculates the

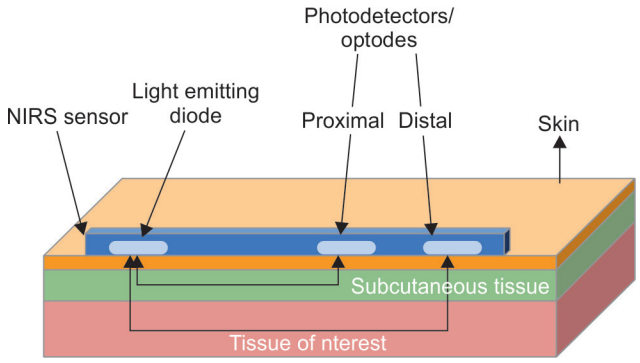

Fig. 1: Technical aspects of NIRS

amount of oxygen extracted from tissues called fractional tissue oxygen extraction (FTOE). FTOE helps to understand the balance between oxygen supply and demand of tissues.[8]

Application in Neonates to Study Splanchnic Oxygenation

The application of NIRS in monitoring splanchnic tissue oxygenation in neonates has been validated by various studies.[23–26] Varela et al. demonstrated the correlation between superior mesenteric artery blood flow and gastric tissue oxygen saturation in an experimental animal model of hemorrhagic shock and abdominal compartment syndrome.[23] Dave et al. reported a rise in splanchnic tissue oxygenation after oral feeding in stable preterm neonates.[26] Some of the studies mentioned above used cerebral oxygenation as a reference assuming that it is kept stable by cerebral autoregulation mechanisms during vascular insults to the gastrointestinal system. However, various studies have reported impaired cerebral autoregulation in clinically sick preterm neonates with variability in systemic blood pressure.[27–29]

Cortez et al., in a prospective cohort study, described the safe and effective use of NIRS to monitor splanchnic tissue oxygenation in the first 2 weeks of preterm neonates' life.[4] This study used arterial oxygen saturation by pulse oximetry as a reference instead of cerebral oxygenation index.[4] The splanchnic tissue oxygenation decreases over the first 9 days of life before increasing till day 14, and the fall in splanchnic rSO_2 leads to a rise in FTOE.[4,30] Mintzer et al. reported considerable variability in the splanchnic rSO_2 readings (~16%), which is higher than that in renal (6%), cerebral (3%), and pulse oximetry (1–2%) rSO_2 values.[31] The NIRS probe is usually applied in a paraumbilical position to avoid interference from the liver and bladder, as it has been demonstrated that supraumbilical and infraumbilical oxygen saturation correlate poorly with each other and cannot be interchanged for measuring splanchnic rSO_2.[32]

NIRS has been extensively studied in neonates to evaluate the significance of cerebral rSO_2 in hypoxic-ischemic encephalopathy, cerebral autoregulation, congenital heart disease, and postsurgical conditions.[8] There have been relatively fewer studies on the application of NIRS in the early diagnosis of NEC and the prediction of its clinical outcomes. However, various animal models and human neonatal studies have validated the reproducibility and feasibility of splanchnic oxygen saturation values and their association with bowel ischemia in NEC.[4,8]

Role in NEC

Role of NIRS in Early Diagnosis and Predicting Outcomes

Patel et al. demonstrated the reduction in splanchnic rSO_2 in neonates with NEC compared to the normal and further reported that rSO_2 equal to or less than 56% was independently associated with around 14 times increased risk of NEC (odds ratio, 14.1; $p = 0.01$).[33] Therefore, the rSO_2 value may be interpreted as an early warning sign of NEC in vulnerable neonates. Due to the brain's higher metabolic activity, it extracts more oxygen from the blood, and consequently, the cerebral rSO_2 is less than the splanchnic rSO_2.[10] The cerebral rSO_2 is generally 5–15% lower than the splanchnic rSO_2.[30] The ratio of rSO_2 of cerebral and splanchnic tissue is called cerebro-splanchnic oxygenation ratio (CSOR). Fortune et al. reported a lower CSOR value in NEC and showed that the measurement of both rSO_2 and CSOR in neonates could predict acute abdomen with a 90% sensitivity and 96% specificity.[34] CSOR below 0.75 indicated a higher probability of the need for surgical intervention.[34] CSOR's reliability in NEC is reduced if concomitant cerebral conditions, such as intraventricular hemorrhage, are present.[4]

NIRS monitoring of preterm neonates with NEC within the first 8 hours of the onset of symptoms may predict complications and outcomes, such as bowel necrosis, perforation, surgical intervention, or death.[19] Schat et al. reported that significantly lower rSO_2 (cerebral rSO_2 <72%, liver rSO_2 <60%) values within the first 8 hours after onset of symptoms predicted complications in NEC with a high sensitivity (100%) and a high specificity (80–100%).[19] A higher cerebral and splanchnic FTOE within the initial 24 hours of symptom-onset also predicted complications.[19] Loss of variability in splanchnic rSO_2 and high signal dropout may also predict the onset of NEC before clinical features become apparent.[4]

A case report on preterm twins by Zabaneh et al. also showed the association between reduced splanchnic rSO_2 values and complications in NEC.[35] Another case report corroborated the presence of splanchnic oxyhemoglobin desaturation in a preterm neonate with congenital heart disease who developed NEC.[36] A piglet model of NEC supported this link between low abdominal NIRS oxygenation values and future development of bowel ischemia and necrosis.[37] The possible reasons for this decline in splanchnic tissue oxygenation are bowel ischemia and necrosis, circulatory insufficiency in NEC compromise blood flow to less essential organs (including bowel), and bowel inflammation in early NEC.[19,38]

Role of NIRS in Transfusion-associated Necrotizing Enterocolitis

A temporal association has been identified between red blood cell transfusion (RBCT) and the development of NEC within 48 hours, which has been called transfusion-associated NEC (TANEC).[39,40] Cerebral and peripheral rSO_2 increases, and FTOE decreases after RBCT in preterm neonates.[41,42] Cerebral and splanchnic rSO_2 in neonates increased after RBCT in neonates with NEC (diagnosed before RBCT) and in neonates without NEC; however, splanchnic rSO_2 subsequently decreased in neonates who developed TANEC.[43] The current evidence in support of TANEC has a "very low" quality, primarily due to the lack of randomized controlled trials (RCTs) supporting the causal association in TANEC.[44,45] Lawrence et al. reported the lack of association between the rise in hematocrit

values following RBCT and TANEC.[46] In very-low-birth-weight infants (birth weight below 1500 g), severe anemia (hemoglobin below 8 g/dL) instead of RBCT was associated with a heightened risk of developing NEC.[47]

The role of enteral feeding during and after RBCT transfusion in the development of TANEC is controversial. It has been hypothesized that enteral feeding during RBCT in preterm neonates may increase the risk of TANEC.[48] A prospective cohort study concluded that enteral feeding is possibly linked to bowel ischemia and TANEC.[49] An RCT conducted by Schindler et al. highlighted the lack of difference in splanchnic rSO_2 regardless of continuing or restricting enteral feeds during RBCT.[50] Further investigation into this phenomenon is warranted in larger RCTs.

Limitations of NIRS

Skin safety, especially in extremely premature neonates, was one of the significant concerns regarding the application of NIRS. Transcutaneous use of NIRS does not cause skin burns even if the probe is applied directly to the skin surface continuously for 48 hours.[32,51] Mepitel barrier on the NIRS sensor was used to rectify this issue.[30] The Mepitel barrier nullified the frequency of adverse skin effects, thereby facilitating the use of NIRS in the long-term monitoring of neonates in the intensive care unit.[30]

Most of the studies included in this review have not accounted for the variability in the splanchnic rSO_2 for changes in gestational age. Increasing gestational age is associated with the growing maturation of splanchnic vasculature and increasing metabolic activity in the gut.[30] The variability in the splanchnic rSO_2 readings was more than that in other tissues.[31] This might reflect uncertainty in the type of intestinal tissue sampled because the intestine is a multilayered, hollow organ with luminal contents and undergoing peristaltic movements. There is also confusion and lack of consensus about the best site over the abdominal wall to place the NIRS probe.[32] Supraumbilical probe placement might sample the liver, spleen, or stomach instead of the intestine; infraumbilical readings might reflect bladder and pelvic wall muscle tissue oxygenations in preterm neonates.[30]

There is also uncertainty about the influence of skin pigmentation and myoglobin over NIRS readings.[51] When assessing peripheral tissues with ample muscular mass, it becomes crucial to account for the contribution of myoglobin to the NIRS results. The heterogeneity in device probes and machine algorithms by various NIRS device manufacturers restricts extrapolating and comparing findings between devices.[8] This also limits the widespread use of the "normal" and "abnormal" values derived by various studies regarding the application of NIRS in NEC. Two observational studies had reported the inability of NIRS monitoring to differentiate between neonates with and without NEC when it was started shortly after the development of clinical features typical of NEC (bloody stools and abdominal distension).[34,52]

Conclusion

Our review highlights the potential use of NIRS in the continuous monitoring of splanchnic tissue oxygenation in preterm neonates to detect early pathogenic changes of NEC. NIRS is a safe and effective modality to incorporate in the neonatal intensive care unit to observe tissue perfusion and oxygenation alterations. It can also differentiate between complicated and uncomplicated NEC, thereby helping us in individualizing the management. Moreover, it might also help to decide feeding protocols in neonates recovering from NEC.

However, there are various limitations to its widespread clinical use. A couple of studies have concluded that NIRS could not diagnose NEC after the onset of clinical features.[34,52] NIRS can alert and warn neonatologists about the beginning of bowel ischemia; however, it might not differentiate between NEC and other intestinal ischemic conditions during the early phase of disease satisfactorily.

There is a lack of consensus among device manufacturers and coordination between researchers worldwide to create a reproducible dataset consisting of "normal" readings for the various tissue oxygenation parameters. Large-scale longitudinal studies are needed to produce such a clinically valuable dataset and standardize the use of NIRS in the diagnosis of NEC.

Recent advancements in NIRS have widened the electromagnetic spectrum of the sensors to create a novel method called broadband optical spectroscopy (BOS).[53] Further research in human neonatal models studying the implementation of NIRS and BOS in the early detection of NEC is required.

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REVIEW ARTICLE

Imaging for Diagnosis and Assessment of Necrotizing Enterocolitis

Vinayak Mishra[1], Alain Cuna[2], Rachana Singh[3], Daniel M Schwartz[4], Sherwin Chan[5], Akhil Maheshwari[6]

Abstract

Necrotizing enterocolitis (NEC) is inflammatory bowel necrosis of preterm and critically ill infants. The disease is seen in 6–10% of preterm infants who weigh less than 1500 g at birth and carries considerable morbidity, mortality, and healthcare cost burden. Efforts focused on timely mitigation remain restricted due to challenges in early diagnosis as clinical features, and available laboratory tests remain nonspecific until late in the disease. There is renewed interest in the radiological and sonographic assessment of intestinal diseases due to technological advances making them safe, cost-efficient, and supporting Web-based transmission of images, thereby reducing time to diagnosis by disease experts. Most of our experience has been with plain abdominal radiography, which shows characteristic features such as pneumatosis intestinalis in up to 50–60% of patients. Many patients with advanced disease may also show features such as portal venous gas and pneumoperitoneum. Unfortunately, these features are not seen consistently in patients with early, treatable conditions, and hence, there has been an unfulfilled need for additional imaging modalities. In recent years, abdominal ultrasound (AUS) has emerged as a readily available, noninvasive imaging tool that may be a valuable adjunct to plain radiographs for evaluating NEC. AUS can allow real-time assessment of vascular perfusion, bowel wall thickness, with higher sensitivity in detecting pneumatosis, altered peristalsis, and characteristics of the peritoneal fluid. Several other modalities, such as contrast-enhanced ultrasound (CEUS), magnetic resonance imaging (MRI), and near-infrared spectroscopy (NIRS), are also emerging. In this article, we have reviewed the available imaging options for NEC evaluation.

Keywords: Diagnostic imaging, Necrotizing enterocolitis, Neonatology, Prematurity, Preterm neonate, Ultrasonography.

Newborn (2022): 10.5005/jp-journals-11002-0002

Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal (GI) complication secondary to preterm birth, with high morbidity and mortality. It is an acute inflammatory bowel disease of preterm infants, and it can affect 6–10% of very-low-birth-weight (VLBW) infants born before 32 weeks of gestation.[1–4] Despite significant advances in neonatal intensive care units (NICUs), the disease still has high mortality rates (30–50%).[5,6] The pathogenesis of NEC is complex, multifactorial, and challenging to predict clinically with sudden onset. Many risk factors for NEC have been identified, such as prematurity, genetic predisposition, chorioamnionitis, perinatal asphyxia, formula feeding, human milk fortifiers, feed thickeners, viral infections, gut dysbiosis, and severe anemia with red blood cell transfusions.[7] However, despite extensive research, a unifying pathophysiological mechanism remains unclear.

Unlike many other organ-specific diseases such as those affecting the brain, lungs, and the urogenital tract, diagnostic imaging of the neonatal gastrointestinal tract has had relatively limited accuracy, and this has constrained improvement in diagnostic efforts and measurement of severity in disorders such as NEC.[6,8] For many decades, the diagnosis for NEC has relied heavily on clinical presentation and abdominal radiographs. The easy availability of portable radiographic machines, cost-effectiveness, and clinicians' familiarity with interpreting results has made abdominal radiographs a definitive part of evaluating preterm infants with suspected NEC.[9] However, even in the best hands, the sensitivity of radiographical approaches has been limited to 55–60% of all patients.

More recently, sonography has emerged as a novel, exciting technological advance. The availability of handheld ultrasound (US) machines has further simplified these approaches and increased the accessibility of such evaluation.[8] These devices are highly portable, provide high-quality images, and can be used for frequent, sequential monitoring with no exposure to radiation. More recent efforts have focused on increasing the sensitivity of sonography by using contrast-enhanced ultrasound (CEUS). Other studies have used magnetic resonance imaging (MRI) or near-infrared spectroscopy (NIRS) for evaluating the anatomy, physiology, and perfusion of the GI tract in relation to NEC.

[1]Department of Pediatrics, Grant Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India

[2]Division of Neonatology, Children's Mercy, Kansas City, Missouri, United States of America

[3]Department of Pediatrics, Tufts Children's Hospital, Boston, Massachusetts, United States of America

[4]Department of Radiology, Tufts University School of Medicine, Boston, Massachusetts, United States of America

[5]Department of Radiology, Children's Mercy, Kansas City, Missouri, United States of America

[6]Global Newborn Society, Baltimore, Maryland, United States of America

Corresponding Author: Vinayak Mishra, Department of Pediatrics, Grant Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India, Phone: +91 8828079692, e-mail: vinayakmishra3009@gmail.com

How to cite this article: Mishra V, Cuna A, Singh R, *et al.* Imaging for Diagnosis and Assessment of Necrotizing Enterocolitis. Newborn 2022;1(1):182–189.

Source of support: Nil

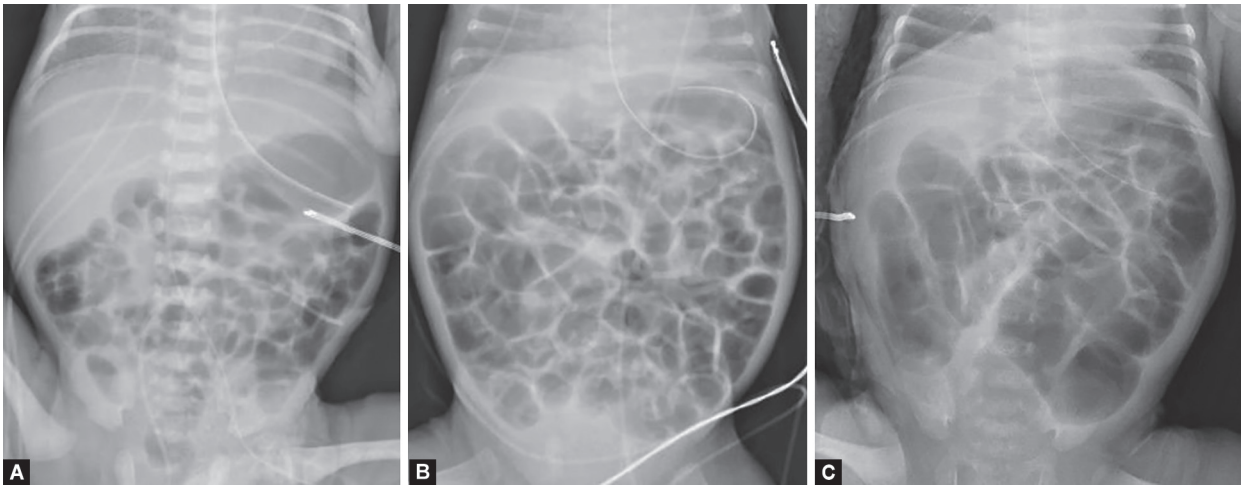
Conflict of interest: None

In this article, we have reviewed the evolving landscape of imaging modalities for NEC evaluation. We begin with a review of traditional abdominal radiographs, focus on the emerging role of abdominal ultrasound (AUS), and conclude with novel modalities for diagnosing and assessing NEC.

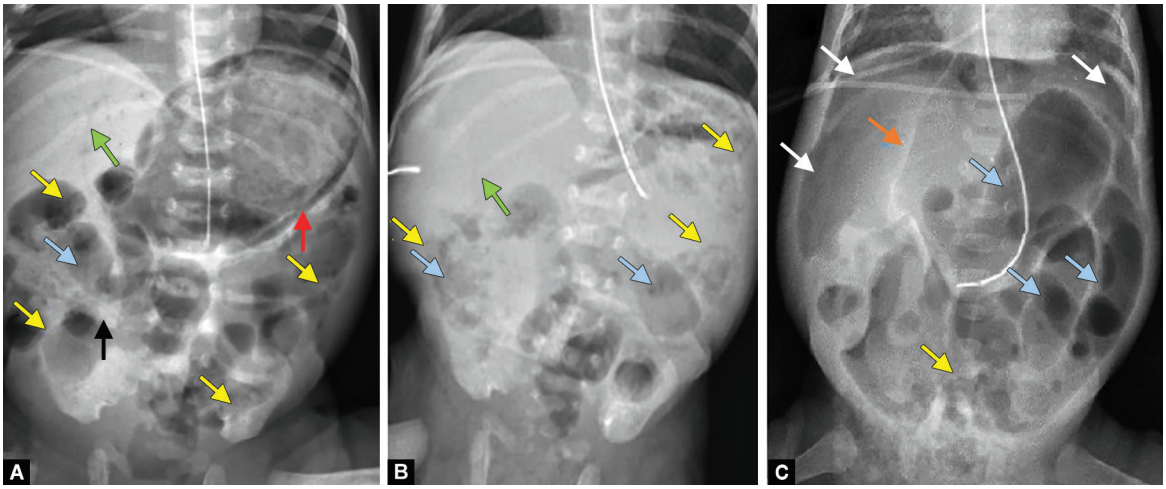
Radiographic Imaging in NEC

The traditional, most widely used technique for evaluation and diagnosis of NEC is plain abdominal radiography. Early, subtle radiographic signs are related to alteration in peristalsis with ileus, such as dilatation and, sometimes, apparent elongation of the bowel loops with the loss of the normal mosaic pattern[10] (Figs 1A to C). Distension of the bowel loops and bowel wall edema, especially if with an asymmetric pattern, is considered more ominous and may suggest impending necrosis in the bowel area.[11] More specific radiographic findings, which have been considered pathognomonic for NEC, include *pneumatosis intestinalis* and the detection of radiolucent gas in the portal venous system (Figs 2A to C). *Pneumatosis intestinalis* refers to gas bubbles/cysts in the mucosal, submucosal, subserosal, or all three bowel wall layers.[12]

Pneumatosis is believed to originate in abnormal bacterial colonization and overgrowth in the bowel wall. Other possibilities rooted in mechanical and biochemical reasons have been considered but never proven.[12] As currently believed, the disruption



Figs 1A to C: Plain anterior-posterior (AP) supine abdominal radiographs demonstrating normal versus abnormal bowel gas pattern; (A) Normal bowel gas pattern with no evidence of NEC; (B) progressively dilated bowel gas pattern, nonspecific for NEC as can be seen in many clinical settings such as swallowed air in a normal infant to inflamed bowel due to NEC and/or with postsurgical ileus. It could be more specific for NEC if bowel dilation is more localized and more severe (bowel lumen diameter measures greater than the width of one vertebral body), or it persists over many subsequent radiographs in the same place and; (C) Advanced dilatation of bowel loops with some evidence of bowel wall edema



Figs 2A to C: Sequential AP supine abdominal radiographs showing the evolution of NEC; (A) NEC totalis with linear lucent shadows in the gastric wall (red arrow), soap-bubble radiolucent intramural gas (pneumatosis intestinalis) in the abdomen (yellow arrows), portal venous gas (green arrow), dilated bowel loops (blue arrowhead) with bowel wall edema/thickening; (B) After 6 hours, the gastric distension seems to have decreased. Intestinal distension, pneumatosis, and a thin line of portal venous gas can still be noticed and; (C) 18 hours later, pneumoperitoneum (indicating intestinal perforation) (white arrows) can be seen with gas between both domes of the diaphragm and the liver and likely forming the relative radiolucency seen in front of the liver. The massive air collection in the peritoneal cavity accounts for the "football sign" (orange arrow). Pneumatosis can still be seen in some areas through the overlying intraperitoneal air

of the mucosal layer allows bacterial translocation into the intestinal wall, and the gaseous products of bacteria metabolism then progressively dissect through the tissues to accumulate in the deeper layers of the injured bowel. Some of these collected gases gradually find their way into the local venules that drain into the portal venous system and can be seen as branching radiolucency against the relatively opaque background of the liver (Figs 2A to C). In severe cases, the necrotic bowel ruptures to release the intraluminal air into the peritoneal cavity. In this pneumoperitoneum, the collection of relatively large amounts of free air can be seen as a "football sign," or the "falciform ligament sign," where the oval abdominal cavity outlined by the lucent intraperitoneal air may be visualized as a football, the longitudinal falciform ligament as the ball's lace, and the transversely transecting ribs as the cross-stitches (Figs 2A to C).

Clinical Use of Abdominal Radiographs in NEC

Several clinical staging systems for NEC incorporate abdominal radiographic findings. Bell's staging, established in 1978, is the most widely used criteria for classifying and managing NEC.[13] Walsh and Kliegman subsequently modified this staging system to make it more contemporary by dividing each stage into two subcategories and incorporating clinical signs indicative of disease severity.[14] In Bell's stage I NEC (suspected NEC), abdominal radiographic findings include nonspecific signs such as intestinal dilatation or mild ileus. Bell's stage II NEC (definite NEC) requires the presence of more specific features such as *pneumatosis intestinalis* and/or portal venous gas. Lastly, Bell's stage III NEC (advanced NEC) includes the finding of pneumoperitoneum or "free" air. Gephart et al. recently described a simple, alternative bedside clinical tool for diagnosing preterm NEC called the "two out of three" rule.[15] This rule is comprised of (1) *pneumatosis intestinalis* and/or portal venous gas at presentation, (2) platelet count below 150,000 for 3 days after diagnosis, and (3) gestational age at disease onset more suggestive of NEC than spontaneous intestinal perforation. Gordon et al. also proposed a new system that utilizes *pneumatosis* and pneumoperitoneum as 2 of 11 diagnostic criteria for classifying NEC and other acute neonatal intestinal conditions.[16] However, it was not widely used in clinical practice, likely due to its complexity.

Limitations of Plain Abdominal Radiographs for NEC Evaluation

Although *pneumatosis* and portal venous gas can be highly specific for NEC, these pathognomonic signs are not always readily evident on plain radiographs. As mentioned above, *pneumatosis* is seen only in 55–60% of all infants with NEC. Occasionally, it also becomes challenging to distinguish between intramural air and air admixed with stool, specifically when the clinical presentation is equivocal. Moreover, nonspecific findings such as gaseous intestinal distension, air-fluid levels, bowel wall thickening, and ascites are common findings on plain radiographs but of unclear usefulness in diagnosing NEC. Sharma et al., in their study of 202 neonates, demonstrated that the clinical and radiographic presentations of NEC are different in extremely preterm infants (gestational age, 23–26 weeks) compared to infants with higher gestational age, highlighting the inadequacy of plain abdominal film in the diagnosis of NEC in extremely premature neonates.[17] Kosloske et al. corroborated this insufficiency by reporting that preterm neonates developed intestinal necrosis before developing diagnostic NEC features in serial abdominal X-rays.[18] Thus, the sole use of abdominal radiographs for the diagnosis and staging of NEC can have numerous demerits.[19,20] A readily available, noninvasive imaging modality that can characterize the state of the intestinal tract in more detail than plain radiographs would help evaluate infants for NEC.

Sonographic Assessment in NEC

Bowel US has been shown to be helpful for the evaluation of NEC since 1984 when Kodroff et al. described "abnormal bowel characterized by a hypoechoic rim with a central echogenic focus."[21] It was suggested that this sign could be used to help identify gangrenous bowel before perforation. Since then, many additional US findings associated with NEC have been identified. A few of these are visible on radiography, including *pneumatosis intestinalis*, portal venous gas, and free intraperitoneal air. However, US may be more sensitive to these findings, and they may be identified earlier in the disease course.[21–24]

US also allows for identifying many additional signs of NEC, which are not apparent on radiography, through real-time imaging of the bowel and peritoneum. The bowel wall can be directly characterized, demonstrating abnormal thickness (increased or decreased) and abnormal echogenicity. Bowel distension with fluid can be seen. Doppler imaging can assess perfusion of the bowel wall (increased or absent). Real-time cine imaging can detect the presence or absence of peristalsis. In addition to the bowel findings, small amounts of free intraperitoneal fluid can be identified, and the fluid can be described as simple or complex. Focal fluid collections or abscesses can be localized.[25–28]

Imaging Findings of NEC Common to Plain Abdominal Radiography and AUS

Pneumatosis Intestinalis

This finding is best identified using high-frequency linear transducers, which allow for higher spatial resolution US. *Pneumatosis* appears as tiny echogenic gas bubbles or granules along the circumference of the bowel wall (Figs 2A to C), dubbed as the "circle sign" with posterior reverberation artifacts.[29,30] When scanning, it can be challenging to differentiate pneumatosis from gas in the bowel lumen, so real-time evaluation and cine imaging can be beneficial in this regard. Kim et al. studied NEC in newborn rabbits and noted that echogenic dots and circumferential granular echogenicity might also correlate with the histopathologic features of ischemic enterocolitis.[31] Intramural gas does not shift position with alterations in the patient's position, bowel peristalsis, respiratory movement, or abdominal compression with the transducer.[32]

Portal Venous Gas

Portal venous *pneumatosis* in neonates can be iatrogenic, resulting from the passage of gas bubbles during umbilical venous catheterization.[33–35] However, portal venous gas is also a frequent finding in infants with NEC.[23,36] It appears as echogenic foci moving as microbubbles with the blood flow inside the lumen of the portal vein on grayscale ultrasonography.[37] These microbubbles in the small intraparenchymal portal vein branches can be seen as hyperechogenic foci in a dendriform granular pattern.[38] On spectral Doppler images, portal venous gas appears as a vertical line in the spectral Doppler waveform tracing and will sound like a popping sound if audio output is enabled on the US machine. In a study including 352 neonates, the presence of portal venous gas in AUS had an 86% specificity and a 45% sensitivity in

NEC diagnosis (Bell's stage II or above).[39] In a more recent study, the combination of portal venous gas on US and *pneumatosis intestinalis* on abdominal X-ray had a diagnostic sensitivity of 89% and specificity of 91%.[40]

Free Abdominal Gas

Bowel perforation in the final stages of NEC, most commonly in the distal ileum and proximal colon, results in free gas in the abdominal cavity. In AUS, free gas appears as bright, linear, or punctate echogenic foci between the anterior abdominal wall and the anterior surface of the liver, between loops of bowel, or floating on peritoneal fluid deep to the abdominal wall.[37,41] The finding of free abdominal gas can be harder to detect on AUS as compared to abdominal radiograph.[35]

Imaging Findings for NEC Unique to AUS

Bowel Wall Thickness and Echogenicity

Increasing bowel wall thickness reflects mucosal hemorrhage and edema at the initial phases of the pathogenesis of NEC. Usually, a bowel wall thickness of more than 2.6 mm is considered pathological.[42] In the later advanced stages, prominent bowel wall ischemia can cause bowel wall thinning. Thickness below 1.1 mm indicates bowel wall ischemia and consequent necrosis.[37,42] The normal echogenicity of the intestinal wall vanishes in case of bowel wall thickening or thinning.[43] The thickened bowel wall appears hypoechoic (black) and contrasts against the increased echogenicity (white) of the *valvulae conniventes*, giving rise to the characteristic grayscale "zebra" or "herringbone" pattern (seen as white stripes/branching surrounded by black) (Figs 3A to C).[43]

Bowel Wall Perfusion

Color Doppler US with the lowest possible pulse repetition rate and the highest Doppler gain settings can be used to assess bowel wall perfusion in NEC.[38] In color Doppler US, bowel wall inflammation in the initial stages of NEC is characterized by ring-shaped signals, a Y-shaped pattern in distal mesenteric and subserosal vessels, and a zebra pattern in longitudinal scans.[42] When there is no color Doppler signal at a velocity of or below 0.029 meters per second, bowel wall perfusion is said to be absent.[42]

Free Abdominal Fluid

In neonates with severe NEC with or without perforation of the bowel wall, free fluid can accumulate in the intraperitoneal cavity. Echogenic ascites is one of the typical findings associated with bowel wall necrosis in ischemic enterocolitis.[44] Low-level echoes and septations within the intraperitoneal fluid that indicates the presence of pus or intestinal contents are more suggestive of perforation of gangrenous bowel.[45] The echogenic foci between bowel loops suggest localized fluid accumulation with or without septations and indicate bowel perforation.[38] It is crucial to appreciate that a small amount of nonechogenic, free abdominal fluid is nonspecific and can be normal in neonates.[37]

Benefits of AUS Over Plain X-ray Examination

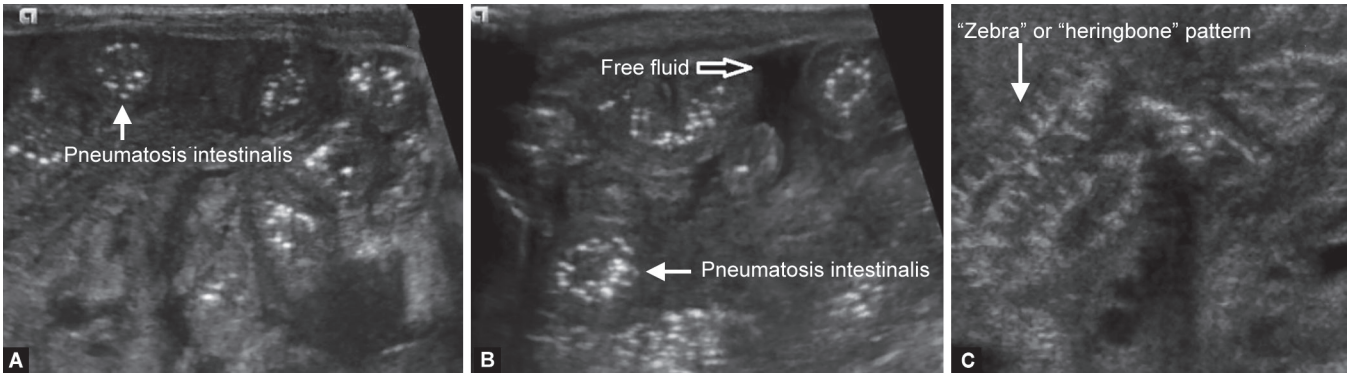
The US examination can display abdominal structures in real time, which allows the assessment of bowel peristalsis and viability. AUS also has significantly higher rates of detection of *pneumatosis* and portal venous gas than abdominal X-ray, permits a more precise assessment of bowel wall thickness, and is superior to plain X-rays in evaluating intraperitoneal fluid.[28] The added capability of color Doppler enables the evaluation of bowel perfusion to recognize bowel ischemia and necrosis.[45,46] Plain abdominal radiographs can also have significant interobserver variability in NEC diagnosis, and the frequent need for multiple serial X-rays exposes preterm neonates to potentially harmful radiation.[47–49]

Several single-center studies have reported the superiority of AUS over plain radiographs in NEC. Dilli et al. showed the benefits of AUS over plain abdominal X-ray as the better demarcation of portal venous gas, intraabdominal fluid, bowel wall thickness, and bowel wall perfusion.[50] Shebrya et al., in a study of 30 preterm neonates, substantiated the superiority of AUS in early diagnosis and detection of complications such as intestinal perforation and, hence, early surgical management associated with better morbidity and mortality rates.[51] Franco and Ramji, in their case report of a preterm neonate, highlighted the importance of AUS in the diagnosis of NEC when there are nonspecific clinical features and an inconclusive plain abdominal X-ray.[52]

Clinical Use of AUS in NEC Evaluation

Diagnosis or Exclusion of NEC

Because of widespread availability and familiarity with clinicians, plain abdominal radiography remains the current imaging modality of choice for the immediate evaluation of infants with suspected NEC. The current role of AUS in NEC evaluation is that of an adjunct to plain X-rays to aid in the diagnosis of infants with clinical suspicion



Figs 3A to C: Sonographic diagnosis of NEC; (A) Multiple punctate, echogenic foci seen within the bowel wall demonstrating pneumatosis (marked by the white arrow), which are distributed circumferentially and in multiple bowel segments; (B) Pneumatosis (white arrow), with some free fluid present between bowel segments (black arrow with white outline) and; (C) Increased echogenicity of the valvulae conniventes (also called the plicae circulares or small bowel folds) has been described as the "zebra" or "herringbone" pattern (white arrow) and is a nonspecific finding of bowel wall edema

of NEC by providing a more detailed evaluation of the intestine. Studies have recommended that the cohesive application of plain abdominal radiographs and AUS in NEC management will improve the diagnostic accuracy and sensitivity.[53–55] The NEC group of the International Neonatal Consortium recommended utilizing AUS to locate pneumatosis and/or portal venous gas as a component of the "two out of three" rule.[56] AUS is also helpful in the differential diagnosis of necrotic bowel conditions. For instance, it can differentiate between NEC and food protein-induced enterocolitis syndrome; the decreased or absent bowel peristalsis is present in the entire gut in NEC, whereas it is present only in an isolated bowel segment in the latter.[57] AUS can also exclude conditions with overlapping clinical presentation, such as neonatal appendicitis and intussusception, better than radiographs.[58,59] Hashem et al. advocated applying color Doppler ultrasonography of the splanchnic circulation to detect NEC early in septic preterm neonates.[40]

Monitor Progression of the Disease

The US has the potential to identify the progression of the disease, with imaging findings ranging from early disease (wall thickening, minimal simple free fluid, and the presence of peristalsis), to more severe disease (increased blood flow, pneumatosis, and portal venous gas), to the most severe disease (bowel wall thinning, decreased perfusion of the bowel wall, and large volumes of complex fluid).[28] Signs of perforation include complex (echogenic) free intraperitoneal fluid and visible free air.[25]

Predicting Outcome with Ultrasonographic Parameters

Predicting patient outcomes is another potential use of AUS. Single-center studies[25,26,60,61] and a recent meta-analysis[62] have identified several ultrasonographic features associated with a strong or moderate association with surgery or poor outcomes, including death. Findings associated with poor outcomes (including surgery or death) include abnormal bowel wall thickness (increased or decreased), pneumatosis intestinalis, absent bowel wall perfusion, bowel dilatation with anechoic contents, complex ascites, a focal fluid collection, and free air. Lack of peristalsis may also predict a poor outcome, while anechoic (simple) free intraperitoneal fluid predicts a better outcome. Portal venous gas and increased bowel wall perfusion did not prove to be helpful with the prediction of outcomes. These studies suggest that AUS may be helpful in risk stratification and identification of infants who may benefit from more aggressive treatment, including surgery.

Technical and Practical Aspects of AUS

AUS to assess bowel viability in NEC is performed in two phases. At first, portal venous gas, free intraperitoneal fluid, free abdominal air, and the relationship between the superior mesenteric artery and superior mesenteric vein are evaluated. Secondly, grayscale and color Doppler AUS are used to assess the bowel loops in the four quadrants of the abdomen.[46] A linear array transducer of frequency 12–20 megahertz (MHz) is used.[48] Color Doppler AUS recommended settings and parameters include lowest possible pulse frequency without aliasing, highest color Doppler gain settings without flash artifacts, and velocity of 2–7 cm/second. Certain conditions can restrict the interpretation of color Doppler AUS signals. These include excessive bowel peristalsis, elevated bowel gas, use of high-frequency ventilation, decreased cardiac output, and use of vasopressors.[42,46]

There are also practical aspects of AUS to consider, as it may not be as readily available as plain abdominal radiographs and involves higher costs, and clinicians may not be as familiar with its use in NEC. AUS also requires sufficient expertise for adequate acquisition and interpretation of images. This expertise is primarily concentrated in pediatric hospitals where pediatric radiologists and US technologists are available. In contrast, most preterm infants at the highest risk for NEC are admitted in level 3 neonatal intensive care units (NICUs) housed within adult hospitals. Radiology services in this setting are often staffed by adult radiologists and US technologists who may not have sufficient expertise with AUS for NEC evaluation. Other practical limitations of AUS include potential intolerance to the procedure in labile, critically ill infants and poor image quality when excessive bowel gas is present.[8]

Emerging Modalities of Assessment in NEC

Several other noninvasive modalities for evaluating NEC have recently been reported in the literature. While not as well-studied as plain radiographs and AUS, these modalities have the potential to be clinically valuable for the assessment of NEC.

Contrast-enhanced US

CEUS is a novel imaging modality increasingly being used to assess pediatric bowel perfusion.[63] US contrast agent consists of microbubbles of gas suspended in a shell of various materials like sulfur hexafluoride, albumin, or lipid. The advantage of US contrast agents over the other more common radiological contrast agents is that they have no renal toxicity and have a lower rate of allergic reaction.

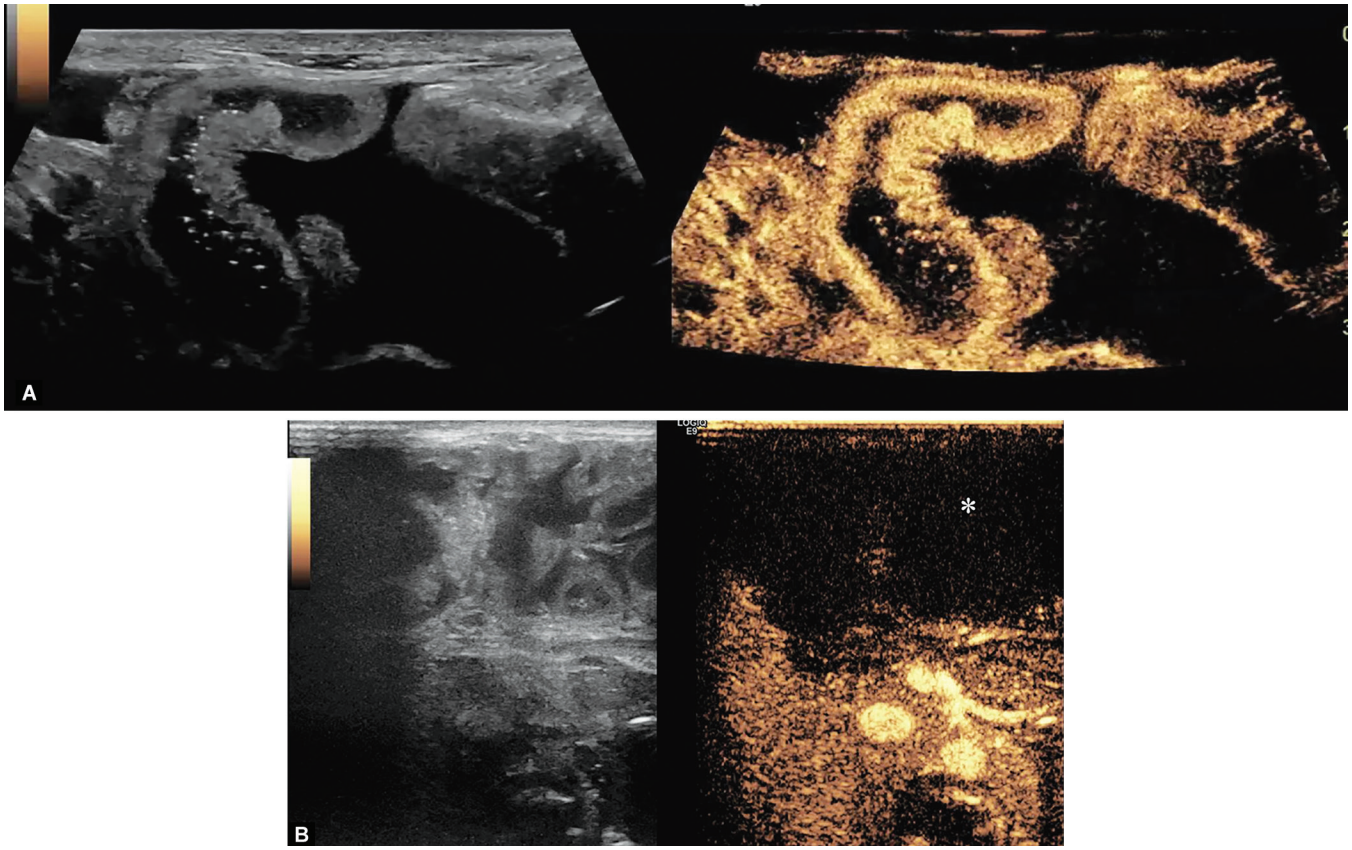
CEUS may act as an alternative to Doppler US to evaluate bowel perfusion, especially in neonates requiring mechanical ventilation, where there are disturbances due to the vibrations conducted from the ventilator to the body. During bowel wall inflammation in the early stages of NEC, CEUS displays hyperenhancement, and during progression to bowel wall ischemia and subsequent necrosis, there is hypoenhancement and eventually no perceptible enhancement in the CEUS (Figs 4A and B).[63,64] These findings are similar to those seen by color Doppler imaging, and the challenge for CEUS will be to prove that it provides increased value in return for the added complexity associated with the US contrast agent.

Magnetic Resonance Imaging

Mustafi et al. demonstrated that advanced magnetic resonance imaging (MRI) methods such as high-resolution magnetic resonance colonography and dynamic contrast-enhanced MRI could ascertain colonic injury in a mouse model.[65] MRI permits the noninvasive diagnosis of bowel necrosis, which appears bubble-like, in preterm neonates.[66] Mustafi et al. expanded the potential application of MRI by reporting its role in the early detection of NEC before clinical features in a neonatal rat model.[67] Although these studies show that NEC can be diagnosed on MRI, many barriers remain to widespread adoption. MRI examinations for NEC are not possible at the bedside, are expensive, and fail to identify any features over those imaged by US and radiograph.

Near-infrared Spectroscopy

Another promising modality is NIRS, which could help distinguish advanced stages of NEC from moderate disease by cerebral and splanchnic oxygenation measures; however, it may not be helpful in early-stage identification.[68,69] Fortune et al. demonstrated that



Figs 4A and B: (A) NEC in a 5-week-old premature infant who was admitted for abdominal distension and bloody stools. Dual-screen grayscale (left) and CEUS (right) still images from cine US show concentric wall thickening with surrounding ascites. The CEUS image shows marked hyperenhancement of the bowel wall compared with that of the surrounding mesentery and adjacent bowel loops, a finding compatible with the hyperperfusion phase of NEC and; (B) Total bowel necrosis in a premature newborn girl who was evaluated for possible in utero volvulus at prenatal US (not shown) and found to have duodenal atresia. Grayscale (left) and CEUS (right) images show that after administration of intravenous contrast material, no enhancement of the bowel wall is visible (*), which is consistent with diffuse bowel necrosis (Republished with permission from: Gokli A, Acord MR, Hwang M, et al. Contrast-enhanced US in pediatric patients: overview of bowel applications. RadioGraphics 2020;40:1743–1762. Copyright RSNA, 2020)

NIRS could be used to detect splanchnic ischemia by comparing cerebro-splanchnic oxygenation ratio (CSOR).[70] Cortez et al. established the feasibility of using NIRS in the early diagnosis of NEC in preterm neonates. They demonstrated that regional splanchnic oxygen saturation ($rsSO_2$) was lower, and fractional tissue oxygen extraction (FTOE) was higher in infants with feeding intolerance than those without feeding intolerance. Additionally, infants with NEC had persistently low $rsSO_2$ with a loss of variability preceded or followed by very high $rsSO_2$.[71] NIRS, alone or with other diagnostic modalities, holds the potential for facilitating early diagnosis and management of NEC[64,72] and may differentiate between complicated and uncomplicated NEC.[68]

Broad Optical Spectroscopy

NIRS involves oximeters that identify light absorbance and reflectance in a narrow wavelength range (700–850 nm); on the contrary, broad optical spectroscopy (BOS) involves spectroradiometers that can detect wavelengths in a much wider range (400–1800 nm).[73] BOS not only analyzes tissue oxygen levels but also evaluates biomarkers involved in the initial pathogenesis and progression of NEC. In a mouse model, it was able to diagnose NEC with 100% sensitivity and specificity.

Conclusion

NEC diagnosis and management continue to be challenging due to the lack of objective imaging methods for early detection with adequate sensitivity and specificity. The traditional use of abdominal radiographs for NEC diagnosis has poor specificity, leading to ambiguity in differentiating it from similar conditions, failure of early detection. By the time the specific diagnostic features become apparent, NEC has already progressed to an advanced, irreversible stage. Recent advancements in this field have identified ultrasonography, both with traditional and handheld probes, as a viable alternative with the potential for earlier diagnosis, improved management, and prognostication of outcomes for preterm infants with NEC. Despite the promising data, further studies are still needed due to lack of consensus, heterogeneous reporting, and a potential bias risk from observational studies. In addition to grayscale and Doppler US, several other modalities are under investigation, such as CEUS, MRI, NIRS, and BOS. Specific focus on optimum timing and frequency of US in preterm neonates with suspected NEC is needed.

Orcid

Rachana Singh https://orcid.org/0000-0001-7783-1214

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REVIEW ARTICLE

Approach to Neonatal Hypocalcemia

Sabitha S Pillai[1], Christy A Foster[2], Ambika P Ashraf[3]

Abstract

Hypocalcemia in neonates is defined as total serum calcium concentration less than 7.5–8 mg/dL and/or ionized calcium less than 4.4 mg/dL in neonates (>1500 g) and total serum calcium concentration less than 7 mg/dL or ionized calcium less than 3.6 mg/dL in low-birth-weight neonates (<1500 g). About 80% of the calcium transfer across the placenta occurs in the last trimester. Parathyroid hormone-related peptide (PTHrP) regulates the positive calcium balance in the placenta. Postpartum serum calcium level in neonates depends on an intricate relationship between PTH and renal and skeletal factors. Based on the timing of the presentation, hypocalcemia can be early onset (develops in the first 72 hours of life) and late onset (occurs after 72 hours of life). Causes of early-onset hypocalcemia include prematurity, SGA, IUGR, birth asphyxia, diabetes mellitus, or toxemia in the mother. Late-onset neonatal hypocalcemia may be caused by increased dietary phosphate content, neonatal vitamin D deficiency, hypomagnesemia, hypoparathyroidism, or parathyroid hormone resistance. We present a neonate with hypocalcemia due to transient hypoparathyroidism secondary to maternal adenoma. A thorough history and physical examination are essential to identify at-risk asymptomatic infants who need screening for hypocalcemia. Neonatal hypocalcemia can be a serious event and can cause serious morbidity and mortality. Majority of the early as well as transient late neonatal hypocalcemia resolves completely, while lifelong treatment may be required in some cases depending on the etiology.

Keywords: Calcium, Hypocalcemia, Neonate.

Newborn (2022): 10.5005/jp-journals-11002-0017

Case Presentation

A 14-day-old male infant was brought to the emergency department with the complaints of worsening seizure-like activity since Day 3 of life. He was born at 39 weeks with a birth weight of 3.09 kg via vaginal delivery to a G3 mother and was sent home on Day 2 of life. Mother noticed shaking of his extremities during sleep on Day 3 of life. These episodes gradually increased in severity and frequency. Mother described these events as diffuse generalized stiffening and shaking, facial grimacing, and gaze abnormalities with cyanosis. There were no constitutional symptoms or sick contacts. Current diet included standard neonatal formula.

Maternal history was significant for parathyroid adenoma detected during her second pregnancy. Surgery was deferred as the size of the adenoma reduced after delivery. During her current pregnancy, mother's serum calcium was elevated, with a maximum of up to 12 mg/dL, requiring hydration.

Initial laboratory studies revealed hypocalcemia (serum calcium 5.3 mg/dL), hyperphosphatemia (8.2 mg/dL), hypomagnesemia (1.34 mg/dL), low serum 25 hydroxy vitamin D (25OHD) (12.9 ng/mL), and inappropriately low intact PTH level (19 pg/mL). Other laboratory studies including hematologic, septic, and metabolic workup were normal. The urine calcium creatinine ratio was 0.3 mg/mg.

Introduction and Definition

Hypocalcemia is a common metabolic problem in neonates. Neonatal hypocalcemia is defined as total serum calcium concentration less than 7.5–8 mg/dL and/or ionized calcium less than 4.4 mg/dL in neonates with birth weights more than 1500 g and total serum calcium concentration less than 7 mg/dL or ionized calcium less than 3.6 mg/dL in neonates with birth weights less than 1500 g.[1]

[1]Department of Pediatrics, The Warren Alpert Medical School of Brown University, Providence, Rhode Island, United States of America

[2,3]Department of Pediatrics, The University of Alabama at Birmingham, Birmingham, Alabama, United States of America

Corresponding Author: Ambika P Ashraf, Department of Pediatrics, The University of Alabama at Birmingham, Birmingham, Alabama, United States of America, Phone: +2055150018, e-mail: AAshraf@uabmc.edu

How to cite this article: Pillai SS, Foster CA, Ashraf AP. Approach to Neonatal Hypocalcemia. Newborn 2022;1(1):190–196.

Source of support: Nil

Conflict of interest: None

Neonatal Calcium Homeostasis

Calcium is the most abundant mineral in the human body. About 99% of the body calcium is in bones and only 1% in serum.[2] Approximately half of the serum calcium is in the ionized form at normal protein concentration and is the physiologically active component. Forty percent of calcium is bound to albumin, and 10% is complexed.[3,4]

Factors that regulate calcium homeostasis include parathyroid hormone (PTH), vitamin D, calcitonin, and calcium-sensing receptors (CaSR). The actions of PTH include bone resorption, phosphate excretion, renal calcium, and magnesium reabsorption and increase 1,25 vitamin D (1,25OHD) levels to adult levels by 48 hours of life by increasing the activity of 1-alpha hydroxylase.[5,6] Most importantly, PTH activates the synthesis of calcitriol (1,25OHD) in the renal proximal tubule.[2,4] At higher serum concentrations, calcitriol causes bone resorption and promotes intestinal absorption of calcium and phosphate. Another key hormone for calcium homeostasis, calcitonin, is secreted by the parafollicular cells of thyroid gland and

decreases bone resorption, reduces gastrointestinal absorption of calcium and phosphate, and increases renal calcium and phosphate excretion.[1] PTH regulates calcium by its action on target cells in bone and kidney that express the PTH/PTH-related peptide (PTHrP) or type I PTH receptor.

Calcium regulation in the fetus and the neonate is markedly different from that in the later life. The calcium concentration is higher *in utero* to attain sufficient bone accretion. Transport of bone minerals across the placenta results in higher concentration in the fetus compared to maternal levels. Eighty percent of the calcium transfer across the placenta occurs in the last trimester.[7–9] Active transport is facilitated by transmembrane calcium selective channel TRPV6, calbindin D_{9k}, and plasma membrane calcium-ATPase.[7]

PTH and calcitonin do not cross the placental barrier. PTHrP regulates the calcium balance in the placenta.[4] Serum levels of phosphate, PTHrP, and calcitonin are greater, while 1,25OHD and PTH are lower in the fetus compared to maternal levels. Levels of 25-hydroxyvitamin D_3 (calcidiol) in the fetus approximate that in the mother.[1] After birth, neonates become reliant on the dietary intake of calcium absorbed from the gastrointestinal (GI) tract and skeletal calcium. The parathyroid glands will then respond to the decreased ionized calcium (iCa), although the response generally is insufficient. Serum total and iCa concentrations reach a nadir by 48 hours before increasing to mid-normal range by 72 hours of life. In the newborn, PTHrP and calcitonin levels decrease, and PTH and calcitriol levels increase over the first 48 hours.[1,4,7]

Other factors that influence neonatal calcium levels include magnesium, phosphate, and other anions in the serum, albumin, and pH. Hypomagnesemia can decrease the production of PTH and thus decrease PTH activity[10] or induce resistance to PTH hormone within the renal tubules and in the bone.[11] Serum anions, such as phosphate, citrate, or bicarbonate, increase the concentration of bound calcium and hence decrease the active iCa levels.[3]

Disturbances in acid-base status can influence iCa levels without affecting total calcium levels. In acidosis, H+ ions bind with albumin thereby reducing available albumin to bind with calcium and hence increases iCa levels, while alkalosis decreases the iCa levels.[12,13] Hypoalbuminemia decreases the total calcium levels, while the iCa level remains normal in the absence of other factors that can affect calcium homeostasis.[3,14,15]

Classification of Hypocalcemia

Neonatal hypocalcemia is a potentially life-threatening condition. When determining the etiology of the hypocalcemia, timing of the presentation is important. Hypocalcemia can be classified as early onset when it develops within the first 72 hours of life and late onset after 72 hours of life and usually by the end of first week after birth.[1,16]

Causes of Neonatal Hypocalcemia

Table 1 illustrates the causes of neonatal hypocalcemia.

Table 1: Etiology of neonatal hypocalcemia

Early-onset hypocalcemia[17]
Fetal/neonatal factors
Preterm infants
Low-birth-weight infants
SGA/IUGR babies
Perinatal asphyxia
Hypomagnesemia
Neonatal sepsis
Renal failure
Latrogenic: lipid infusions, citrated blood, bicarbonate
Maternal factors
Maternal diabetes mellitus
Maternal vitamin D deficiency
Toxemia of pregnancy
Maternal hyperparathyroidism
Late-onset hypocalcemia[1,5]
Increased dietary phosphate
Neonatal vitamin D deficiency
Hypomagnesemia
Hyperbilirubinemia and phototherapy
Hypoparathyroidism
Parathyroid hormone resistance

Early-onset Hypocalcemia

Early-onset hypocalcemia results from an exaggerated reduction in serum calcium that physiologically develops within the first 72 hours of life. It is often transient.

Roughly one-third of preterm babies and majority of the very low-birth-weight infants develop early neonatal hypocalcemia.[9,17] The causes of hypocalcemia in premature neonates include premature discontinuation of calcium transfer across the placenta, rapid and more significant fall in serum calcium from intrauterine levels, reduced nutritional intake, delayed secretion of PTH in response to low calcium levels, and decreased target organs response to PTH.[18] Hypocalcemia in low-birth-weight neonates may be secondary to the increased calcium accumulation in bones and resistance to vitamin D action resulting in reduced intestinal calcium absorption and skeletal calcium reabsorption.[9] The mechanism of hypocalcemia in asphyxiation is likely multifactorial, including greater phosphate load secondary to cell death, decreased calcium intake, greater calcitonin secretion, along with concurrent renal failure, and metabolic acidosis.[9,18,19]

Early neonatal hypocalcemia occurs in about half of infants born to mothers with diabetes mellitus. Women with diabetes will have higher serum calcium levels during pregnancy compared to healthy controls.[20,21] Hence, higher serum calcium seen in these babies *in utero* results in suppression of endogenous PTH secretion. Decreased maternal-fetal transfer of magnesium due to increased maternal urinary excretion of magnesium also leads to functional hypoparathyroidism in these neonates.

Late-onset Hypocalcemia

Late-onset neonatal hypocalcemia usually occurs 5–10 days after birth. The etiology is broad.[1,5]

Phosphate content is about seven times greater in cow's milk compared to that in breast milk (956 vs 140 mg/L in breast milk).[9] Infants taking milk formula or evaporated milk with high phosphate load develop hypocalcemia due to poorly soluble calcium salt formation. Increased phosphate will lead to increased PTH secretion or function or may cause increased skeletal deposition of calcium and phosphate causing hypocalcemia.[18,22]

Neonatal vitamin D deficiency may result from vitamin D deficiency in the mother, malabsorption, renal failure, and chronic liver diseases.[23,24] Hypophosphatemia usually accompanies hypocalcemia in these infants.[9]

Magnesium plays an important role in calcium homeostasis.[25] Hypomagnesemia causes PTH resistance and impaired PTH secretion resulting in hypocalcemia.[26] Most hypomagnesemia seen in neonates are transient.[25,27] Defects involving intestinal or renal tubular magnesium transport can result in hypomagnesemia, such as the mutations in the transient receptor melastatin 6 (TRPM6) and CLDN16 genes.[28–30]

Hypocalcemia accompanied by hyperphosphatemia should prompt the evaluation for hypoparathyroidism where PTH level may be low or inappropriately normal.[31] Hypoparathyroidism can be primary or secondary. Primary hypoparathyroidism can be isolated or associated with syndromes such as DiGeorge syndrome.[9] Maternal hyperparathyroidism also may lead to an impairment of the neonatal parathyroid gland's response. Maternal hypercalcemia due to hyperparathyroidism causes increased transfer of calcium. This increased fetal serum calcium suppresses fetal PTH synthesis and stimulates calcitonin secretion.[32]

Isolated causes of hypoparathyroidism include *GMC2*, PTH-gene mutations, autosomal dominant CaSR or *GNA11* mutations, and X-linked *SOX3* mutations.[33,34] Gain-of-function mutations in CaSR reduce the setpoint of CaSR, resulting in no PTH secretion at low calcium levels that would normally trigger PTH secretion.[35,36] These infants have an inappropriately normal-high urinary calcium excretion, in the setting of hypocalcemia due to increased CaSR activity in the kidney.[7]

Syndromic causes of hypoparathyroidism include 22q deletion syndrome, CHARGE association (CHD7), autoimmune polyglandular syndrome type I, and hypoparathyroidism, sensorineural deafness, and renal dysplasia (HDR) syndrome.[34] Mitochondrial cytopathies (Sanjad-Sakati and Kenney-Caffey syndromes) can also lead to hypoparathyroidism.[7,28,34,37] The most common syndromic cause of hypoparathyroidism is DiGeorge syndrome (DGS); the most severe phenotype includes cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, and hypocalcemia.[7,38,39]

Pseudohypoparathyroidism with end-organ PTH resistance may be transient as seen in babies with renal dysplasia or obstructive uropathy.[40] Permanent pseudohypoparathyroidism is seen in those with GNAS mutations causing pseudohypoparathyroidism type Ia (Albright's hereditary osteodystrophy).[41–43]

Infants with hyperbilirubinemia requiring phototherapy may develop hypocalcemia possibly due to reduced melatonin secretion resulting in increased skeletal uptake of calcium and increased urinary excretion of calcium.[44,45]

Clinical Signs of Hypocalcemia

Many neonates with hypocalcemia are asymptomatic, especially during the initial 72 hours of life. Notable symptoms include neuromuscular hyperexcitability manifested as jitteriness, jerking, tremor, hyperacusis, and focal or generalized seizures.[46] These infants may also exhibit apnea, cyanosis, reduced feeding, tachycardia, and congestive heart failure. Other less common symptoms include inspiratory stridor due to laryngospasm, wheezing due to bronchospasm, or vomiting due to pyloric spasm.[7,9]

Screening: Whom and When

As most infants with hypocalcemia are asymptomatic, serum or preferably ionized calcium should be measured in those at risk for hypocalcemia, such as preterm neonates, infants born SGA/IUGR, infants with a 1-minute APGAR score <4, and infants of diabetic mothers. For babies with extremely low birth weight (birth weight less than 1000 g) or infants with underlying sepsis, ionized calcium should be monitored at 12, 24, and 48 hours of life. For babies with gestational age less than 32 weeks and preterm babies with a birth weight between 1000 and 1500 g, iCal should be checked at 24 and 48 hours of life.[47] Any infant having symptoms consistent with hypocalcemia should be screened.[9] Calcium monitoring should be continued until calcium levels normalize and oral intake is adequate.[47]

Approach to Hypocalcemia in Neonates

A thorough history and physical examination are essential to identify at-risk asymptomatic infants who need screening for hypocalcemia (Tables 2A and B).

Laboratory Workup

Initial laboratory studies include total and iCa and serum phosphorus[9]—see Flowchart 1. As total calcium in serum includes both the free (biologically active) and protein-bound components, iCa should be measured, particularly in the setting of acute illness, premature or ill infants, malnutrition, or hypoalbuminemia. For each unit increase in pH, iCa falls by 0.16 mg/dL. Serum total calcium levels must be corrected for the albumin level (plasma calcium concentration falls by 0.8 mg/dL for every 1.0 g/dL fall in the plasma albumin concentration).

Normal or low phosphate level is seen in vitamin D deficiency, vitamin D resistance, renal loss as in renal tubular defects, and iatrogenic causes, such as administration of citrated blood and bicarbonate therapy. High phosphate concentration may be due to increased phosphate load in the feedings, renal failure, hypoparathyroidism, or pseudohypoparathyroidism.[9] Low PTH can be seen in hypoparathyroidism and hypomagnesemia, while marked or persistent elevation in PTH is seen in vitamin D deficiency and pseudohypoparathyroidism. Serum magnesium level should be checked. Mothers of neonates with low PTH and with normal magnesium should be screened for maternal hypercalcemia and hyperparathyroidism. Infants with primary hypoparathyroidism should undergo detailed genetic and metabolic workup. Urine calcium, magnesium, phosphate, and creatinine levels should be checked in neonates with suspected renal tubular defects.[48]

Imaging studies include chest X-ray to look for absent thymic shadow (in DGS) and status of aortic arch, and echocardiography to evaluate for cardiac truncal defects which can occur in 22q11.2 deletion disorders.[7] Fluorescence *in situ* hybridization (FISH) studies assess for the genetic deletion in 22q11.2. Electrocardiographic (EKG) changes associated with hypocalcemia include a prolonged corrected QT interval, prolonged ST segment, and T-wave abnormalities.

Treatment

Table 3 details the management of hypocalcemia. Asymptomatic infants with hypocalcemia can be managed with oral calcium supplementation. The goal is to ensure adequate calcium intake by initiating early feedings. Formula that provides calcium and phosphorus in 2:1 ratio is optimal. Oral calcium supplements can be given that increases the calcium phosphorus ratio to 4:1 till hypocalcemia is corrected and PTH function is normalized.[9]

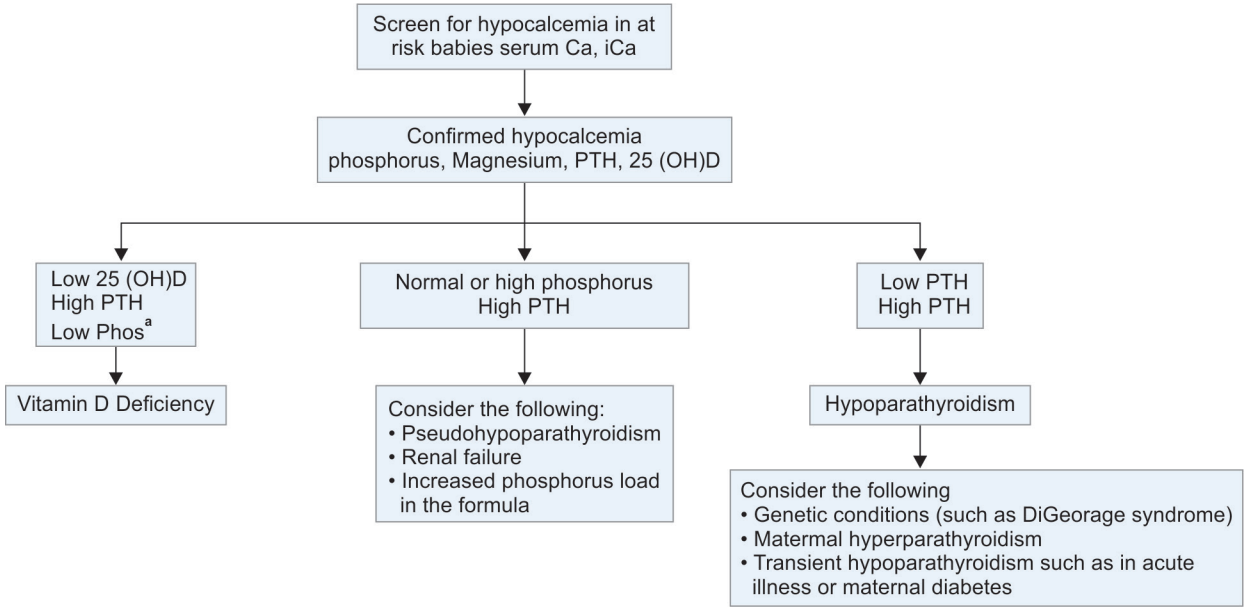
Table 2A: Salient points in the history for neonatal hypocalcemia

History	*Salient points*
Antenatal history	Gestational/permanent forms of diabetes with degree of glycemic control, toxemia of pregnancy, maternal vitamin D deficiency, maternal hyperparathyroidism, maternal medications (excess alkali), and fetal growth restriction
Natal history	Gestational age, birth weight, mode of delivery and associated complications, and perinatal asphyxia
Postnatal history	Day of presentation, feeding method, phosphate content of the formula used, calcium content of the TPN, neonatal sepsis, phototherapy, renal failure, hepatobiliary disease, blood transfusion, and medication review[9]
Family history	DGS, renal calculi, rickets, seizures due to hypocalcemia, and skeletal abnormalities[9]

Table 2B: Key physical examination elements for neonatal hypocalcemia

Vitals: Tachycardia, apnea
Cyanosis, hyperbilirubinemia
Growth parameters: IUGR, SGA
Cardiac failure
Cardiac murmur
Examine for stigmata of 22q11.2 deletion syndrome: Cleft palate, bifid uvula, enophthalmos, ear anomalies, prominent nasal bridge, micrognathia, asymmetric crying facies and craniosynostosis, anteriorly placed or imperforate anus, cardiac defects, infantile hypotonia, congenital talipes equinovarus, polydactyly, cryptorchidism, and hypospadias.[50,51]
Examine for clinical features of Type Ia—Albright's hereditary osteodystrophy: Short fourth, and fifth metacarpals and metatarsals or short fourth metacarpal only "Knuckle, knuckle, dimple, dimple" sign on closed fist.[50,51]

Flowchart 1: Diagnostic evaluation for hypercalcemia[9,50]



For those who are on total parenteral nutrition, 10% calcium gluconate can be added to the solution (50 mg/kg of elemental calcium/day). Treatment is also directed toward other underlying etiologies, such as hypomagnesemia, vitamin D deficiency, and hyperphosphatemia.

Symptomatic infants with tetany/seizures should be managed with 10% calcium gluconate solution 1 mL/kg intravenously over 5–10 minutes with careful monitoring of heart rate and infusion rate. Calcium gluconate at 1 mL/kg can be repeated if no response seen in 10 minutes. Calcium chloride at a dose of 0.2 mL/kg is another preferable alternative. To avoid precipitation of calcium salts, phosphate and bicarbonate should not be infused concomitantly with the calcium. Following the administration of calcium as a bolus, 50–150 mg/kg/day elemental calcium infusion should be initiated until the patient can tolerate oral calcium supplementation.[5]

Once the symptoms are controlled and the patient is able to tolerate supplements by mouth, oral calcium supplementation should commence.[16] Calcium carbonate and calcium citrate have the greatest proportion of elemental calcium (40 and 21% elemental calcium by weight, respectively); they are considered the supplements of choice.

Table 3: Acute management of hypocalcemia

Acute management of hypocalcemia[*5,9]	
Asymptomatic	
Calcium supplementation	Enteral: 50 mg/kg elemental calcium
	TPN: 10% calcium gluconate added to the solution
Symptomatic[α]	
Calcium supplementation	IV calcium gluconate 1 mL/kg over 10 minutes, repeat PRN
	Bolus followed by elemental calcium infusion 50–150 mg/kg/day IV until enteral feeds can be tolerated.
Vitamin D supplementation[β]	
	Vitamin D2/D3 2000 IU daily × 6–12 weeks, then maintenance dose, 400 IU/day
	Calcitriol[£] 0.08–0.1 µg/kg/day

[*]Total calcium <8 mg/dL; iCal <1.2 mmol/L; [α]Seizures or abnormal EKG; [β]Vitamin D2 or 3 in vitamin D deficiency or hypoparathyroidism; [£]Calcitriol short term to correct hypocalcemia in transient or permanent hypoparathyroidism

EKG monitoring is recommended as dysrhythmias can occur if correction is too rapid.[9] Due to potential risk of extravasation of calcium into subcutaneous tissues causing tissue necrosis and calcification of subcutaneous tissues, oral calcium supplementation should be initiated as soon as possible. Other feared adverse effects include hepatic necrosis as a result of infusion via an umbilical venous catheter with tip in a portal vein branch and arterial spasm following intraarterial infusion.[16]

Additional management depends on the underlying etiology. Vitamin D deficiency (low serum 25OHD) is treated by either ergocalciferol (D2) or cholecalciferol (D3). If renal 1 alpha-hydroxylation is impaired, such as hypoparathyroidism or PTH resistance or the vitamin D–dependent rickets syndromes, metabolites that do not require this enzymatic activation should be administered (calcitriol).

For infants with late-onset hypocalcemia, vitamin D3 400 international units/day is given for 1–2 weeks. Calcitriol at a dose of 0.08–0.1 µg/kg can be given to facilitate intestinal calcium absorption and to release calcium from the skeleton.[16] Calcitriol is an active form of vitamin D which has a number of *direct* effects, including (1) increasing intestinal calcium and phosphorus absorption, (2) increasing renal calcium reabsorption, (3) bone resorption and release of calcium, and (4) suppressing parathyroid gland PTH secretion via transcriptional downregulation.[49] Except in cases of hypoparathyroidism, calcitriol is not continued long term.

In hypoparathyroidism, in addition to calcium supplementation and calcitriol, a low phosphate formula is preferred. Those with hyperphosphatemia are given a diet high in calcium and low in phosphorus in addition to oral calcium supplements.[50] Treatment can be further intensified with thiazide diuretics (increasing distal renal tubular calcium reabsorption) and a low salt (dietary sodium restriction). The primary goals of management include symptom control, maintaining serum calcium in the low-normal range, reducing serum phosphorus to a normal range, and maintaining a calcium-phosphate product below 55 mg^2/dL^2. Complications of current therapies for hypoparathyroidism include hypercalciuria, nephrocalcinosis, and soft tissue calcification. Duration of treatment may last from weeks to months in cases of transient hypoparathyroidism and can be permanent in cases of primary hypoparathyroidism.[9]

Follow-up

The neonate in the above case received intravenous (IV) calcium gluconate bolus followed by continuous calcium carbonate (125 mg of elemental calcium/kg/day) via nasogastric (NG) tube as there was no IV access. He also required repletion of magnesium sulfate. Infant was also started on 800 IU vitamin D and 0.05 µg bid calcitriol. His formula was changed to PM 60:40. His further workup showed normal EEG, CT brain, and chest X-ray.

The continuous calcium carbonate supplementation via NG tube was changed to intermittent enteral calcium carbonate as his calcium stabilized over 72 hours. The calcitriol dose was reduced to 0.05 µg once daily. The calcitriol was stopped when the serum calcium reached >9.0 mg/dL. The infant was weaned from calcium supplements by 5 weeks of life and vitamin D by 8 weeks of life. He remained stable and symptom-free with normal serum biochemistries during follow-up.

Conclusion

The patient in the vignette had hypocalcemia, hyperphosphatemia, and inappropriately low intact PTH for the hypocalcemia consistent with hypoparathyroidism. Maternal history of parathyroid adenoma with hypercalcemia suggested transient hypoparathyroidism secondary to chronic maternal hypercalcemia. The concomitant vitamin D deficiency and hypomagnesemia exacerbated the clinical picture. We were able to wean him off all the supplementation by 6–8 weeks confirming a diagnosis of transient hypoparathyroidism. Consideration of the differential for neonates should be kept broad and key history, and physical findings can illuminate the most likely etiology.

Orcid

Ambika P Ashraf https://orcid.org/0000-0003-0692-6624

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ARTICLE COMMENTARY

Rethinking the Paradigm: The Evolving Care of Children with Trisomy 13 and 18

Kimberly L Spence[1], Erica K Salter[2]

Abstract

A chromosomal evaluation should be used to provide better care for a child and their family, not limit it. However, in many pediatric institutions, the diagnosis of a chromosomal abnormality automatically circumscribes the medical and surgical options made available to the family. For example, alongside many other comorbidities (including severe cognitive impairment), infants diagnosed with trisomy 13 or 18 (T13/18) often have cognitive heart defects (e.g., atrial or ventricular septal defects, patent ductus arteriosus, atrioventricular septal defects) that can be successfully repaired or palliated in the general population. However, because T13/18 have historically been considered "lethal" diagnoses or "incompatible with life", surgical correction of these defects is not frequently offered, and instead infants with these diagnoses are managed with a noninterventionist, "comfort care" approach in which the infant is simply allowed to expire after birth. In recent years, however, more data have emerged from centers that regularly pursue medical and surgical interventions in this population, demonstrating improved outcomes in both quality and quantity of life. Simultaneously, the pediatric ethics literature has argued that treatment decisions for infants with T13/18 are frequently informed by unfounded biases concerning disability and quality of life. Now that neonatology is equipped with improved medical and ethical evidence, the practice of categorically excluding infants with a T13/18 diagnosis from life-saving interventions should be challenged, and instead, parents of these infants should be offered targeted interventions, including corrective and palliative procedures, and included in the process of shared decision-making about which interventions best meet the family's goals of care.

Keywords: Ethics, Trisomy 13, Trisomy 18.

Newborn (2022): 10.5005/jp-journals-11002-0007

Introduction

A chromosomal evaluation should be used to provide better care for a child and their family, not limit it. However, in many pediatric institutions, the diagnosis of a chromosomal abnormality automatically circumscribes the medical and surgical options made available to the family. For example, alongside many other comorbidities (including severe cognitive impairment), infants diagnosed with trisomy 13 or 18 (T13/18) often have cognitive heart defects (e.g., atrial or ventricular septal defects, patent ductus arteriosus, atrioventricular septal defects) that can be successfully repaired or palliated in the general population. However, because T13/18 have historically been considered "lethal" diagnoses or "incompatible with life",[1] surgical correction of these defects is not frequently offered, instead infants with these diagnoses are managed with a noninterventionist, "comfort care" approach in which the infant is simply allowed to expire after birth. In recent years, however, more data have emerged from centers that regularly pursue medical and surgical interventions in this population, demonstrating improved outcomes in both quality and quantity of life.[2–4] Simultaneously, the pediatric ethics literature has argued that treatment decisions for infants with T13/18 are frequently informed by unfounded biases concerning disability and quality of life. Now that neonatology is equipped with improved medical and ethical evidence, the practice of categorically excluding infants with a T13/18 diagnosis from life-saving interventions should be challenged, and instead, parents of these infants should be offered targeted interventions, including corrective and palliative procedures, and included in the process of shared decision-making about which interventions best meet the family's goals of care.

[1]Department of Pediatrics, Saint Louis University School of Medicine, Saint Louis, Missouri, United States of America

[2]Center for Health Care Ethics, Saint Louis University, Saint Louis, Missouri, United States of America

Corresponding Author: Kimberly L Spence, Department of Pediatrics, Saint Louis University School of Medicine, Saint Louis, Missouri, United States of America, Phone: + 314-577-5642, e-mail: Kimberly.spence@health.slu.edu

How to cite this article: Spence KL, Salter EK. Rethinking the Paradigm: The Evolving Care of Children with Trisomy 13 and 18. Newborn 2022;1(1):197–200.

Sources of support: Nil

Conflict of interest: None

Precedent for Cultural Shift

Even relatively recent events demonstrate the ways in which the medical ethos around individuals with disabilities can shift dramatically. For example, consider the Baby Doe Regulations of 1984, catalyzed in part by the widely publicized case of Baby Doe, in which life-saving treatment options were withheld from an infant with Trisomy 21 (T21), primarily because of judgments about his quality of life. These federal regulations prohibit medical providers from withholding medically indicated treatment from disabled infants with life-threatening conditions when that treatment would likely be effective at correcting or ameliorating all such conditions.[5] The regulations included four situations in which treatment would not be required: (1) to the chronically or irreversibly comatose, (2) if futile, (3) if inhumane, or (4) would merely prolong dying.[5] The

legacy of Baby Doe has been improved survival rates and quality of life for more patients with T21 and their families.[6] Alongside a significantly improved life-expectancy (from 25 years in 1983 to 49 years in 1997), care providers were newly able to study the long-term effects of T21 and the unique physiology of infants with T21, providing optimized and individualized care.[6,7] For example, the increased recognition of pulmonary hypertension as a complicating factor of congenital heart disease led to more nuanced care for these children with altered timelines of repair of cardiac defects as well as increased surveillance for certain complications.[8] This improved understanding of the unique physiology translated to increased rates of survival and improved outcomes for children with T21. These significant medical developments only occurred because the Baby Doe ruling required health care institutions to offer otherwise medically indicated treatment to a population that had been regularly excluded from intervention.

Improving Outcomes

The original survival data published reported survival rates of approximately 10% at one year of age for infants with T13/18.[9] This led physicians to consider these chromosomal defects "lethal" and to recommend comfort measures for these families. In fact, most textbooks of pediatrics and neonatology report that most babies with T13 and T18 die in the first year of life and that, for the rare survivor, quality of life is unacceptably poor.[10] As a result, many medical textbooks explicitly recommend against life-saving interventions in this population. However, time has revealed this prognosis to be a self-fulfilling prophecy. If babies are expected to die with or without treatment, they will not be treated. And if not treated, they will certainly die, thus fulfilling the prophecy of lethality. While there remain some cases in which certain life-saving interventions for T13/18 infants would be futile toward relevant family goals (e.g., continued survival, living at home with family, better quality of life), this is no longer the case for all T13/18 patients, as targeted interventions are frequently able to meet these goals. An unbalanced or apparently hopeless clinical presentation will cause many parents to find alternative sources of information, including social media groups dedicated to parent support. These groups typically offer parents a more positive outlook, including first-hand accounts of parents of children with T13/18 with very good outcomes, causing some to worry these groups cause unrealistically positive expectations.[10,11] It should not surprise us, however, that parents will seek out more balanced information and the possibility of hope when it is not presented by their clinical team.

As occurred with infants with T21, interventional studies largely from the United States and Japan have demonstrated improved survival in children offered medical and surgical interventions.[3,12–14] Congenital heart disease is very common in infants with T13/18 and one of the primary sources of mortality.[15] Given the shortened lifespan, comorbidities as well as cognitive impairments, the surgical treatment of congenital heart disease has become very controversial. However, recent data concerning cardiac surgical outcomes in infants with T13 and T18 are promising. For example, in Japan about 94% of infants with T13/18 had congenital heart disease; patients who underwent operative repair survived longer than those who did not have surgery.[14] Furthermore, recent studies have demonstrated improved outcomes (longer lengths of survival and higher rates of survival to discharge) for patients who underwent complete repair versus a palliative procedure.[3] Expectant management of congenital heart disease led to death prior to discharge about 50% of the time.[15] Though some infants might not benefit from cardiac interventions, many will and a categorical exclusion of T13/T18 infants from surgical intervention only perpetuates injustice against disabled populations.

Other surgical interventions have shown similar successes. Nishi et al. reported on 24 patients with T18 who underwent tracheoesophageal fistula repair, a potentially fatal complication that usually requires surgery shortly after birth (r17). Of these patients, 17 went on to tolerate enteral feeds, none suffered intraoperative deaths or anesthetic complications.[16] Of note, the 1 year survival rate was 17% for those infants managed with palliative procedures and 27% of those who underwent complete repair, emphasizing the benefits of complete repair over palliative options in some cases.[16] The steepest part of the survival curve for these patients occurs in the first few months with approximately 40% of T13 and about 35% of T18 alive at 30 days.[2] However, those who make it to a month of life then have a 60% (T13) and 71% (T18) probability of survival to one year.[17,18] Among the most common situations motivating an institutional transfer are infants who survived the first few months of life, and now have conditions that would benefit from surgical intervention, but are being treated at an institution that has a philosophy of care that excludes T13/18 infants from aggressive intervention. These patients with T13/18 represent a subgroup most likely to benefit from more aggressive interventions, having "beaten the odds" of an early death from apnea. More facilities should consider reevaluating institutional practices around T13/18 interventions in general, but especially this sub-group, for whom the benefits are even more likely to exceed the risks.

It is critical to do more research and identify factors influencing outcomes in infants with T13/18 to best serve this population. A common goal of many families is to simply take their child home. It would seem from this preliminary data that many fairly routine medical and surgical interventions such as fetal monitoring, cesarean sections for fetal distress, resuscitation, gastrostomy tubes, and ventricular septal defects (VSD) repairs achieve this goal better than the traditional non-interventionist philosophy of care. And most importantly, these decisions should be made with, not simply for, the parents of these infants.

Ethical Considerations: Quality of Life, Disability, and Shared Decision-making

As presented above, medical training (via analysis of medical textbooks) almost uniformly presents T13/18 as a lethal diagnosis resulting in a very short lifespan characterized by poor quality of life. Judgments about the quality of a T13/18 infant's life is likely informed by evidence that these diagnoses are usually accompanied by comorbidities of physical (e.g., limb malformations, growth restriction, rocker bottom feet, and myelomeningocele) and developmental disabilities (e.g., hearing and vision deficiencies, communication difficulties, and low IQ). Judgments or assumption about what life with these disabilities might be like can result in a significant bias against offering medical or surgical interventions. However, as has been well documented, children and adults with disabilities experience a much higher quality of life than others assume for them. This phenomenon of under-estimation of quality of life by outside observers—known as the "disability paradox"—is exhibited by not only strangers, but by clinicians, caregivers, and even parents of the disabled individual.[19–21] Simply put, assumptions

T13/T18 babies experience a uniformly low quality of life are likely wrong. In fact, despite significant delays, children who survive past 1 year of age show continued achievement of developmental milestones across their lifespan, and there are some older children who even grow to exceed the average developmental quotient.[11,22]

Further, even severe developmental disabilities do not eliminate an individual's ability to give or receive love, nor do they impair a child's ability to relate to their parents, siblings, or loved ones. Studies show that children with T13/18 can communicate in a variety of ways including smiling, laughing, reaching, and vocalizing, and the majority (66%) of parents of children with T13/18 reported their child produced at least one word, gesture, or augmentative and alternative communication form.[23–25] Further, families describe their child's life as "significant," "valuable," and "transformative to the lives of those around them," no matter how short or how disabled.[26,27]

Although the data suggest that (contrary to the conventional presumption of medicine) many children with T13/18 will experience improved outcomes in the quality (and quantity) of their life with the provision of targeted medical and surgical interventions, this should not lead us to presume that all parents would, or should, choose aggressive interventions for their child. Instead, shared decision-making should remain the model for care for these patients. Shared decision-making requires the presentation of accurate, balanced, and up-to-date information about the possible implications of a T13/18 diagnosis, including both the possible negative consequences (which have been traditionally over-emphasized) and the possible positive consequences (traditionally under-emphasized, or absent altogether). Families that are told by medical providers that their child's life lacks value or will only be characterized by suffering and burden may cause parents to "fight back," and escalate care, even in situations where it does not serve the child's well-being. Thus, medical providers should acknowledge the impact of their own biases and value judgments on parental decision-making and seek to create a therapeutic alliance that is marked by honesty, transparency, and empathy.

The medical team then should explore each family's unique preferences, values, hopes, and fears, including any relevant emotional, financial, relational, and practical aspects of the diagnosis.[28] In addition, the family should be encouraged to explore, and in some cases challenge (when affected disproportionately by the disability paradox) their own notions of quality of life. Further, medical decision-making about a complex diagnosis like T13/T18 is never a single, one-size-fits-all encounter; providers should anticipate many ongoing conversations and, as circumstances change, revisit the family's goals and the interventions best suited to meet those goals. Only when informed by a specific family's experiences, challenges, and goals of care can clinicians cultivate a trusting, therapeutic relationship with families and recommend interventions that are truly family centered.

Conclusion

The legacy of Baby Doe provides historical precedent for a shift in the paradigm of care for all infants with a chromosomal abnormality, but specifically those with T13/18. Indeed, T13/18 have been called the "next Baby Doe."[29] Baby Doe demonstrates that medical knowledge and the resulting decision-making structures evolve over time with the emergence of new data, new evidence about quality of life based on those data, and thus, new ethical principles undergirding decision-making. Therefore, rather than categorically limit care, genetic diagnosis of T13 or T18 should provide clinical insight and help inform therapeutic interventions the same way a diagnosis of T21 is able to guide targeted and compassionate care today. With the parent's unique goals of care in mind, the individual patient's distinct pathophysiology should guide management, not the medical care team's judgment about the child's future cognitive abilities. Management should be targeted to a family's specific goals of care, pragmatic and guided by the same ethical principles by which other children with the same underlying conditions are managed. Certainly, there will be babies who die shortly after birth, but there is a significant number who survive and should be given the same opportunities for care, treatment, and dignity as other children.

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ARTICLE COMMENTARY

Current Understanding of Transfusion-associated Necrotizing Enterocolitis: Review of Clinical and Experimental Studies and a Call for More Definitive Evidence

Minesh Khashu[1*], Christof Dame[2*], Pascal M Lavoie[3], Isabelle G De Plaen[4], Parvesh M Garg[5], Venkatesh Sampath[6], Atul Malhotra[7], Michael D Caplan[8], Praveen Kumar[9], Pankaj B Agrawal[10], Giuseppe Buonocore[11], Robert D Christensen[12], Akhil Maheshwari[13]

Abstract

Introduction: The association between red blood cell (RBC) transfusions and necrotizing enterocolitis (NEC), so-called transfusion-associated NEC (ta-NEC), was first described in 1987. However, further work is needed to confirm a causal relationship, elucidate underlying mechanisms, and develop possible strategies for prevention. We performed an extensive literature search in the databases PubMed, EMBASE, and Scopus.

Areas covered: Although multiple retrospective human studies have strongly suggested an association between blood transfusions and subsequent occurrence of NEC, meta-analyses of randomized controlled trials (RCTs) testing RBC transfusion thresholds or the use of recombinant erythropoiesis-stimulating growth factors did not confirm an association of anemia with ta-NEC. These conflicting data necessitated the development of an animal model to elucidate mechanisms and causal factors. Data from this recent mouse model of ta-NEC highlighted the importance of sequential exposure to severe anemia followed by transfusion for development of ta-NEC.

Expert opinion: This review summarizes current human and experimental data, highlights open questions, and suggests avenues for further research aimed at preventing ta-NEC in preterm infants. Further studies are required to delineate whether there is a tipping point, in terms of the level and duration of anemia, and to develop an effective strategy for blood management and the quality of RBC transfusions.

Keywords: Anemia, Necrotizing enterocolitis, Preterm infants, ta-NEC, TANEC, TRAGI, Transfusion.

Newborn (2022): 10.5005/jp-journals-11002-0005

Introduction

Premature infants are a heavily transfused population.[1,2] Up to 90% of extremely low birth weight infants, and approximately 60% of preterm infants born at <32 weeks of gestational age receive red blood cell (RBC) transfusions during the neonatal period.[3] In several reports, up to 25–40% of infants who developed necrotizing enterocolitis (NEC) may have received RBC transfusion(s) in the preceding 48 hours, and this connection has been referred to as transfusion-associated NEC (ta-NEC).[4–26] However, these results have not been conclusive in systematic reviews of case-control or observational studies.[6,27–29] In contrast, ta-NEC has not been associated to anemia in RCTs on RBC transfusion thresholds and in studies on the efficacy of recombinant erythropoiesis-stimulating factors.[30,31] These problems indicated a need for focused preclinical and clinical studies. However, the interest in the association between anemia and ta-NEC has been rekindled following a recent comprehensive animal study.[32]

Herein, we review the current data on ta-NEC and its possible mechanisms and then recommend future direction of research and clinical practice. We performed an extensive literature search in the databases PubMed, EMBASE, and Scopus. To minimize bias, key words from the medical subject heading thesaurus on PubMed were shortlisted prior to the actual search and combined with text words used in titles and abstracts.

[1]University Hospitals Dorset, Poole, United Kingdom
[2]Charité–Universitätsmedizin Berlin, Berlin, Germany
[3]University of British Columbia, Vancouver, British Columbia, Canada
[4]Northwestern University, Chicago, Illinois, United States of America
[5]University of Mississippi, Jackson, Mississippi, United States of America
[6]University of Missouri–Kansas City, Kansas, United States of America
[7]Monash University, Melbourne, Australia
[8]University of Chicago, Chicago, Illinois, United States of America
[9]Postgraduate Institute of Medical Education and Research, Chandigarh, Punjab, India
[10]Boston Children's Hospital, Harvard University, Boston, Massachusetts, United States of America
[11]University of Siena, Siena, Italy
[12]University of Utah, Salt Lake City, Utah, United States of America
[13]Global Newborn Society, Baltimore, Maryland, United States of America
[*]Drs Khashu and Dame contributed equally to this work

Corresponding Author: Akhil Maheshwari, Global Newborn Society, Lily Garden, Clarksville, Maryland, United States of America, Phone: +1 708-910-8729, email: akhilglobalnewborn@gmail.com

How to cite this article: Khashu M, Dame C, Lavoie PM, *et al*. Current Understanding of Transfusion-associated Necrotizing Enterocolitis:

RBC Transfusions as a Potential Trigger of NEC in Very Preterm Infants

The association between transfusions and NEC was first observed by McGrady et al.[33] in 1987, who investigated an outbreak of 33 cases of NEC in their neonatal intensive care unit (NICU) and reported that many cases followed RBC transfusions (odds ratio [OR] = 15.1, confidence interval [CI]: [2.59–92.51]).[33] In 1998, Bednarek et al.[34] noted that NICUs with fewer transfusions had less NEC (OR = 0.3, CI: [0.1–0.8]). Several subsequent studies confirmed that 25–40% of very preterm infants who developed NEC had received one or more RBC transfusions in the preceding 2–48 hours, generally about 12 hours, before the onset of NEC.[5,11,13,21,23,25,26,33–41] These studies suggested that ta-NEC occurred (a) in infants born at earlier gestational ages than those who developed NEC unrelated to transfusion;[5,11,13,21,23,25,26,33–39] (b) at 3–5 weeks after birth, later than NEC unrelated to transfusions seen at 1–3 weeks;[5,13,21,23,25,26,33–39] (c) in neonates who had received 1–3 RBC transfusions;[5,25] and (d) in infants who may have had a higher acuity of illness prior to developing NEC.[6,11,13,23,26,39,40] The postnatal age of infants who developed ta-NEC was higher than those who developed NEC without a temporally proximate transfusion.[5,13,25] However, the storage age of donor blood transfused into infants who developed ta-NEC was not different from that in matched controls who did not develop NEC.[4]

Most studies of ta-NEC were based on a small number of patients, and therefore, lack generalizability and the statistical power to adjust for confounders. Therefore, there have been several efforts focused on meta-analysis of pooled data. In one of these early reports, Mohamed and Shah[6] systematically reviewed 11 observational studies of NEC and confirmed increased odds of NEC within a 48-hour period following an RBC transfusion. Another meta-analysis of five studies showed increased risk of NEC following transfusions in the previous 48 hours (pooled OR = 3.91, 95% CI: [2.97–5.14]; $I^2 = 58\%$).[5,11,21,25,42] A meta-analysis of four studies also showed increased risk of NEC (pooled OR = 2.01, 95% CI: [1.61–2.50]; $I^2 = 91\%$).[25,38,43] Another study that combined seven case-control studies (480 blood transfusion cases, 2,845 control cases) showed similar results in a random-effects model (OR = 3.35, 95% CI: [1.54–7.27]).[27] Sensitivity analysis showed an increased OR for NEC within 48 hours after transfusion at 4.21 (95%

Review of Clinical and Experimental Studies and a Call for More Definitive Evidence. Newborn 2022;1(1):201–208.

Source of support: This article was supported in parts by an Investigator Grant from the British Columbia Children's Foundation (PML); and NIH awards DK116568 (IGDP); GM115428 (PMG); DK117296 (VS); HL124078 and HL133022 (AM).

Conflict of interest: None

Table 1: Summary of studies Included in the meta-analysis by Garg et al.

Authors of study	*Type of study*	*Gestational age*	*Birth weight*	*No. of infants case*	*No. of infants control*	*Hematocrit NEC*	*Odds ratio (OR, 95% confidence interval) RBC transfusion*
Patel et al.	Case control	27.9 ± 3.3	1015 ± 273	40	554		For transfusion-0.4 (0.17–1.1), for anemia-5.9 (2–18; $p = 0.001$)
AlFelah et al.	Retrospective case control	28	1,042	40	112		OR = 0.39, (0.18–0.84), $p = 0.02$
Sharma et al.	Case control	27 ± 2	983 ± 333	42	42		1.4 (0.4–5.6)
Wallenstein et al.	Retrospective cohort	27 IQR 3	790 IQR 290	24	390	29	0.6 (0.2–1.7)
Bak et al.	Retrospective case control	27.6 ± 2.2	1027 ± 343	18	162	46.9 ± 4.1	1.63 (1.14–2.3)
Gomez-Martin	Retrospective case-control			30	30		1.5 (1.0–2.2)
Wan-Huen et al.	Case control	26 ± 2.4	840	49	97	31.4 ± 3.7	3.0 (1.7–5.5)
Demirel et al.	Case control	28.4 ± 1.4	1078 ± 236	96	551	30 ± 4.4	OR not mentioned
Stritzke et al.	Case control	25.8 ± 2.6	885 ± 446	927	2,781	NA	2.2 (1.8 0 2.8)
El-Dib et al.	Phase 1: Retrospective case-control; Phase 2: comparison study of incidence of NEC	26.8 ± 2.5	935 ± 350	25	25	NA	5.1 (1.4–17.9)
Paul et al.	Case control	26.8 ± 2.4	969 ± 239	33	30	28.6 ± 5.2	2.5 (0.8–8.3)
Singh et al.	Case control	26.9 ± 2.5	969.7 ± 309.0	111	222	29.9 ± 5.6	5.6 (3.2–10.2)
Christensen et al.	Retrospective case control	27 (26–28)	981 (835–1,128)	62	248	Not mentioned	11.8 (4.6–30.4)
Josephson et al.	Case control	27.7 (25.7–30.7)	1030 (740–1,410)	93	91	31.8 ± 7.8	0.7 (0.4–1.3)
Binder et al.			<1,500	78	783		0.07 (0.03–0.14)
Harsono et al.			<1,500	43	2,080		0.3 (0.2–0.6)
Garg et al.	Retrospective cohort	27.3 ± 2.5	992 ± 377	99		27.4 ± 4.5	2.83 (0.97–8.9)

NEC, necrotizing enterocolitis; RBC, red blood cell; IQR, interquartile range; NA, not applicable

CI: [2.17–8.16]). The OR was 4.29 (95% CI: [1.39–13.24]) after factors such as gestational age and birth weight were deconfounded.[27]

The evidence for an association between RBC transfusions and NEC became less convincing following a comprehensive meta-analysis of 17 observational studies by Garg et al.,[44] who did not find supportive results (OR = 0.96, 95% CI [0.53–1.71], $p = 0.88$) (Table 1). Rai et al.[45] performed a meta-analysis with 10 studies, and actually noted a 45% reduction in the unadjusted odds of NEC in infants exposed to a recent RBC transfusion (OR = 0.55, 95% CI: [0.31–0.98]).

A prospective, matched-pair comparison of 42 NEC cases and their controls at three centers found no association between transfusions with NEC in the subsequent 48 h or 7 days.[46] Elabiad et al.[22] reviewed a large cohort of 3060 infants and identified 174 infants (5.7%) with NEC. They noted that 116 (67%) infants had been exposed to RBC transfusions; infants with BW ≤750; 751–1,000; 1,001–1,250; and 1,251–1,500 g had a relative risk of 0.14, 0.46, 1.83 and 1.78, respectively, to develop NEC following transfusions. They concluded that RBC transfusions significantly reduced the risk of NEC in ≤1,000 g infants, but noted a trend towards increased risk of NEC in infants with a birth weight of 1,001–1,500 g.

Anemia as Risk Factor for NEC in Very Preterm Infants

In a prospective, multicenter observational cohort study, Patel et al.[12] evaluated 598 very low birthweight infants and noted that 44 (7.4%) developed NEC. In this cohort, however, 319 (53%) infants were exposed to RBC transfusions. The unadjusted cumulative incidence of NEC at 8 weeks after birth was 9.9% (95% CI [6.9–14.2%]), which was higher than the 4.6% (95% CI [2.6–8.0%], $p = 0.02$) incidence among those who were not transfused with RBCs ($p = 0.02$). In multivariable analysis, RBC transfusion in a given week was not significantly related to the rate of NEC (adjusted cause-specific hazard ratio 0.44 (95% CI [0.17–1.12]), $p = 0.09$). Based on the evaluation of 4,565 longitudinal measurements of hemoglobin concentrations (median = 7 g/dL per infant), they associated NEC with severe anemia (adjusted cause-specific hazard ratio 5.99 (95% CI [2.00–18.0]); $p = 0.001$), but not with RBC transfusions.[12] A recent retrospective single-center cohort study in 207 extremely premature infants (23–27 wk gestation) identified a portion of 46% (13/28) of infants with ta-NEC and 54% (15/28) with non–ta-NEC. The incidence of ta-NEC, however, did not correlate with the number of antecedent pRBC transfusions or the pretransfusion median hemoglobin levels.[40]

These reports of the association of NEC with anemia were interesting. However, the impact of anemia on the risk of NEC remains uncertain. Two recent studies (ETTNO, TOP) compared liberal (higher) and restrictive (lower) RBC transfusion thresholds in extremely low birthweight infants, but did not find an association between NEC and low pretransfusion hematocrit/hemoglobin values.[47] In the ETTNO trial, including 1013 infants, the absolute difference in the incidence of NEC (modified Bell stage ≥ IIa) in the liberal (hematocrit on day >21: <34% or <28% in critical or non-critical state) vs restrictive (hematocrit on day >21: <27% or <21% in critical or noncritical state) transfusion threshold was −0.9 (95% CI [−3.8–2.0]).[48] The TOP trial included 1,824 infants; the adjusted relative risk for NEC (Bell's stage ≥2) at high (weeks ≥3: 11.0 g/dl with or 10.0 g/dL without ventilator support) vs low (weeks ≥3: 8.5 g/dL with or 7.0 g/dL without ventilator support) hemoglobin thresholds was 0.95 (95% CI [0.73–1.25]).[47]

Some studies have evaluated the impact of recombinant human erythropoietin (rEpo), or its derivative darbepoietin, given to reduce anemia and the need for transfusions, on NEC. Ohlsson and Aher[49] performed a meta-analysis to evaluate 3,643 preterm or low birth weight infants who had received early (at ≤8 days of age) received rEpo or darbepoetin vs others treated with placebo. As anticipated, early rEpo treatment reduced the numbers of RBC transfusions and donor exposures. A subanalysis of 15 studies reporting 2,639 infants showed that rEpo or darbepoetin administration reduced the rate of NEC (RR = 0.69, 95% CI [0.52–0.91]; $p = 0.01$; number needed to treat to benefit = 33). In another prospective (not double-blinded) randomized clinical trial of 1,285 infants, rEpo reduced the incidence of NEC (3% vs 5.4%, $p = 0.027$).[50] The recent Preterm Erythropoietin Neuroprotection Trial (ett), a phase 3 RCT designed to assess the safety/efficacy of early high-dose rEpo for neuroprotection in 941 extremely preterm infants born at 24 weeks and 0 days to 27 weeks and 6 days of gestation, showed only a trend towards a decreased frequency of NEC in the rEpo group (RR = 0.87; 95% CI [0.60–1.27]; not significant).[51]

We were not able to find consistent clinical evidence to determine whether anemia and/or RBC transfusions could be clearly associated with NEC. In search of insights, we therefore also revisited other analogous conditions marked by severe anemia with a need for transfusion(s), and the risk of intestinal injury. Twin-to-twin transfusion syndrome or hemolytic disease of the newborn are known to trigger intestinal injury, particularly after RBC transfusion.[52–54]

The importance of severe anemia in intestinal injury also finds credence in the typically delayed occurrence of ta-NEC beyond four weeks of postnatal age, when anemia of prematurity is often concordant with nutritional deficiencies and inflammatory conditions such as bronchopulmonary dysplasia.[13] NEC-like bowel injury has also been seen in other populations of critically ill infants such as those receiving cardiopulmonary bypass or extracorporeal membrane oxygenation, particularly when they receive top-up transfusions to treat severe anemia.[55] These findings are consistent with at least two clinical studies that have noted the importance of anemia in risk-stratification for ta-NEC.[12,21] The association between packed RBC transfusion and splanchnic perfusion after feeds has been studied to understand ta-NEC better, but in multivariate analysis no overall association was found between splanchnic fractional tissue oxygen extraction (FTOE) and fasting perfusion in a multivariate repeated-measures model that accounted for transfusion epochs (primary analysis approach). However, exploratory analyses of postprandial changes in FTOEs undertaken for each transfusion epoch separately showed increasing postprandial FTOEs with repeated transfusions.[56,57]

More generally, close examination of the literature shows important differences when the observational studies were compared with the meta-analyses of RCTs. The interest in ta-NEC, as judged by the number of publications/year, began to wane, and this was possibly related to the lack of new insights from associative clinical studies. However, a recent preclinical study in newborn mice has shed some novel mechanistic insights and showed that severe anemia, followed by RBC transfusions may cause intestinal injury. This has rekindled the ta-NEC controversy. In the following sections, we recapitulate the experimental findings from this study.

A Murine Model of Transfusion-associated NEC

Maheshwari's team developed a mouse model to evaluate the association between transfusions and NEC. They phlebotomized mouse pups on postnatal days 2, 4, 6, 8, and 10 to induce severe anemia (hematocrit 20–24%), and transfused these anemic pups and controls on postnatal day 11 with 20 mL/kg leukoreduced, packed (70%) RBCs that had been stored at 4°C for 7 days, recapitulating

current transfusion practices in neonates.[58] Control pups remained unharmed. However, the anemic-transfused pups developed intestinal injury in the ileocecal and mid-colonic segments between 18 and 28 h after transfusion. The histopathological features in the affected intestine were characteristic of NEC.[59,60] Consistently, animals with intestinal injury showed increased plasma levels of the gut epithelial injury marker fatty acid-binding protein 2,[61] loss of intestinal barrier function,[62] and developed a severe systemic inflammatory response syndrome.

Both anemic and the anemia-transfused mice showed monocyte/macrophage infiltration in the affected intestine.[63] RBC transfusions contained free hemoglobin and heme,[64] which activated the newly recruited monocytes and macrophages in the intestine by activating Toll-like receptor 4-mediated signaling, redox cycling,[65] and downstream NF-κB pathways.[66] The role of Toll-like receptor 4 in NEC pathogenesis has been noted in other NEC models,[67] and these seminal findings in ta-NEC highlighted the importance of these pathways as a unifying mechanism in NEC. The requirement of macrophages in ta-NEC was notable because the macrophage depletion prior to transfusions was protective. Blocking the NF-κB pathway in macrophages by administering specific inhibitory nanoparticles was also protective against ta-NEC.

The severity of anemia was important in murine ta-NEC. Mouse pups defined to have severe anemia (hematocrit 20–24%) developed more severe bowel injury as compared with those with moderate anemia (hematocrit 25–30%). The duration of anemia was also important; mice transfused on P10 (soon after the last phlebotomy) sustained less bowel damage than those transfused 24 hr later, on P11. If the transfused RBCs were leukoreduced, washed, and resuspended in saline prior to storage, the severity of ta-NEC was decreased. Pups that received multiple transfusions showed more severe injury. However, the duration of RBC storage (7 days vs 14 days) prior to transfusions did not change the severity of ta-NEC.

Discussion

A number of clinical studies suggests an association between RBC transfusions and NEC, although these findings have not been consistently proven in meta-analyses.[4–26] In some premature infants, severe anemia may even be the predominant, possibly even sufficient, factor in the causation of NEC.[12] However, the exact contribution of anemia and RBC transfusions to the development of bowel injury is not certain. In animal models, RBC transfusions can alter splanchnic autoregulation and cause at least transient intestinal ischemia.[24,68] The murine model that we described above suggests that neither anemia nor RBC transfusions may be independently sufficient to cause NEC, but the risk may increase with a sequential exposure to these two factors. A dual-causation or possible multihit model may possibly explain the conflicting conclusions from many of the clinical studies and two of the meta-analyses.[6,44] However, even though the experimental and laboratory findings in these animal studies are informative, there is a need for a cautious, refined approach to understand ta-NEC, thus using these findings only as a basis for designing confirmatory human studies.

There has been some difficulty in identifying the thresholds of low hemoglobin/hematocrit, at which the risk of NEC related to severe anemia may be higher than that related to corrective RBC transfusions.[69–71] Four peer-reviewed national transfusion guidelines have been developed in Australia, Canada, and Europe (Fig. 1). These recommendations have found support from the broader scientific communities in neonatology, hematology, and transfusion medicine in their respective countries. In the United States, several single-center

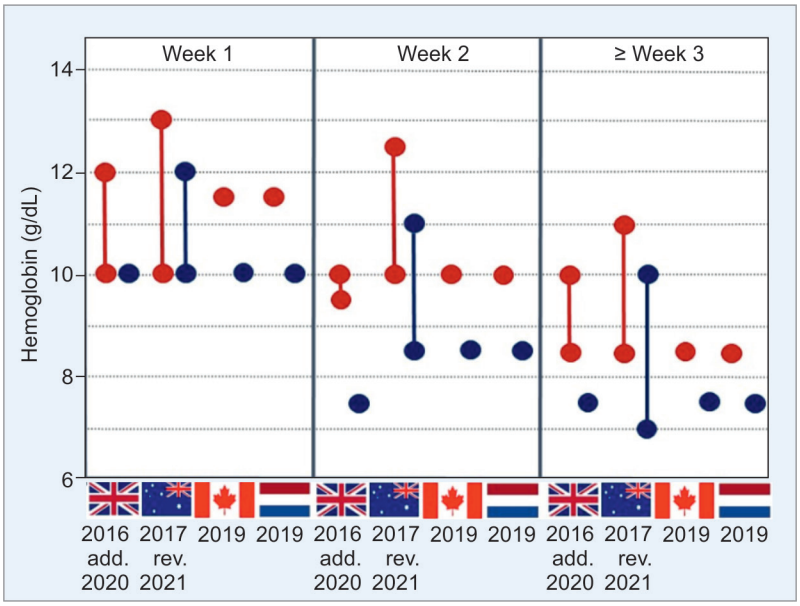

Fig. 1: This graph summarizes current national guidelines (the United Kingdom, Australia, Canada, and the Netherlands as indicated by their flags) for RBC transfusions in VLBW infants with respect to age and the need of respiratory support (red dots). The wide variation in these recommendations highlights the need for further research to identify more definitive thresholds for transfusion. Blue dots indicate thresholds for infants without respiratory support. Recommended ranges or point thresholds of hemoglobin at which transfusions may be considered are shown. The year of publication is provided to visualize the trend towards more restrictive transfusion thresholds. Please note that we have not yet considered the impact of specific conditions such the type of blood sampling for measurement of hemoglobin values (vascular or capillary blood draws), precision of laboratory measurement, and the implications of physiological changes such as with altitude or the intravascular volume status

guidelines are in use that show some heterogeneity;[13,48,72,73] this is highlighted in a *post hoc* analysis of data from the 19 participating sites in the preterm erythropoietin neuroprotection trial (PENUT).[74] As discussed above, the adoption of clinical practices to minimize iatrogenic blood loss and possibly, (early) administration of rEpo/darbepoietin to prevent anemia, or other strategies to reduce the need for transfusions, is logical. However, further evidence is needed before firm recommendations can be made.[75,76]

Eventually, a range, not clear tipping points, of hematocrit/hemoglobin levels may have to be considered based on individual physiological and environmental factors. The impact of the duration of anemia on subsequent risk of NEC also needs investigation. A combination of a transfusion threshold plus another marker of end organ oxygen delivery or perfusion may help to define better when to or when not to transfuse. Given the multifaceted nature of the problem, we see a need to combine data from multiple centers across the world to inform well-designed clinical trials.

While we ponder on these questions, we need to ask ourselves whether we can do anything immediately to minimize the serious morbidities and mortality associated with transfusion and possibly with ta-NEC. The answer to this, from our perspective, is in the affirmative. Even though we acknowledge the disparity between the observational evidence and the randomized trials and would consider the latter to provide more reliable evidence, data from the rodent model of ta-NEC and the available observational studies have led us to come to the following considerations for the "best clinical practice" at the present time.

Expert Opinion

Current evidence favors minimizing the exposure of premature infants who may be at high risk of NEC, to prolonged periods of severe anemia. Four sets of national guidelines for RBC transfusions are currently available (Fig. 1),[77–80] which show considerable differences. To understand the urgency of correcting anemia, we need further research to identify safe thresholds of hematocrit/hemoglobin.[48] We should also continue to evaluate the potential benefits of rEpo and darbepoietin, at least in specific subsets (such as extremely low birth weight infants), if not in all at-risk infants. The exact thresholds of hematocrit/hemoglobin levels when RBC transfusions become necessary are still unclear. The animal models suggest that increased risk of NEC at hematocrit levels ≤24%,[4] but some human studies suggest that organs such as the brain may be even less tolerant to anemia, with functional changes becoming evident at hematocrits ≤28%.[81] RBC transfusion thresholds may have to be viewed as a range, not exact levels (Fig. 1), of hematocrit/hemoglobin based on the corrected gestational age, altitudes of residence, comorbidities, and the functional status of the microcirculation in various organ systems. Several interventions in a patient's blood management are now possible to avoid severe anemia and to reduce the need for transfusions.

Thus, we conclude that only a minority of severely anemic premature infants develop ta-NEC.[4] There is a need to identify the predisposing (genetic) and clinical factors that may increase the risk of ta-NEC. Considering the typically delayed onset of anemia of prematurity after birth, studies may need to focus on the identification of periods of increased vulnerability. Splanchnic vascular autoregulation deserves further evaluation. The availability of portable ultrasound machines may enhance the feasibility of such studies within the neonatal intensive care units.[82] The association of ta-NEC with stimuli that increase intestinal oxygen consumption, such as feeding, need evaluation in high-risk infants.[83] Murine and human studies have shown no harm in transfusions with RBCs stored for short periods,[84] which is reassuring.[85,86] However, further preclinical studies and models could be used to test potential interventions.[84] We also do not believe irradiation to have a possible protective effect; current evidence suggests a pathophysiological role of macrophage-mediated innate, not lymphocyte-mediated adaptive immune response. The pathophysiological concept of anemia needs additional evaluation in premature infants.[4] Fetal and adult hemoglobin have different oxygen carrying capacities. Therefore, low hematocrit levels in an infant who has previously received transfusions of RBC from adult donors may have different physiological implications than in a nontransfused infant.[87] Thus, a detailed analysis infants recruited in the aforementioned clinical trials, who developed NEC/ta-NEC, would be of particular interest. The advantages and disadvantages of washing RBCs prior to storage or before transfusion needs study. Many studies suggest that washing may reduce the half-life of transfused RBCs.[88] The findings in the murine model suggest that subtle hemolysis and extravasation of RBC contents may increase the risk of ta-NEC. The relationship between enteral feedings and ta-NEC needs more evaluation. Although the feeding during red cell transfusion (FEEDUR) trial, an open, multi-arm, parallel-group, single-center RCT did not show any difference in splanchnic oxygenation,[89] the WHEAT (withholding enteral feeds around packed red cell transfusion) study[90] may still yield important insights. Nutritional interventions, such as the use of pasteurized human donor milk as opposed to formula, enteral substitution of lactoferrin and/or L-arginine, and preventive application of multiple-strain probiotics deserve further investigation as strategies to reduce the rates of ta-NEC.[30,91,92] Further study is needed to identify infants who might have ongoing subtle hemolysis either due to low-grade immune responses related to genetic defects or due to blood group incompatibility, as suggested by elevated reticulocyte counts and/or the presence of spherocytosis on peripheral blood smear.[93] These infants may have anemia, activation of monocytes and macrophages, factors that have been found to be associated with ta-NEC. Effects of simultaneous transfusions with other blood products such as platelets, needs further evaluation as additional or synergistic risk factors for ta-NEC.[94] In addition to larger, more definitive trials, some of the relevant questions in this domain may be amenable to scrutiny through use of big data and machine learning/AI.[95–98]

Article Highlight Box

- Several retrospective studies have associated RBC transfusions with necrotizing enterocolitis (ta-NEC) in very preterm infants.
- Randomized controlled trials on transfusion thresholds and treatment with recombinant human erythropoietin or darbepoetin have not provided significant evidence for this association.
- A murine model of RBC transfusion-associated NEC-like pathology showed that sequential exposure to anemia followed by RBC transfusion is a risk factor for ta-NEC.
- Experimental and clinical data suggest that strategies of personalized blood management (including late cord clamping/milking and reduction of iatrogenic blood loss) in very preterm infants may help prevent ta-NEC.

Orcid

Parvesh M Garg https://orcid.org/0000-0001-5949-7304

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