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# newborn

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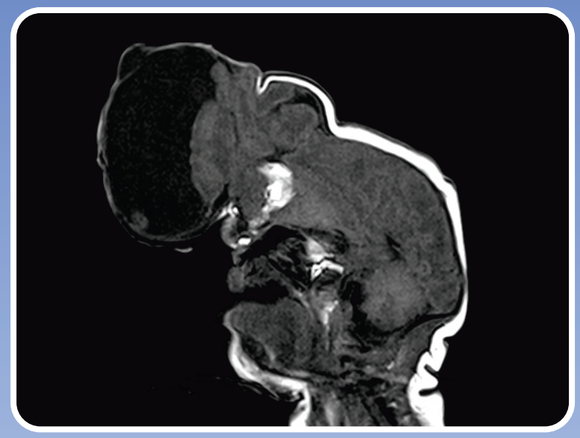
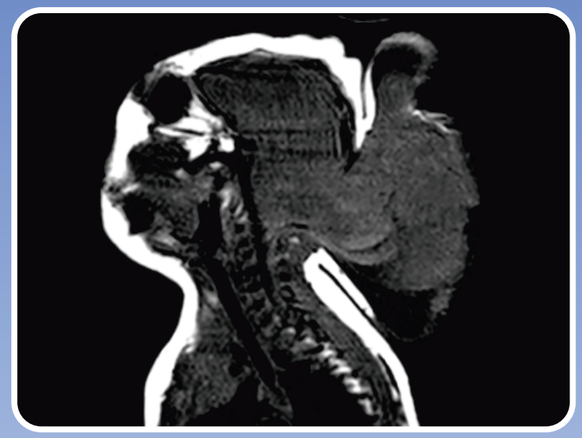


Fronto-ethmoidal Encephalocele



Occipital Encephalocele

Neurological Abnormalities in Infants of Mothers with Diabetes Mellitus

**Other highlights:**

The First 1000 Days: Assembly of the Neonatal Microbiome and Its Impact on Health Outcomes

Approach to Neonatal Alloimmune Thrombocytopenia: The Perspective from a Transfusion Medicine Service

Also available online at
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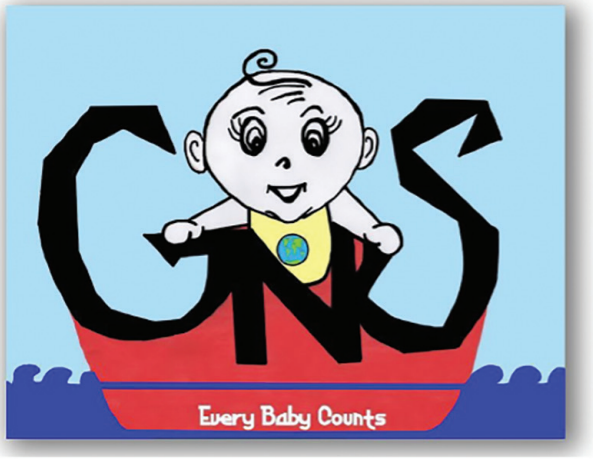


**Global Newborn Society**

**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 yet. 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 small size of a lovable little newborn baby. The issue editors chose three 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*

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# Contents

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## Care of the Newly Born Needs to Begin Prior to Birth and to Continue then After

Recent scientific progress has brought increasing clarity in our understanding of the etiopathogenesis, diagnosis, and the optimal management of many structural abnormalities and diseases that we see in newborn infants.[1] Many of these abnormalities have a prenatal onset and can now even be monitored *in utero* for progression and complications.[2,3] In these efforts, advanced imaging and analysis of fetal DNA in fetal/maternal blood has facilitated diagnosis, grading of severity, and, in many conditions, helped define indications for temporizing or definitive treatment prior to birth.[4-7] Such progress has been possible most notably in genetic, neurological, hepatobiliary, and hematological problems.[6] Initiation of care before such disorders get established and/or the onset of secondary complications can possibly make a difference.[8] In many conditions, there is now encouraging evidence for effectiveness of specific management performed *in utero* on immediate and medium/long-term outcomes.[9]

Early diagnosis is a key determinant of outcomes in most neonatal conditions.[10] The timing of disease onset may be particularly important because of the possibility of interruption in the structural and functional changes that are going on during that period of development.[3] Hence, serial imaging and/or laboratory tests can be used not only for monitoring fetuses/infants with known disease conditions but also for the evaluation of normal or hitherto asymptomatic fetuses/infants who have known familial or genetic risk factors.[3] Knowledge about the severity and extent of various abnormalities/diseases can help prepare for treatment procedures and counseling the families.[11] Most of these procedures are complex and need a multi-disciplinary approach for optimizing the outcomes.[12] The importance of preparing a closely knitted team and establishing a close relationship with the families is important as the possibility of adverse outcomes and even reproductive grief cannot be overlooked.[13] Transparency is a key word in this process. A paternalistic physician attitude is not acceptable; evidence-based, collaborative approaches are more appropriate for families and care-providers handling adverse outcomes.[7]

In the *Newborn*, our aim is to cover problems that a baby might develop *in utero*, the perinatal period, following birth, and the implications of these abnormalities during the first 1000 days after birth. In this 2nd issue of our journal, we present a set of articles that represent each of these subsets. The risk of perinatal mortality is a matter of universal concern. Shukla and Carlo[14] have reviewed the predictive accuracy of machine-learning statistical models for intrapartum stillbirth and neonatal mortality. Another article summarizes the information we have on the association between chromosomal abnormalities and neonatal necrotizing enterocolitis (NEC); the authors noted a possible association with abnormalities in chromosomes 1, 6, 15, 21, and 22.[15] The findings are not conclusive as the cohorts are not numerically-adequate in a statistical sense, but show the need for translational studies. In another study, Sun and Romano-Keeler[16] have reviewed the impact of early life microbial colonization on the development of the immune system, postnatal growth, and long-term health and disease.

Morotti *et al*.[17] examined the records of 289 infants with early-onset sepsis and noted the characteristics of neutrophil cell populations as recorded in automated hematology analyzers. The changes in neutrophil volumes showed moderate accuracy in identifying early-onset sepsis, but the high negative predictive value can be useful in reducing unnecessary administration of antibiotics. These observations are exciting as the information can be obtained as an extension of the complete blood counts that are performed routinely in the evaluation of infants with suspected sepsis. No extra blood samples will be needed.

Grewal *et al*.[18] discuss how neonatal acute liver failure is distinct from acute liver failure in older children and adults. There are important differences in etiology, clinical presentation, and the response to therapeutic interventions. In another article, Sharma and her colleagues[19] have reviewed normal platelet counts in neonates, the epidemiology and pathogenesis and of neonatal alloimmune thrombocytopenia (NAIT), the specific platelet antigens identified as targets in NAIT, and the approach for laboratory diagnosis of NAIT. The article is particularly interesting as it presents a detailed, methodological approach used in hematology laboratories.

Mishra and coworkers[20] review the most frequently-seen structural neurologic anomalies in infants of mothers with diabetes mellitus. The authors have provided information on structural neurologic malformations, cognitive disorders, motor deficits, and psychosocial disorders in these infants. And finally, there is an expert review on compassionate organization of grief care in the neonatal intensive care units.[21] Kathryn Grauerholz highlights the "human" components of treatment, and the supportive measures that the patients and their families need. The experience of parenting a critically-ill infant can be overwhelming and traumatic. These difficulties can be particularly difficult for parents who have previously endured a reproductive loss, as the distress can get compounded with lingering grief from a prior perinatal loss.

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**Akhil Maheshwari MD**
Editor-in-Chief

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Issue Editors

ORIGINAL RESEARCH

# Automated Cell Counter-derived Neutrophil Cell Population Data by VCS Technology as a Marker of Early-onset Neonatal Sepsis

Francesco Morotti[1], Gilberto F Candela[2], Giovanni Martellosio[3], Federico Serana[4], Moira Micheletti[5], Duilio Brugnoni[6], Francesco M Risso[7], Mario Motta[8]

## Abstract

**Aim:** Early-onset neonatal sepsis (EONS) occurring within the first 72 hours after birth is a common, life-threatening disease in neonatal intensive care units (NICUs). The limited accuracy of diagnostic tools makes the diagnosis of EONS difficult, and the quest for new markers remains open. Automated hematology analyzer-derived neutrophil cell population data (N-CPD) have been identified as a potential marker of neonatal sepsis, but their role for EONS has not been elucidated yet. Our aim is to explore the role of automated hematology analyzer-derived N-CPD as a marker of EONS.

**Methods:** We prospectively evaluated a cohort of 289 neonates admitted to the NICU with clinical signs of sepsis, and checked if N-CPD from the Beckman Coulter UniCel DxH 800 device could help identify those who would develop culture-proven EONS. Clinical characteristics, sepsis markers, blood culture results, and N-CPD were recorded. The diagnostic accuracy of N-CPD was tested using receiver-operator curves (ROCs).

**Results:** Receiver-operator curves of the standard deviation of neutrophil volume (SD-V) showed moderate accuracy in identifying EONS (AUC 0.74), with a high negative predictive value (NPV 98.6%) for cut-off values >21.76 arbitrary units. Accuracy was higher with VCS at 12–48 hours of life (AUC 0.8). Standard deviation of neutrophil volume accuracy was independent from gestational age (GA), birth weight, and timing of test execution (OR 1.14, $p = 0.002$; AUC 0.71).

**Conclusion and significance:** Our study confirms the role of N-VCS in the diagnostic workup for EONS. High NPV values may be useful as they suggest a role as an adjunctive marker useful for ruling-out EONS and support early empirical antibiotic withdrawal.

**Keywords:** Automated cell counter, Case-control study, Cell population data, Diagnosis, Early-onset neonatal sepsis, Markers, Neutrophils.

*Newborn* (2022): 10.5005/jp-journals-11002-0030

## Introduction

Early-onset neonatal sepsis is defined as a positive blood or cerebrospinal fluid bacterial culture at less than 72 hours of age and is the most common life-threatening, vertically-transmitted bacterial infection in NICUs.[1] The overall estimated incidence of EONS is 0.98 per 1,000 live births, with 10.9% mortality. Both the incidence and mortality are inversely proportional to the infants' GA and birth weight.[2,3] Given the ambiguity in the clinical picture and the risk of rapid progression, neonatal sepsis needs prompt investigation and treatment.

Currently, the diagnosis of neonatal sepsis requires a positive blood culture. However, its power is undermined by difficulties in obtaining adequate blood volumes, levels of bacteriemia below a certain detectable threshold, and exposure to antenatal antibiotics that may limit bacterial growth. Hence, there is a need for adjunctive diagnostic tests that are not based on blood culture. Some of these tests include the measurement of the total and differential white blood cell (WBC) counts, the absolute neutrophil counts (ANCs), and the ratio of immature-to-total (I/T) neutrophils.[4,5] However, these tests are limited by low sensitivity and empiric antibiotic therapy still remains necessary for neonates with clinically suspected EONS.[6]

Leukocyte quantitative parameters such as the cell population data (CPD) can be measured using automated hematology analyzers.[7] The Beckman Coulter UniCel DxH 800 is an example; it obtains CPD such as cell volume, conductivity, and scatter (VCS). The VCS technology examines the biophysical properties of 8,000 leukocytes in each specimen, refining the output for increased accuracy. A link between acute bacterial infection and morphologic changes of reactive neutrophils, detected by the VCS technology, has been demonstrated in adults.[8] Preliminary studies show similar changes in neonatal sepsis, but most studies have included both EONS and late-onset neonatal sepsis (LONS).[9–14] In this study, we focused on EONS and evaluated neutrophil VCS parameters to assess the diagnostic accuracy of these parameters in these infants.

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**Source of support:** Nil

**Conflict of interest:** None

# Methods

## Study Population and Sepsis Screening

The recruitment was conducted prospectively from January 2018 to June 2019 at the NICU of the Children's Hospital of Brescia, Italy. The study received approval from the Ethics Committee of ASST-Spedali Civili of Brescia, Italy. Eligibility criteria included inborn neonates admitted to NICU, with clinical suspicion of sepsis within the first 72 hours after birth. We included infants in who a blood culture had been obtained at the time of NICU admission, and they had at least one complete blood count (CBC), including the ANC and VCS parameters, manual I/T ratio, and the C-reactive protein levels measured within the first 72 hours after birth; and (c) The availability of parental written informed consent. Neonates with congenital or chromosomal abnormalities, isoimmunization, or maternal preeclampsia were excluded.

Recruited neonates were observed for 72 hours after birth. There were two groups: (a) Those with blood culture-proven EONS; and (b) Controls with negative blood culture and negative sepsis screening defined as C-reactive protein ≤10 mg/L, WBC counts ≥5000/mm$^3$, ANC >1000/mm$^3$, and I/T ratios ≤0.2. Neonates with mixed results such as a positive sepsis screening but negative blood cultures were considered to be potential confounders, and therefore, excluded from the analysis.

## Neutrophil Cell Population Data by VCS Analysis

We used the UniCel DxH 800 hematology analyzer (Beckman Coulter, Miami, Florida, USA) to perform CBCs and CPD by VCS analysis. The VCS technology uses three independent energy sources to evaluate the biophysical properties of leukocytes, namely, direct current impedance to measure cell volume (V); radio frequency opacity that is related to intracellular features such as the cytoplasmic/nuclear ratio, to characterize conductivity (C); and a laser beam to measure multiple angles of light scatter (S) for cytoplasmic granularity and nuclear structure.[15] Four angles of light scatter were available, including the median angle light scatter (MALS), the upper median—(UMALS), lower median—(LMALS), low angle—(LALS), and the axial light loss (ALL). With these parameters, the VCS analysis provides information on cell volume (V), cell conductivity (C), and scatter (S), which are reported as mean (MN) and standard deviation (SD).

## Statistical Analysis

Statistica (StatSoft Inc., Tusla, Oklahoma, USA) and MedCalc (MedCalc Software, Mariakerke, Belgium) software were used. The characteristics of the two groups of neonates were reported using descriptive statistics. Continuous variables were presented as the median and interquartile range (IQR) and compared by the Mann–Whitney *U* test. Categorical variables were presented as absolute numbers and percentages and compared using Fisher's exact test. Receiver operating characteristic (ROC) curves were analyzed to estimate the accuracy of VCS parameters in predicting EONS. The overall test performance was expressed as the area under the ROC curve (AUC) with a 95% confidence interval (CI). Youden index and its associated cut-off value, for which both sensitivity and specificity are maximized, were determined.[16] A prevalence value of 3.8%, corresponding to the local area-based average value of proven EO sepsis, was used to calculate the positive and negative predictive values. Finally, multivariate logistic regression analysis was done to evaluate the association between VCS parameters and the occurrence of EONS, independently from possible confounders. All statistical tests were considered significant for *p*-values <0.05.

# Results

## Demographic Characteristics

During the study period, 544 neonates were admitted to NICU. We found 289 to be eligible for the study, and 262 were excluded. Fifteen were excluded because of consent denial, 63 were out-born, 24 had congenital or chromosomal abnormalities, 13 had isoimmunization, 10 were born to mothers with pregnancy-induced hypertension, 7 because of missing data, and 137 did not receive sepsis workup.

Among the 289 enrolled infants, 31 (10.7%) with positive blood cultures were included in the sepsis group, and 198 (68.5%) with negative sepsis screening and negative blood culture and were labelled as controls. To minimize confounders, 58 neonates (20.4%) with positive sepsis screening but negative blood culture were excluded from the data analysis. One more neonate (0.4%) was excluded from data analysis because of overt blood culture contamination.

## Clinical Characteristics

Clinical and laboratory characteristics of neonates in the sepsis or control group are summarized in Table 1. Cesarean section (CS) was the mode of delivery in 75% of the control group and 55% of the sepsis group ($p = 0.02$). Neonates in the sepsis group had significantly lower GA and birth weight compared to controls. The timing of sepsis screening was similar between the two groups. Among laboratory tests, values of red blood cells, I/T ratio, and C-reactive protein were significantly different, whereas WBC, ANC, and platelets values were not.

Blood culture results are shown in Table 2. Gram-negative bacteria were detected in 25 cases (80.6%) of positive blood cultures. The gram-positive bacteria included group B Streptococci (five cases, 16%), coagulase-negative *Streptococcus* (five cases, 16%), and *Enterococcus faecalis* (five cases, 16%). Gram-negative bacteria were present in six cases (19.4%), with *E. coli* being the most frequent (four cases, 12.9%). No fungi were detected.

## Neutrophil—CPD Data

Patients received a total of 315 CBC with VCS parameters analysis. The cumulative distribution of CBC tests over time is shown in Figure 1. We compared the neutrophil VCS parameters in the sepsis and control groups. Among the mean channel values of VCS, all the parameters were significantly different, except for the MN-LALS. In addition, all parameters of VCS standard deviations resulted significantly different between the two groups, except for conductivity. VCS parameters with statistically significant differences ($p$ <0.01) between the two groups are summarized in Table 3.

## Receiver Operating Characteristic (ROC) Curves

Results of the ROC curve analysis of VCS parameters are summarized in Table 4. According to the Swets classification,[17] the accuracy of SD-V results was moderate in predicting EO sepsis (AUC of 0.74) with a sensitivity of 76.9% and an NPV of 98.6% for a cut-off value of >21.76 au (arbitrary unit). All the other VCS parameters were less accurate (AUC ≤0.7). Similarly, the accuracy of WBC and ANC values was low, while the accuracy of the I/T ratio resulted in moderate (AUC of 0.71).

Standard deviation of neutrophil volume ROC curve was analyzed at different timing of sepsis screening, as reported in Table 5. The cut-off value of SD-V, calculated at different cumulative times of sepsis screening, remained the same over the period of 72 hours after birth. Furthermore, the AUC values

**Table 1:** Newborn characteristics

| | *Sepsis (n = 31)* | *Controls (n = 198)* | *p value* |
|---|---|---|---|
| Male/female | 19/12 | 116/82 | 0.776 |
| Birth cesarean/vaginal | 17/14 | 148/50 | 0.021 |
| Gestational age, weeks | 30 (26–34) | 33 (31–35) | 0.004 |
| Birth weight, gm | 1,465 (860–2,000) | 1,855 (1,461–2,423) | 0.008 |
| Sepsis work-up time after birth, hours | 22 (11–34) | 19 (2–31) | 0.222 |
| WBC, *n*/μL | 11,058 (8,452–16,686) | 11,017 (7,985–15,032) | 0.825 |
| RBC, $N \times 10^3$/μL | 4,066 (3,463–4,421) | 4,405 (3,931–4,869) | 0.004 |
| PLT, $n \times 10^3$/μL | 205 (163–269) | 219 (177–263) | 0.666 |
| ANC, *n*/μL | 6,152 (3,769–10,662) | 5,825 (3,535–9,190) | 0.453 |
| I/T ratio | 0.08 (0.06–0.18) | 0.04 (0.03–0.07) | <0.001 |
| CRP mg/L | 3.7 (0.0–10.4) | 0.0 (/) | <0.001 |

Values are reported as median, (interquartile range). ANC, absolute neutrophil count; CRP, C-reactive protein; I/T, immature to mature neutrophils ratio; RBC, red blood cells; WBC, white blood cells

**Table 2:** Blood culture results; number and (percentage)

| | *N (%)* |
|---|---|
| Gram-positive organisms | 25 (80.6) |
| *Group B streptococci* | 5 (16.1) |
| *Coagulase-negative staphylococci* | 5 (16.1) |
| *Enterococcus faecalis* | 5 (16.1) |
| *Gram-positive cocci (other)* | 4 (12.9) |
| *Staphylococcus lugdunensis* | 2 (6.5) |
| *Corynebacterium* sp. | 2 (6.5) |
| *Turicella otitidis* | 1 (3.2) |
| *Lactobacillus jensenii* | 1 (3.2) |
| Gram-negative organisms | 6 (19.4) |
| *Escherichia coli* | 4 (12.9) |
| *Haemophilus influenzae* | 2 (6.5) |

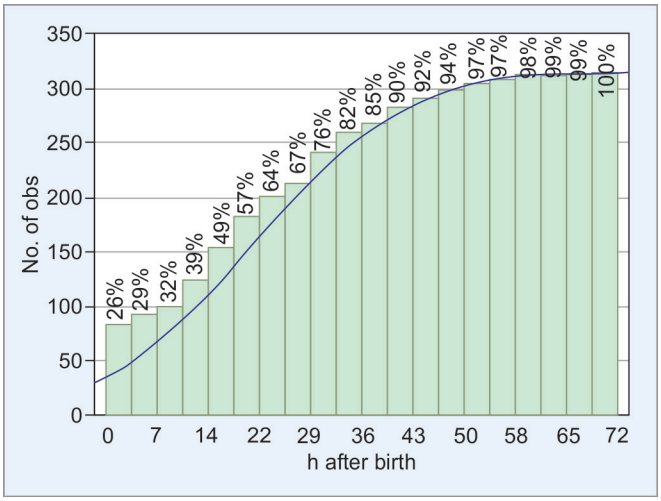



**Fig. 1:** Cumulative distribution of CPD determination over time

remained greater than 0.70, with the best performance for tests taken between 12 and 48 hours, with an AUC of 0.8 (Fig. 2). Although the positive predictive value was 24.2% at best, the negative predictive value was stably higher than 95%. By logistic regression, SD-V was significantly associated with EO sepsis independently from GA, birth weight, and timing of test execution [OR 1.14 (95% CI 1.05–1.24), $p = 0.002$; AUC 0.71].

## Discussion

In EONS, the relatively-modest diagnostic accuracy of CRP and the long turn-around time of blood cultures makes prompt diagnosis a difficult task.[18] To overcome such limitations, several EONS management strategies have been evaluated, including clinical algorithms[19,20] and logistic regression models (Kaiser Permanente, California, USA). Despite some limitations, these efforts have helped reduce unnecessary examinations and empirical antibiotic therapy in at-risk late preterm or term newborn infants. Unfortunately, these methods have been designed primarily for near-term infants who are usually healthier and more mature than the premature infants admitted to the NICUs.

We are still looking for novel, accurate diagnostic methods to screen less mature preterm infants who are at risk of EONS. Several markers have been investigated so far, such as procalcitonin (PC), interleukin 6 (IL-6), presepsin (PS), or CD64, but a reliable, cost-effective test has not been found.[18] Along with CRP, CBC and peripheral blood smears are routinely used as tests for neonatal sepsis workup, with I/T ratio being a useful additional sepsis marker (sensitivity 90%, NPV 98–99%, PPV 25% for I/T >0.2).[18] Nevertheless, the manual examination is usually conducted with a time-consuming, direct observation of only 100–200 cells, is operator-dependent, and is subject to sampling variations. The optimal diagnostic assay is still far from being identified.

Modern hematology analyzers use multiple physical parameters to evaluate and classify the cells of a specimen. The Coulter UniCel DXH800 employs the VCS technology to examine CPD. When applied to leukocytes, the VCS parameters can be considered as a fully-automatized, highly objective, and reproducible equivalent of morphological parameters. The initial data are exciting for sepsis evaluation.[8] To our knowledge, six studies assessing N-CPD performance in neonatal sepsis are available so far.[9–14] Raimondi et al.[9] found MN-V and neutrophils SD-V to be useful in excluding bloodstream infection in LONS, with AUC, sensitivity, specificity and NPV of 0.92, 95.0, 88.0, 98.9% and 0.75, 80.0, 52.0, 92.8%, respectively. The other authors described N-CPD performance in mixed early and late-onset sepsis cohorts. With some differences, all found MN-V interesting, with a mean cut-off >155.9 au (range 151–157.1 au), and

**Table 3:** Neutrophil VCS parameters

| *VCS parameters (AU)* | *Total (315)* | *Sepsis (n = 31)* | *Controls (n = 198)* | *p value* |
|---|---|---|---|---|
| MN-V | 141 (136–148) | 146 (137–158) | 141 (136–147) | <0.001 |
| SD-V | 21.9 (19.6–23.2) | 23.86 (21.8–26.4) | 20.92 (19.5–22.6) | <0.001 |
| MN-C | 139 (136–142) | 137 (133–142) | 139 (137–142) | 0.006 |
| MN-MALS | 130 (127–135) | 127 (122–131) | 131 (128–135) | <0.001 |
| MN-UMALS | 136.9 (133–142) | 133 (129–140) | 138 (134–142) | 0.001 |
| SD-UMALS | 15.4 (12.9–15.9) | 15.58 (13.7–20) | 14.01 (12.9–15.6) | 0.002 |
| MN-LMALS | 119 (115–125) | 116 (112–120) | 120 (116–125) | <0.001 |
| SD-SAL2/ALL | 16.5 (14.7–17.3) | 17.00 (15.9–21.5) | 15.72 (14.5–17) | <0.001 |

Measurement units were arbitrary units (AU). Values are reported as median, (interquartile range). ALL, axial light loss; C, cell conductivity; LMALS, lower median angle light scatter; MALS, median angle light scatter; MN, mean channel; SD, standard deviation; UMALS, upper median angle light scatter; V, cell volume

**Table 4:** ROC curve analysis for selected VCS parameters and white blood count (WBC), absolute neutrophils count (ANC), immature to mature neutrophil ratio (I/T)

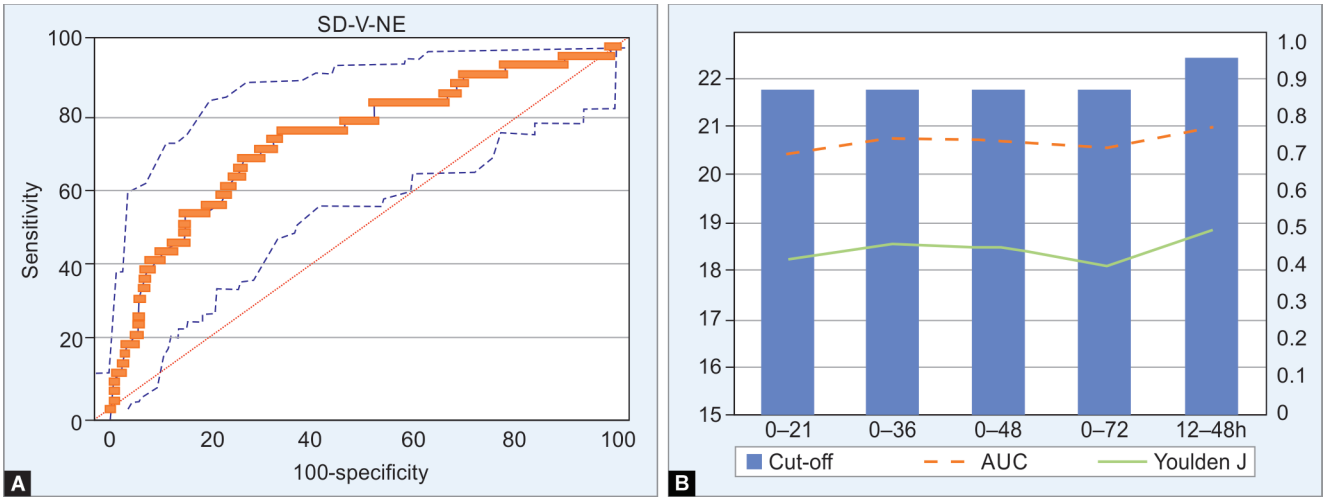
| *VCS parameters* | *Cut-off (AU)* | *AUC* | *Sensitivity (95% CI)* | *Specificity (95% CI)* | *PLR (95% CI)* | *NLR (95% CI)* | *PPV (95% CI)* | *NPV (95% CI)* |
|---|---|---|---|---|---|---|---|---|
| MN-V | >149 | 0.67 | 48.7 (32.4–65.2) | 85.9 (81.2–89.8) | 3.5 (2.2–5.3) | 0.6 (0.4–0.8) | 12 (4.4–24.5) | 97.7 (95.1–99.1) |
| SD-V | >21.76 | 0.74 | 76.9 (60.7–88.9) | 65.9 (60.0–71.5) | 2.3 (1.8–2.9) | 0.4 (0.2–0.6) | 24.2 (17.0–32.7) | 95.3 (91.2–97.8) |
| MN-C | ≤134 | 0.63 | 38.5 (23.4–55.4) | 88.4 (84.0–91.9) | 3.3 (2.0–5.5) | 0.7 (0.5–0.9) | 11.6 (3.6–25.7) | 97.3 (94.7–98.9) |
| MN-MALS | ≤126 | 0.68 | 48.72 (32.4–65.2) | 81.88 (76.8–86.2) | 2.69 (1.8–4.0) | 0.63 (0.5–0.9) | 9.6 (3.5–19.9) | 91.9 (87.7–95.0) |
| MN-UMALS | ≤133 | 0.66 | 51.28 (34.8–67.6) | 78.26 (72.9–83.0) | 2.36 (1.6–3.4) | 0.62 (0.4–0.9) | 8.5 (3.2–17.5) | 97.6 (94.8–99.1) |
| SD-UMALS | >14.44 | 0.65 | 74.36 (57.9–87.0) | 51.09 (45.0–57.1) | 1.52 (1.2–1.9) | 0.50 (0.3–0.9) | 5.7 (2.6–10.5) | 98.1 (94.5–99.6) |
| MN-LMALS | ≤118 | 0.67 | 69.23 (52.4–83.0) | 60.87 (54.8– 66.7) | 1.77 (1.4–2.3) | 0.51 (0.3–0.8) | 6.5 (2.9–12.3) | 98 (88.6–96.5) |
| SD-SAL2 | >16.46 | 0.69 | 66.67 (49.8–80.9) | 64.86 (58.9–70.5) | 1.90 (1.4–2.5) | 0.51 (0.3–0.8) | 7 (3.1–13.3) | 98 (94.9–99.5) |
| WBC (cell/µL) | >21,220 | 0.5 | 18.4 (7.7–34.3) | 94.2 (90.8–96.7) | 3.18 (1.4–7.2) | 0.87 (0.7–1) | 11.2 (1.6–33.4) | 96.7 (94–98.4) |
| ANC (cell/µL) | >15,586 | 0.53 | 18.4 (7.7–34.3) | 95.6 (92.5–97.7) | 4.24 (1.8–10) | 0.85 (0.7–1) | 14.3 (2.1–41.3) | 96.7 (94–98.4) |
| I/T (ratio)* | >0.05 | 0.71 | 75.8 (56.5–89.7) | 62.6 (54.8–69.2) | 2 (1.5–2.5) | 0.39 (0.2–0.7) | 7.3 (2.8–15.1) | 98.5 (94.6–99.8) |

*I/T values were provided only for 29 cases, 185 controls. NLR, neutrophil to lymphocyte ratio; NPV, negative predictive ratio; PLR, platelet to neutrophil ratio; PPV, positive predictive ratio

**Table 5:** SD-V ROC curve analysis for different timings of sampling

| *Timing* | *Number Case/ctrl* | *Cut-off (AU)* | *AUC* | *J* | *Sensitivity (95% CI)* | *Specificity (95% CI)* | *PLR (95% CI)* | *NLR (95% CI)* | *PPV (95% CI)* | *NPV (95% CI)* |
|---|---|---|---|---|---|---|---|---|---|---|
| 0–24 hours | 20/176 | >21.76 | 0.72 | 0.44 | 75 (50–91.3) | 69.32 (61.9–76.0) | 2.44 (1.7–3.4) | 0.36 (0.2–0.8) | 8.8 (3.2–18.6) | 98.6 (94.8–99.8) |
| 0–36 hours | 32/230 | >21.76 | 0.77 | 0.48 | 80.6 (62.5–92.5) | 67.4 (60.9–73.5) | 2.47 (1.9–3.2) | 0.29 (0.1–0.6) | 8.9 (3.9–16.8) | 98.9 (95.9–99.9) |
| 0–48 hours | 35/259 | >21.76 | 0.76 | 0.47 | 80 (63.1–91.6) | 67.18 (61.1–72.9) | 2.44 (1.9–3.1) | 0.3 (0.2–0.6) | 8.8 (4.1–16.1) | 98.8 (96.1–99.8) |
| 0–72 hours | 39/276 | >21.76 | 0.74 | 0.42 | 76.9 (60.7–88.9) | 65.9 (60.0–71.5) | 2.3 (1.8–2.9) | 0.4 (0.2–0.6) | 24.2 (17.0–32.7) | 95.3 (91.2–97.8) |
| 12–48 hours | 25/164 | >22.4§ | 0.8 | 0.52 | 80 (59.3–93.2) | 72.53 (65.1–79.2) | 2.92 (2.1–4) | 0.28 (0.1–0.6) | 9.3 (3.2–20.2) | 99 (95.6–100) |

Measure unit: arbitrary unit (AU). §Value of 21.76 not included in sample. NLR, neutrophil to lymphocyte ratio; NPV, negative predictive ratio; PLR, platelet to neutrophil ratio; PPV, positive predictive ratio




**Figs 2A and B:** (A) ROC curve, SD-V; (B) SD-V performance at different sample timings

mean AUC of 0.84 (0.8–0.99), sensitivity, specificity, and NPV of 75.9% (35–97%), 85.2% (71.9–96%), 80.5% (65–98%), respectively. Standard deviation of neutrophil volume was also indicated by four studies, with a mean cut-off >29.7 AU (range 21.5–37.4), and mean AUC of 0.76 (0.68–0.9), sensitivity, specificity, and NPV of 70.4% (60–88%), 72.8% (64–78%), 74.7% (48–92%), respectively.[11–14] However, the authors considered both EONS and LONS together: Their results may not be fully applicable to EONS, as during the early neonatal period the neutrophil kinetics is known to show high variability and physiologic changes.[5] Moreover, most authors included infants with both culture-proven and variously defined suspected/clinical sepsis in the "sepsis group". This approach may have diluted the diagnostic accuracy of various kinetic parameters of neutrophils.[9,12,14]

To our knowledge, this is the first reported study specifically evaluating neutrophil CPD for predicting EONS. Our sample includes 31 culture-proven EONS cases, matched with 198 controls. Blood samples were collected as per routine internal protocol or clinical indication. Cases and controls significantly differed for GA, birth weight, and mode of delivery. Nevertheless, none of these data, as well as the timing of blood sampling, had an influence on the N-CPD predictive value, as shown by logistic regression. Among sepsis screening tests, I/T ratio, CRP, and several N-CPD showed a significant difference between the sepsis group and controls. Our NICU can rely on long-time experienced physicians for I/T ratio execution. The close similarity of I/T and SD-V performance at the ROC curve suggests a biological link between the two measurements.

In most previous studies, MN-V and SD-V were identified as best performing parameters. We focused on the best-performance VCS parameter SD-V and MN-V. Standard deviation of neutrophil volume >21.76 au showed an AUC of 0.74, 76.9% sensibility, and 65.9% specificity, with an NPV of 95.6%. The cut-off value, AUC, and Youden index's J remained constant when analyzed for different time slots (Fig. 2). Interestingly, the best performance was obtained with VCS analysis performed at 12–48 hours, with AUC of 0.8 and NPV of 99%, suggesting that the timespan is optimal for N-CPD evaluation.

Compared to existing data, our focused analysis of N-CPD in infants with EONS has shown interesting results. Standard deviation of neutrophil volume was more accurate than previously reported, showing more variability of neutrophils in EONS due to enhanced margination of neutrophils at diverse stages of immaturity. In contrast, the lower AUC values of MN-V for EONS could be explained by the higher number of large, immature neutrophils physiologically present in the circulation in the first hours after birth. These results highlight the biological difference between early and late neonatal sepsis response, which should be taken into account when studying potential neutrophil morphology derived parameters. Our study does have some limitations, particularly in its small population size. Nevertheless, these preliminary data that are specifically oriented to EONS suggest that the results could be reliable for clinical use. Standard deviation of neutrophil volume showed moderate accuracy along with a high negative predictive value, as well as stability over the first 48 hours. Compared to other laboratory tests, N-CPD can be generated automatically with routine CBCs and does not require additional blood specimens, are available within a few minutes, and once established, there are no additional costs. This could very well develop into an important adjunctive marker in the work-up for EONS.

## Conclusion

Neutrophil cell population data can be a cost-effective and time-sparing laboratory evaluation that could provide important adjunctive markers for routine EONS workup. The high negative predictive value makes SD-V a useful parameter for ruling-out EONS. The low PPV and specificity can limit its use as an individual test, but it could be very useful in combination with other sepsis workup examinations to improve EONS diagnosis and refine antimicrobial stewardship programs.

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REVIEW ARTICLE

# Risk Prediction for Stillbirth and Neonatal Mortality in Low-resource Settings

Vivek V Shukla[1], Waldemar A Carlo[2]

## Abstract

High stillbirth and neonatal mortality are major public health problems, particularly in low-resource settings in low- and middle-income countries (LMIC). Despite sustained efforts by national and international organizations over the last several decades, quality intrapartum and neonatal care is not universally available, especially in these low-resource settings. A few studies identify risk factors for adverse perinatal outcomes in low-resource settings in LMICs. This review highlights the evidence of risk prediction for stillbirth and neonatal death. Evidence using advanced machine-learning statistical models built on data from low-resource settings in LMICs suggests that the predictive accuracy for intrapartum stillbirth and neonatal mortality using prenatal and pre-delivery data is low. Models with delivery and post-delivery data have good predictive accuracy of the risk for neonatal mortality. Birth weight is the most important predictor of neonatal mortality. Further validation and testing of the models in other low-resource settings and subsequent development and testing of possible interventions could advance the field.

**Keywords:** Low- and middle-income countries, Mortality fetal, Mortality neonatal, Neonatal, Newborn infant, Preterm infants, Perinatal mortality, Resuscitation, Stillbirth.

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## Introduction

Advances in perinatal and neonatal care, including the implementation of programs directed at improving perinatal care, have decreased stillbirth and neonatal mortality globally.[1] However, global stillbirth and neonatal mortality rates remain high with an increasing proportion of the under-5 child mortality.[2,3] Stillbirth and neonatal mortality are concentrated in low-resource settings of LMICs.[4,5] Approximately 98% of all stillbirths and neonatal deaths occur in these low-resource settings,[6] despite the sustained focus of many local or global public health organizations over the last few decades.[7]

A large number of deliveries occur at home in the absence of trained birth attendants that can provide an appropriate level of care.[7,8] Inadequate access to appropriate health care at delivery[9–11] is one of the leading factors responsible for preventable stillbirth and neonatal mortality.[12] Lack of optimal resources combined with varying social issues, cultural practices, and health literacy lead to wide healthcare disparities. The barriers to healthcare at different stages[13] and available resources vary between sites[14–16] making universal access to quality perinatal health care at delivery a difficult aim to achieve.

Timely identification of at-risk pregnancies and neonates is critical for reducing stillbirth and neonatal mortality in low-resource settings. It has been estimated that 0.8 million stillbirths and 1.9 million neonatal deaths per year could be prevented by optimizing the coverage and quality of prenatal, intrapartum, and neonatal care.[10] Unfortunately, studies focused on the quantification of stillbirth risk have been hampered by small event rates, a limited range of predictors that typically exclude obstetric history, lack of validation, and restriction to a single classifier (logistic regression). Consequently, predictive performance remains low, and risk quantification has not been adopted into antenatal practice.[17] Improved risk stratification and triaging of pregnancies and neonates at higher risk of mortality could lead to optimal resource utilization, ensuring an appropriate level of care for at-risk pregnancies and neonates. Analyses of data of pregnancies and neonates with a higher risk of stillbirth or neonatal mortality can inform the field and lead to improved resource allocation to improve care. A recently published study suggests that models that include delivery and post-delivery variables had good predictive accuracy for the risk of neonatal mortality and that birth weight was the most important predictor for neonatal mortality.[18] The current review highlights the available evidence for stillbirth and neonatal mortality risk prediction and identifies possible future research and implementation directions.

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### Risk Prediction for Stillbirth and Neonatal Mortality

Many studies report associations of pregnancy, delivery, and neonatal variables with stillbirth or neonatal mortality.[19,20] Specific variable associations with mortality do not help in identifying individual-level risk assessment. Multivariable risk prediction models provide individual-level risk assessment, which could be helpful for clinical interpretation and intervention.[21,22] Only a few investigators have published studies designed to develop models to predict the risk for stillbirth or neonatal mortality. The majority

of the modeling studies for neonatal mortality risk prediction used data from extremely low birth weight neonates (ELBWs) who received intensive care in high-income countries (HIC).[23–27] The prediction models are specific for the type of population and settings from which they are developed, so the models from HICs cannot be extrapolated to low-resource settings of LMICs.

There is a paucity of outcome prediction models for intrapartum stillbirth and neonatal mortality in either HICs or LMICs. A few outcome prediction modeling studies from HICs[23,24] used machine learning-based modeling techniques as they are thought to perform better than conventional models when applied to relatively large data sets because these models may improve the ability to delineate complex relationships and identify novel interactions between variables. However, machine-learning models had not been performed with data from low resource settings of LMICs.[28]

## Stillbirth and Neonatal Mortality Risk Prediction in Low-resource Settings in LMICs

Prediction models for intrapartum stillbirth and neonatal mortality in low-resource settings in LMICs have been reported recently.[18] This study had the largest sample size stillbirth or neonatal mortality prediction study to date with 502,648 prospectively enrolled pregnancies over 9 years from six countries in South Asia (India and Pakistan), Africa (Democratic Republic of Congo, Zambia, and Kenya), and Latin America (Guatemala) using a high-quality population-based vital registry (Global Network Maternal Neonatal Health Registry, GN Registry).[19] The investigators reported a comparison of predictive accuracies of conventional and advanced machine learning predictive modeling techniques to identify the best predictive model for intrapartum stillbirth and neonatal mortality in low-resource settings. The availability of a large sample size allowed building models using rigorous modeling techniques with independent training, test, and validation data sets to ensure their generalizability. Using the most important predictive variables and variable interactions from the best-identified model, which was a machine learning model, mortality risk prediction scores were developed. The study also identified important predictors for intrapartum stillbirth and neonatal mortality (Tables 1 and 2). This study determined that models based on prenatal or pre-delivery data have low predictive accuracy for intrapartum stillbirths, whereas neonatal mortality models that include delivery and post-delivery data had a good predictive accuracy of the risk for neonatal mortality. A similar finding of improved predictive accuracy with the addition of delivery and post-delivery variables has been reported in relatively small studies from both HICs[23] and LMICs.[22] Birth weight was identified as the most important predictor of neonatal mortality (Table 2). Birth weight has also been reported in small studies to be associated with the risk for neonatal mortality in studies from HIC,[23,26,29,30] although these studies did not identify that birth weight was the best predictor. An innovative easy to use mortality risk-prediction tool using birth weight as a continuous measure was developed for use by healthcare workers for point of care risk assessment (Fig. 1).

**Table 1:** Top predictors for intrapartum stillbirth and neonatal mortality

| *Rank* | *Predictor* | *AUC* | *AUC increase* |
|---|---|---|---|
| Prenatal | | | |
| 1 | Cluster perinatal mortality | 0.6 | X |
| 2 | Gestational age at enrollment | 0.61 | 0.008 |
| 3 | Maternal age | 0.61 | 0.003 |
| 4 | Birth order | 0.62 | 0.008 |
| 5 | Parity | 0.63 | 0.009 |
| Pre-delivery | | | |
| 1 | Antepartum hemorrhage | 0.56 | X |
| 2 | Cluster perinatal mortality | 0.64 | 0.086 |
| 3 | Gestational age at enrollment | 0.64 | 0.002 |
| 4 | Hypertension/pre-eclampsia/ eclampsia | 0.66 | 0.018 |
| 5 | Maternal age | 0.66 | 0.002 |

Predictors are added consecutively using the gradient boosted ensemble model; then AUC is calculated. Intrapartum stillbirth was defined as non-macerated stillbirth occurring presumably during labor, and neonatal mortality was defined as mortality from birth to 28 days. Cluster perinatal mortality is the perinatal mortality rate within each distinct geographical area (cluster) of the sites as defined by the GN Registry. Hypertensive disease/severe pre-eclampsia/eclampsia is defined as blood pressure >140/90 mm Hg, proteinuria, and seizures[18]

**Table 2:** Top predictors for neonatal mortality

| *Delivery/day 1* | | | |
|---|---|---|---|
| 1 | Birth weight | 0.78 | X |
| 2 | Bag and mask resuscitation | 0.81 | 0.039 |
| 3 | Gestational age | 0.81 | 0.003 |
| 4 | Cluster perinatal mortality | 0.82 | 0.001 |
| 5 | Maternal age | 0.82 | 0.004 |
| *Post-delivery/day 2* | | | |
| 1 | Birth weight | 0.76 | X |
| 2 | Neonatal hospitalization | 0.81 | 0.05 |
| 3 | Neonatal antibiotics | 0.84 | 0.032 |
| 4 | Gestational age | 0.84 | 0.003 |
| 5 | Bag and mask resuscitation | 0.85 | 0.01 |

Predictors are added consecutively using the gradient boosted ensemble model; then AUC is calculated. Neonatal mortality was defined as mortality from birth to 28 days. Cluster perinatal mortality is the perinatal mortality rate within each distinct geographical area (cluster) of the sites as defined by the GN Registry[18]

## Future Directions for Retrospective or Prospective Assessment of the Risk Score and Tool

The study from the GN Registry[18] was done using a prospectively collected high-quality population-level research database, so the study results are likely to be reproducible in communities in resource-limited regions similar to those of the study settings. However, retrospective and prospective studies could be done to evaluate the predictive accuracy of the risk score and risk assessment tool in communities outside of the GN Registry and adapt them as needed. The score was developed using readily available clinical data; data on stratification of individual risk factors as per illness severity, details of treatments received, laboratory variables, and clinical response were not included for ease of use and because of unavailability of such complex variables. It may be possible to further improve the score predictive accuracy by including complex variables, but such an attempt should be made carefully as that may need model reconfiguration with changes in the variable coefficients.[31] The incremental benefit of such an exercise may not be reasonable as the risk prediction score and

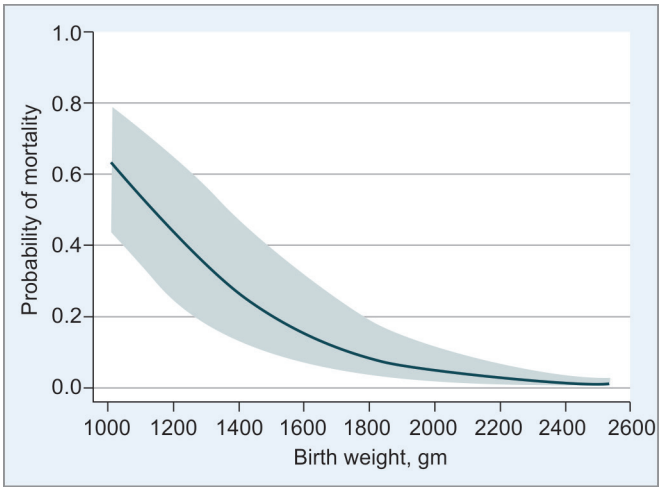


**Fig. 1:** Probability of mortality as a function of birth weight. The risk for neonatal mortality increased with decreasing birth weight. Birth weight was the most important predictor of neonatal mortality in both the delivery/day 1 and post-delivery/day 2 scenarios and explained a large percentage of the variance of mortality. The probability of neonatal mortality with decreasing in birth weight occurred in both the delivery/day 1 and post-delivery/day 2 scenarios. Reproduced with permission[18]

tool had good predictive accuracy using readily available clinical variables. After ensuring the applicability of the risk score and risk tool, implementation research assessing mortality risk-based triage, referral, and management for reducing the number of stillbirths and neonatal deaths in low-resource settings of LMICs can be tested. Pre-delivery estimates of birth weight could also be tested as a strategy for pre-delivery triage and referral.

## Conclusion

Timely identification of at-risk pregnancies and neonates is critical for the reduction of stillbirth and neonatal mortality in low-resource settings of LMICs. Risk stratification and triage of pregnancies and neonates at higher risk of mortality could help to reduce the burden of stillbirth and neonatal mortality in low-resource settings LMICs. The available risk assessment score and tool can be tested further using retrospectively or prospectively collected data. Implementation research should also be used to assess and improve the utility of these models. A population-based, multi-country, and high-quality database would assist and promote future outcome prediction research in low-resource settings.

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REVIEW ARTICLE

# The First 1000 Days: Assembly of the Neonatal Microbiome and Its Impact on Health Outcomes

Joann Romano-Keeler[1], Jun Sun[2]

## Abstract

Early life microbial colonization is critical for the development of the immune system, postnatal growth, and long-term health and disease. The dynamic and nascent microbiomes of children are highly individualized and are characterized by low bacterial diversity. Any disruptions in microbial colonization can contribute to shifts in normal microbial colonization that persist past the first 1000 days of life and result in intestinal dysbiosis. Here, we focus on microbiome-host interactions during fetal, newborn, and infant microbiome development. We summarize the roles of bacterial communities in fetal development and adverse health outcomes due to dysbiosis. We also discuss how internal and external factors program the microbiome's metabolic machinery as it evolves into an adult-like microbiome. Finally, we discuss the limits of current studies and future directions. Studies on the early-life microbiome will be critical for a better understanding of childhood health and diseases, as well as restorative methods for the prevention and treatment of diseases in adulthood.

**Keywords:** Bacteria, Fungi, Immunity, Microbiome, Necrotizing enterocolitis, Probiotics, Virome.

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## Introduction

With the application of culture-independent techniques and high throughput technologies over the last decade and a half, the significance of the intestinal microbiome has been revealed. Alongside this discovery, has been an appreciation for the dynamic and complex colonization during the first 1000 days after conception, including pregnancy and the first 2 years of life.[1–3] Microbiome studies have defined the concepts of the microbiota (microorganisms present in a given environment) and the microbiome (genes and genomes of the microbiota, including products of the microbiota and the host environment).[4] Though initially perceived as chaotic and haphazard, research has demonstrated that early life bacterial succession is in fact a well-orchestrated series of events that when occurring correctly, can result in intestinal eubiosis.[5,6] In such cases, the intestinal microbiome can impart optimal health outcomes, not only during the first 3 years of life but also throughout later childhood and adulthood. Early-life microbial programming of the developing infant immune system is now considered a key component of the Developmental Origins of Health and Disease (DOHad), also known as the Barker hypothesis.[7,8] The DOHad hypothesis, which existed well before the advancement of human microbiome research, describes variations in health outcomes, including allergies, asthma, and hypertension, due to a cadre of environmental factors during a critical window of development. This concept now extends to the specific role that microbes may have in populating infants during early life, including their interactions with the host's developing immune system, postnatal growth, and their subsequent role in long-term health and disease.

Focusing on early life microbial colonization is critical because the early life microbiome is not stable during this window of development.[9] In contrast, the adult microbiome, is minimally impacted by external factors, including antibiotics, diet, and interval illnesses. The dynamic and nascent microbiomes of children are highly individualized and are characterized by low bacterial diversity with fewer overall bacterial species.[9] In the case of newborns and infants, any disruptions in bacterial colonization can contribute to shifts in normal microbial colonization that are not transient but persist past the first 1000 days of life. These disruptions, often result in dysbiosis, which is defined as an imbalance in the composition and metabolic capacity of microbiota and can increase the risk of chronic health conditions. Though human microbiome studies have typically focused on bacterial colonization, other organisms, including viruses (virome), fungi (mycobiome), archaea (archaeome), and bacteriophages are also dynamic during this period. All of these

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organisms establish themselves in the gut through interactions with bacteria and the host immune system.[10]

For the purposes of this review, we will focus on microbiome-host interactions during fetal, newborn, and infant microbiome development. We will discuss bacterial communities that may play a role in fetal development. We will describe the dynamic changes in the microbiome during the first year of life in term and preterm infants and the second year of life after the introduction of an expansive diet. We will also identify areas where we have observed adverse health outcomes due to intestinal dysbiosis early in life. Finally, we will discuss how internal and external factors program the microbiome's metabolic machinery as it evolves into a more mature, adult-like microbiome.

## Factors Affecting Intrauterine Colonization and the Fetal Microbiome

### Maternal Factors during Pregnancy

Many maternal factors affect the newborn microbiome, including interactions between the maternal and fetal genome that contribute to bacterial colonization and unique cross-talk between pioneer organisms and the *in utero* environment. Pregnancy is associated with a shift in the mother to a proinflammatory state which is associated with metabolic dysfunction, including insulin resistance, dyslipidemia, and hypertension.[11] It is unclear whether these changes in maternal metabolic machinery, which are beneficial to the developing fetus, are modulated by a shift in the maternal intestinal microbiome and whether subsequent effects are observed in the fetus. In some studies, despite developing a proinflammatory metabolic profile during pregnancy, the maternal microbiome remains unchanged, while in other studies an increase in the abundance of Actinobacteria and Proteobacteria, a decrease in butyrate-producing bacteria, and a decline in bacterial diversity are reported.[11,12] Further research is necessary to define how any of these changes in the maternal intestinal microbiome affect fetal development and newborn intestinal colonization (Fig. 1).

Maternal antibiotic use during pregnancy has been associated with alterations in the maternal microbiome, including increases in vaginal colonization by Staphylococcus. It is also accompanied by derangements in the newborn bacterial ecosystem, increasing the childhood risk of developing allergies, otitis media, and obesity. Another maternal factor affecting the newborn microbiome includes maternal obesity, which is correlated with a shift in the infant microbiome that can increase their risk of obesity. The intestinal dysbiosis associated with obesity in pregnant and nonpregnant adults is characterized by an imbalance in the Firmicutes-to-Bacteroidetes ratio.[13,14] The microbiome of infants born to obese mothers had a similar derangement with an increased abundance of Firmicutes, particularly from the Lachnospiraceae family. These infants also had a greater

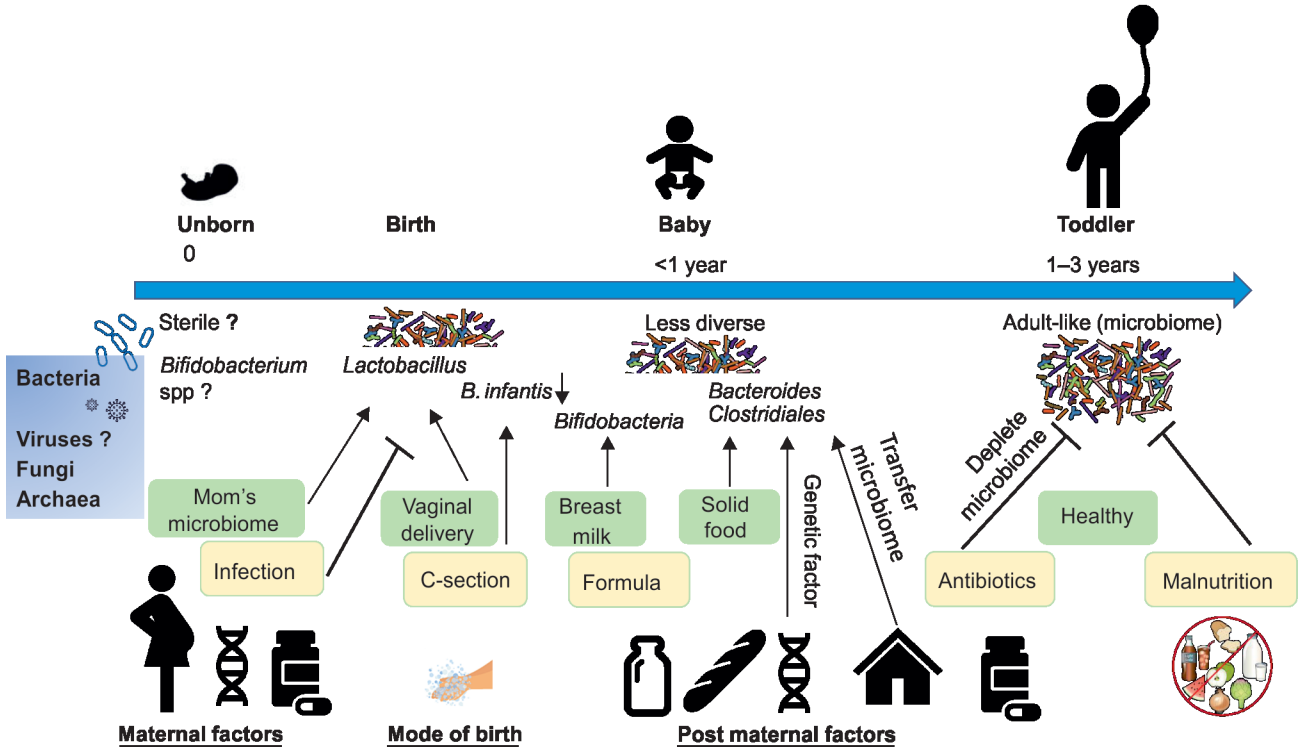


**Fig. 1:** Maternal factors, modes of delivery, and postnatal factors determine the microbiome during early life. The fetus may be associated with microbes before birth. Mom's microbiome could be transported through the bloodstream to the fetus. Maternal vaginal infections could result in bacteria invading the uterine environment. The delivery method shapes the initial microbial inoculum of the newborn. Vaginally delivered infants had more *Lactobacillus and Bacteroides*. C-sections had lower levels of *Bacteroides longum* subspecies *infantis* (*B. infantis*). Postnatal factors such as antibiotic use, diet (such as human milk vs formula, and introduction of solid food), genetic factors, and environmental exposure further configure the microbiome. By age 3, the microbiome gradually shifts toward an adult-like profile. The green box indicates the positive factor to promote beneficial bacteria, and the yellow box indicates the negative factor reduces the bacterial diversity. " ? " indicates controversial results or unknown. Individual bacteria associated with the different processes are indicated. The roles of viruses, fungi, and other microbes are still unknown

risk of becoming overweight at 1–3 years of age.[15] Mothers with gestational diabetes mellitus (GDM) have an altered gut microbiota, including *Bifidobacterium* spp., which was heritable by the fetus during pregnancy.[16,17] In one study by Wang et al., samples across multiple body sites were collected from pregnant women and their neonates. Bacterial communities in mothers with GDM and their offspring were similarly altered across body sites, consistent with an intergenerational concordance between GDM mothers and their offspring. Other associations between maternal complications, including HIV, group B Streptococcus, irritable bowel disease, and intra-amniotic infection, have been associated with inconsistent shifts in the infant intestinal microbiome. However, most studies on the fetal inheritance of the maternal microbiome demonstrate a well-orchestrated process with a lifelong impact on the newborn.

### Fetal Microbiome

While the *in utero* environment was traditionally viewed as sterile, controversy now exists over whether a fetal microbiome modulated by maternal factors, including diet and immunogenetics, develops during pregnancy and impacts childhood and adult health outcomes.[18,19] With conflicting evidence on this issue, we cannot accurately identify the initial timepoint when bacteria stimulate the development of different organ systems, including mucosal immunity. Other complexities in this debate are whether bacterial DNA that is detectable in the placenta or amniotic fluid, represents viable or dead organisms and whether its presence is sufficient to corroborate not only the presence of these microorganisms but a role in fetal development. Thus, studies have emerged on both sides of this discussion to further investigate whether bacteria influence *in utero* development.

Studies supporting colonization *in utero* include work from Aagaard et al., in which approximately 320 placental samples were collected under sterile conditions. The microbiome of these samples was compared with samples from different body sites (nares, mouth, skin, gut) in nonpregnant patients enrolled in the NIH-sponsored human microbiome project.[20] Gram-negative intracellular organisms were detected in placental samples from this study in the absence of chorioamnionitis. In addition, based on Bray Curtis similarity indices, bacterial communities in the placenta shared the greatest similarity with the oral microbiome of nonpregnant subjects.

Maternal-fetal microbiota transmission *in utero* is also supported by the detection of bacterial DNA in meconium (first stool passed by a newborn in the first few hours to days of life) from organisms that were also detected in amniotic fluid, an expected finding given how the fetus swallows amniotic fluid during gestation. A study comparing fluid and meconium demonstrated that approximately 30% of detected species were shared between amniotic fluid and meconium samples, while 30% were only found in amniotic fluid and 40% were unique to meconium. However, the 30% of shared species accounted for up to 95% of total reads and only a small percentage of reads was found in either the amniotic fluid or meconium alone.[21]

Microbiome studies of meconium shortly after delivery were also investigated in a cohort of infants undergoing bowel resections due to anatomic anomalies without evidence of infection. Unique intestinal microbial communities were identified as early as the first day of life, even in those infants delivered operatively without ever having received enteral feeds.[22]

Further studies on *in utero* colonization that define the origins of the nascent microbiome in fetuses and newborns are imperative for understanding disease processes, such as preterm labor and necrotizing enterocolitis (NEC). All of these studies will need to address concerns about contamination and incorporate negative controls into analyses to withstand the scrutiny of the scientific community. However, should fetal inheritance of the maternal microbiome prove true, it would be a unique opportunity to modulate fetal outcomes through modifications in maternal diet and other lifestyle choices during pregnancy.

## Perinatal and Newborn Microbial Colonization

### Delivery Mode

A large source of pioneer bacteria colonizing newborns is acquired during delivery with the mode of delivery playing a major role in the type of bacteria inherited. In one of the initial studies of ten infants, including four delivered vaginally and six delivered via cesarean section (C-section), maternal samples (skin, oral mucosa, vagina) were collected prior to delivery along with neonatal samples (oral mucosa, nasopharynx, meconium) and environmental samples from the operating room.[23] Though bacterial communities of newborns were highly undifferentiated, vaginally delivered infants had microbiomes similar to that of maternal vaginal samples, including a higher abundance of Bacteroides. Conversely, infants born via C-sections had microbiomes similar to maternal skin and hospital organisms, including those detected in the operating room. Such studies demonstrate a shift toward aberrant gut colonization after C-sections and raise concerns about the impact of a worldwide rise in the rate of C-sections performed without maternal health indications.[24,25] Studies describing the association of operative deliveries with adverse health outcomes during childhood, including delays in cognitive development, autoimmune diseases, and atopic disorders, have generated questions about whether aberrant postnatal colonization contributes to these outcomes.[26,27] Infants born via C-section also had lower levels of *Bacteroides longum* subspecies *infantis* (*B. infantis*), a key symbiont and a common strain contained in probiotics.[28,29] *B. infantis* has co-evolved with mother-infant dyads to assist with a host of metabolic and immune functions, including complex carbohydrate digestion. In one study, infants delivered via C-sections had decreased colonization with *B. infantis* and microbial diversity. This was associated with reduced Th1 responses and neonatal immunity during the first 2 years of life.[30]

Another area related to the delivery mode that is under active debate is the question of whether the maternal vaginal vs rectal microbiome is the key driver of a newborn's postnatal colonization across all delivery modes. To investigate whether maternal or vaginal microbiome impacts colonization of the newborn after delivery one study examined 75 infants born either vaginally ($n = 40$) or by planned or emergent C-sections ($n = 35$) and found virtually undetectable levels of Lactobacillus in vaginally delivered patients, despite Lactobacillus being the most common member of the vaginal microbiome. Vaginally delivered infants were in fact seeded by more bacterial strains, however, the strains matched maternal rectal swabs.[31] Transplantation of the maternal fecal microbiota has been investigated as a potential way to reverse detrimental effects on the gut microbiota observed in infants delivered by C-sections and restore a normal gut microbiome. In

a recent proof of concept study, researchers identified a cohort of seven mothers with planned C-section delivery, and maternal fecal samples were collected approximately 3 weeks prior to delivery.[32] Infants received these maternal fecal samples orally after delivery and after approximately 3 months, infants demonstrated restoration of their intestinal microbiome to mimic that of infants delivered vaginally. These studies have provided promising insight into how to reestablish normal gut colonization after an important early life event.

## Differences between the Preterm and Term Infant Microbiome

Distinct differences exist between the term and the preterm intestinal microbiome, which serves as the foundation for normal immune development, postnatal growth, and subsequent health outcomes. Immaturity of the intestinal microbiome in preterm infants affects their normal metabolic capacity with impaired nutrient absorption and delayed intestinal motility. Subsequently, more than half of preterm infants are discharged with postnatal growth failure.[33] The preterm gut also has poorly developed barrier function, motility, and immune function, which makes it a source of infections and inflammation, including NEC, a devastating bowel condition that impacts approximately 7% of preterm births. Up to 5% of all neonatal intensive care unit (NICU) admissions are affected by NEC and it is associated with significant mortality and morbidity in up to 35% of cases.[34] The gut microbiota of preterm infants is characterized by delayed bacterial colonization including decreased microbial diversity, decreased levels of commensals and obligate anaerobes, and increased detection of potentially pathogenic organisms and opportunistic bacteria, including *Enterobacter*, *Enterococcus*, and *Staphylococcus*.[35,36] Studies have demonstrated that NEC patients, compared with preterm infants without NEC, have an even more dramatic drop in microbial diversity, which may be in part due to their increased exposure to antibiotics. This significant drop in diversity allows for individual bacterial species to have a greater impact on intestinal microbial communities, which can drive the pathogenesis of NEC.[37] Other common practices that preterm infants may be introduced to which can disrupt the intestinal microbiome include C-sections, antibiotic use, and formula feedings. In the prospective fecal collection from 45 preterm infants aged birth to 60 days, gestational age was the major determinant for preterm infants' ability to achieve normal and mature gut microbiomes, including a shift towards a *Bifidobacterium* dominant microbiome. All preterm infants in this study received human milk, which is known to increase the abundance of *Bifidobacteria* in newborns. Research continues to focus on how to promote healthy gut colonization after preterm delivery, including access and utilization of breast milk (expressed or donor) and indications for probiotics.[38]

## Breast Milk and Formula's Influence on the Infant Microbiome

The American Academy of Pediatrics recommends the exclusive use of human milk for the first 6 months of life.[39] This policy statement reflects the importance of early nutrition for optimal development of the immature intestinal microbiome and immune function of the gastrointestinal tract.[1] The robust composition of human milk includes but is not exclusive to antimicrobial and immunological components, prebiotic substances (lactose, human milk oligosaccharides), and live microorganisms.[40] Metabolites and microbes present in breast milk are responsible for promoting tolerance to self-antigens and the gastrointestinal tract's response to potential human pathogens. Specifically, microorganism-associated molecular pattern signaling with toll-like receptors (TLRs) promotes a tolerogenic environment with the expansion of T regulatory cells (T regs) within the intestines.[41]

In a large, multi-center study, The Environmental Determinants of Diabetes in the Young study (TEDDY), in which stool samples collected monthly from 903 children between 3 and 46 months of age were analyzed by 16S rRNA gene sequencing, infants receiving breast milk had higher levels of *Bifidobacterium* species, specifically *B. breve* and *B. bifidum*.[42] *Bifidobacterium* spp. and *Lactobacillus* spp. were present and viable in breast milk. The ability of these two organisms, transferred from breast milk, to flourish is in part due to the initial colonization of the newborn intestine by aerobic bacteria, including *Enterobacteria, Staphylococcus,* and *Enterococcus*. These aerobic organisms create ideal conditions for anaerobic *Lactobacillus* and *Bifidobacteria* to thrive. Breastfeeding during this window had a comparable effect on the microbiome composition, regardless of whether it was combined with formula or solids. Conversely, early cessation of breast milk was associated with faster maturation of the gut microbiome, including accelerated colonization by Firmicutes. Infants not breastfed at all were rapidly colonized with *Escherichia coli.* While breast milk itself is a source of microbes, contact with human skin and the areola, along with the retrograde flow of the infant's oral microbiota that occurs during nursing, plays an important role as well.[43] The TEDDY study also demonstrated that *Staphylococcus* was the main organism transferred to the newborn during contact with the areola.

## Environmental Factors

The importance of environmental factors, including a newborn's immediate surroundings, his family's lifestyle, and geographic location, is well-demonstrated, particularly in preterm infants residing in the NICU, who have an increased likelihood of inheriting flora from this hospital setting.[44] Inherited organisms are often those associated with nosocomial infections due to their extended exposure to the hospital environment during a critical window of development for their immune systems.[45] Outside of the hospital, an infant's environment remains an important contributor to intestinal gut colonization and has been validated in stool studies. In such studies, bacteria isolated in infant fecal samples matched those identified in their immediate environment. Family members and siblings have been shown to affect an infant's microbiome and initial colonization. For example, the number of older siblings positively correlates with bacterial diversity and richness at 18 months of age, with the increasing relative abundance of *Firmicutes* and *Bacteroidetes* in infants with more siblings.

Other differences in microbial composition between infants with siblings and those without have been observed.[46] For example, in the KOALA Birth Cohort from the Netherlands, infants with sibling exposure had a higher number of *Bifidobacteria* in their gut than those without siblings.[47] In another cohort study, infants with no siblings had an increased number of non-*E. coli enterobacteria* along with clostridia in the gut in tandem with a lower anaerobe-to-facultative anaerobe ratio.[48] More studies in this area of the "sibling effect" are warranted in order to better define the specific role of siblings during early life development.

Geographic locations are also a key consideration and differences are likely secondary to different ethno-geographic populations with distinct regional diets and cultural practices.[49] When studies of infants' gut microbiota were conducted in infants living in rural Africa, comparisons of their fecal microbiome amongst these rural babies with those living in urban Italy demonstrated the dissimilarity between the different communities.[50] Similar differences amongst the infant microbiome in other regions of the world are also apparent when exploring functional differences in bacterial communities in urban vs rural environments.[51] All of these studies demonstrate the inability to generalize microbiome studies across different family structures, countries, and home environments without taking into account such variables.

## Evolution of the Microbiome during the Second Year of Life

The introduction of solid foods and cessation of breast milk or formula feeds in the second year of life is responsible for a rapid expansion in the structure and function of infants' microbiomes as their diets gradually transition to an adult-like composition.[2,52,53] This evolution in the microbiome of infants' intestinal tracts is essential for bacteria to assume new digestive roles, including the breakdown of glycans, mucin, and complex carbohydrates, along with the production of short-chain fatty acids. In a large study of 330 Danish children whose microbiome was evaluated at 9, 18, and 36 months, as formula and breast milk were gradually discontinued, *Lactobacillaceae*, *Bifidobacteriaceae*, *Enterococcaceae*, and *Enterobacteriaceae* species abundance decreased. Simultaneously, an increase in the abundance of *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae* species occurred.[54] Such findings were concordant with another large cohort study conducted in five different countries in which similar changes were observed anywhere from 4 to 6 weeks after transitioning from milk-based feedings to a family diet.[55]

Challenges exist in discerning the changes in the nascent microbiome that are due to a shift in dietary intake with alterations in the composition and function of bacterial communities due to advancing age. With increasing age, infants have more environmental exposures (daycare and school settings, travel) and have begun to move independently with frequent oral contact-generating a constant source of microbes that they can acquire. This confounding variable was explored by the same Danish investigators in two independent cohorts of infants aged 9 months in which a comprehensive analysis of the infants' diets was conducted in parallel with 16S rRNA gene analysis of infants' fecal samples. These analyses demonstrated that microbial diversity significantly correlated with progression toward family foods. In addition, family food dietary patterns in both cohorts, including foods rich in protein and fiber, were associated with increased alpha diversity.[56] Questions remain with regard to whether a delayed introduction of solid foods and sustained breastfeeding into the first year of life compromises this growth in bacterial diversity. Conversely, it is unclear whether this expansion of bacterial species which occurs as an infant's diet diversifies is what drives the development of a healthy microbiome in early childhood.

## Colonization Beyond the First 1000 Days and Its Impact on Childhood Diseases

The importance of early life colonization is secondary to the foundation it provides for optimal lifelong health. Disrupted microbial colonization in the first 2 years of life contributes to the onset of pediatric and adult diseases, including gastrointestinal pathologies, metabolic disturbances, and neurological disorders. A common feature of many of these disorders is a loss of microbial diversity and key beneficial microbes. While the predominance of *Bifidobacterium* during the first year of life in infants on a breast milk diet is a key driver of immune protection, a pivot towards a more diverse and robust microbiome in the second year of life after the introduction of complementary foods is equally as important.[3,57] The timing of this transition is exquisitely sensitive as evidenced by adverse health outcomes that result from the early loss of *Bifidobacterium*, the premature introduction of new bacterial species, or loss of microbial diversity secondary to poor-quality diets or antibiotic use.[58]

Frequent exposure to antibiotics during the first 2 years of life, even at low doses, is one of the greatest insults to the nascent microbiome. Antibiotic stewardship has grown in attempts to preserve microbial diversity during this critical window. Specifically, the use of antibiotics during this developmental window is associated with asthma, atopic dermatitis, multiple sclerosis, inflammatory bowel disease, juvenile idiopathic arthritis, and obesity.[59,60] The "hygiene hypothesis," which describes limited exposure to microorganisms that are important for shaping the maturing immune system, has been suggested to be responsible for the increased incidence of childhood asthma and allergies. With aggressive hygiene measures that decrease the risk of early-life infections, immune tolerance is not induced, and infants have an increased risk of developing an auto-immune disorder.[61] This situation with decreased microbial exposure, decreased microbial diversity, and impaired immune tolerance, may be further exacerbated by increased hygiene measures associated with the COVID-19 pandemic.[62]

## Conclusion and Future Directions

Growing recognition of how important early life intestinal colonization has improved our understanding of disease pathologies in pediatric patients as well as our ability to identify restorative methods in instances where the nascent microbiome has been disrupted, including interventions such as prebiotics, probiotics, and fecal microbial transplantation. However, many studies investigating the neonatal microbiome are limited by the homogeneity of the population sampled because recruited subjects are typically from a single-center or geographic region. Comparisons across studies are challenging due to the use of different sample types, collection methods, and omics platforms. Finally, microbiome studies of low biomass samples, such as those collected during fetal and neonatal development, are fraught with concerns for environmental and reagent contamination.[63]

Future directions should incorporate studies that explore not just interactions between the host immune system and pioneer bacteria, but also those involving other eukaryotes, including viruses and fungi. For example, while we have defined the role of mode of birth in shaping the microbiome, few studies to date have

examined how birth mode affects the diversity of the early-life gut viral microbiome or "virome."[64] Viral transmission and colonization of the newborn intestinal tract, which may be modulated by similar drivers as the gut microbiome, may share an important role in facilitating bacterial colonization and promoting bacterial diversity. Conversely, maternally-derived viruses that require maternally-derived bacteria for replication (bacteriophages) might only be able to proliferate weeks to months after bacterial populations are established, as demonstrated in prospective twin studies.[65] Growth of these bacteriophage communities might occur during the first 2 years of life alongside burgeoning bacterial diversity. Over the next decade, defining these intricate interactions between viruses and bacteria may provide valuable insights into how to improve health outcomes for newborns and infants.

Another emerging area of research that may improve our understanding of this critical developmental window is the regulation of epigenetic mechanisms through crosstalk with gut microbial metabolites. Epigenetics, which is the study of phenotypic changes due to modifications in gene expression, are not directly driven by an individual's genome. Modifications do not alter the host's nucleotide sequences but involve methylation, posttranscriptional histone alterations, chromatin restructuring, and regulation of non-coding RNA.[66] While interactions between an individual's genome and environmental factors are critical in the regulation of these epigenetic mechanisms, gut microbial metabolites may also play a role.[67,68] Further studies that elucidate interactions between an individual's microbiome and epigenome - all epigenetic marks on the DNA of a single cell - may provide the most comprehensive understanding of risk factors for adult-onset metabolic disorders.

The establishment of a biobank with multicenter participation and collection of a variety of sample types is essential for moving many of these translational research goals forward. Sites that have developed infrastructure for creating a neonatal biobank have described the feasibility of doing so through strengthening relationships between parents and clinical research teams to maintain sample stability between transfer from the clinical laboratory to the biorepository to encourage long-term participation in sample collection.[69] Large scale initiatives, including the integrative human microbiome project, which include prospective sample collection of fetal membranes, amniotic fluid, placenta, and postnatal stool, have the potential to improve our clarity of these dynamic early life events.[70]

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REVIEW ARTICLE

# Tasked for Compassion: Initiating Reproductive Grief Care in the Neonatal Intensive Care Unit

Kathryn R Grauerholz

## Abstract

The experience of parenting a premature or ill infant in the neonatal intensive care unit (NICU) can be overwhelming and traumatic. Parents who have previously endured a reproductive loss may find that an accumulation of escalating distress related to nurturing a neonate while receiving care in intensive care compounded with lingering grief from a prior perinatal loss can overwhelm their capability to cope. The ambiguous nature of perinatal loss and societal disenfranchisement of the grief often results in a prolonged or complicated bereavement trajectory which can inhibit bonding, mental health, and physical wellness. The frequent contact and perinatal conversations between parents and clinicians provide opportunities for essential discussions about emotional vigor, grief, and bereavement. A review of the literature and current research found that initiating conversations and care modalities that facilitate Worden's "tasks of grieving" can foster a necessary healing pattern for bereaved parents. These efforts will theoretically nurture parent–child bonding and promote desirable neonatal outcomes.

**Keywords:** Bereavement, Fetal demise, Miscarriage, Neonatal intensive care unit, Perinatal loss, Reproductive grief, Stillbirth.

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## Fundamentals of Reproductive Loss and Trauma

Reproductive loss is common, occurring in up to a quarter of all pregnancies, and recurrent pregnancy loss is also related to subsequent neonatal morbidity and mortality.[1,2] Parents experiencing difficulty grappling with an infant enduring significant morbidity in the intensive care are more likely to have experienced reproductive loss or issues with infertility.[3] Additionally, advanced maternal age, multiple gestation pregnancies, and reproductive technology have been linked to increased neonatal morbidity and mortality.[4–8]

In the past two decades, with the emergence of perinatal palliative care, more efforts have been made to implement efficacious perinatal bereavement into maternity services.[9,10] However, recent studies have shown that the progression to provide sufficient perinatal grief support in NICU is still slow in meeting the need.[11–17] Currently, in the United States, there is no standard of care for reproductive grief after miscarriage (<20 weeks gestation), which often occurs in the home or emergency setting and where healthcare support for grieving, though necessary, is typically absent.[18,19] Because gestational age is not necessarily linked to the intensity of the emotional reaction to the loss, the impact of grief subsequent to miscarriage should not be underestimated.[20–23] Complicated reproductive grief reactions also pose a compelling risk for maternal–fetal attachment problems for subsequent pregnancies.[24,25] It is highly probable that NICU parents with a history of successive reproductive loss will be more at risk for prolonged, complicated grief reactions and attachment challenges in bonding with a fragile and/or ailing neonate.

## Reproductive Story and Ontological Death

Developmental psychologists have described how the unconscious narrative and attachment to future children begins long before conception.[26–30] In childhood, desires and expectations for future offspring are envisioned and integrated into an individual's reproductive story, but this longstanding aspiration can be shattered with experiences of miscarriage, stillbirth, or infertility.[28] Often those impacted by reproductive loss experience waning trust in their bodies, health, healthcare, and ability to procreate their longed for progeny.[28,31] This wounding experience can have long-standing implications for the individuals' mental health, wellbeing, relationships, and future bonding with subsequent children.[32] There are very few longitudinal studies on reproductive grief reactions, but one study reported that the risk for complicated grief after perinatal loss is 59% or greater when evaluated two years after the loss.[33]

The loss of a pregnancy or infant can disrupt or crush an individual's view of the world and fundamental beliefs about their place and purpose of existence. Reproductive loss is often associated with ontological death, resulting in the disruption of long-standing beliefs about the meaning of life which can have far-reaching implications on future relationships, intimacy, spirituality, and self-actualization.[34,35] Frequently, self-blame and shame surround the experience of loss. These reactions which are reported by parents experiencing any form of reproductive loss can further hinder the parents' ability to reconstruct their reality without the desired child.[36–40] Reproductive grief reactions can be lengthy, spanning more than ten years in some studies.[18,41] Furthermore, in the

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absence of healing bereavement support, subsequent adversities experienced in the perinatal periods can trigger self-preservation and detachment which can in turn impact bonding and optimal neonatal trajectory.[41–45] When multiple factors indicating parental wellness and adjustment after the loss of a child were looked at simultaneously, one study demonstrated that indicators of actual resilience could be attributed to only about 5% of the parents.[46]

## Barriers to Grieving Reproductive Loss

*Ambiguity* – Experiences of loss during the perinatal period lack the physical evidence and shared memories that are usually associated with death and mourning. Reproductive morbidity is an ambiguous loss that hinders the grieving trajectory for the bereaved.[23,47] Lang and her colleagues[48] found several factors that contributed to the ambiguity and suffering of perinatal loss, including aspects of viability, disposition of fetal remains, the painful or traumatic physical process of losing the pregnancy, and the disclosure of the loss to others.

*Disenfranchisement* – Healthy coping in response to loss is heavily reliant upon societal support and acknowledgement of the grief experience.[49] In Western society, the loss of a pregnancy or infant is often associated with secrecy and cultural silence.[36] For instance, first trimester losses are not openly discussed in social spheres, and parents are often told by other family members or close friends not to share news of a pregnancy until the second trimester (when the risk of miscarriage has significantly lowered). In a 2008[50] article in *The New York Times*, a father wrote after experiencing the stillbirth of his son, "When a parent dies or a partner–when we lose someone who has lived in the world–there are customs, worn paths to follow, ways to talk about it. But I didn't see any path with this. Was I supposed to keep quiet and pretend nothing had happened? I couldn't accept that."

*Paradox of Reproductive Loss* – Contrary to common societal beliefs, the attachment to an expected child usually endures after a perinatal loss. Researcher Berry and her colleagues[51] (p. 25) found that "perinatal loss is a paradoxical experience in which parents often feel misunderstood and alone". There is a disconnection and often a disparity between the grief that parents may experience years or decades after a loss and the support they receive from care providers, family members, and encountered social spheres.[34] Disenfranchised reproductive grief is typically met with social assimilations that cling to expressions that minimize or ignore the experience rather than compassionately meeting the patients' needs.[51] Those providing care need to be aware of their own assumptions, and in turn strive to provide a more holistic approach to care. This requires planning to meet the parents' needs and affirm the intrinsic values they associate to their loss.

*Trauma Informed Reproductive Bereavement Care* – The value of efficacious bereavement care, provided at any point after a reproductive loss, cannot be underscored enough. Decades of research have demonstrated the potential for chronic maladaptive dysfunction of the sympathetic nervous system in reaction to trauma and lasting biopsychosocial impact which can reduce an individual's capacity to cope, adapt, and learn.[52–55] Many situations of reproductive loss are traumatic and require support from healthcare professionals to foster wellbeing and resilience. Bereavement modalities that authentically focus on emotional healing and affirm the dignity of the human experience of loss are needed rather than shifting to an atomistic physiological focus.[54–57] Active and engaged professional and family enfranchisement of grief are associated with better long-term parental outcomes.[58–61]

## Simplicity yet Difficulty of a Compassionate Approach

Misconceptions about how the grief experience can impact physical or mental well-being are evidenced in inadvertent yet hurtful language patterns that frequently accompany reproductive loss and include comments like "it's common" or "you can just have another one".[62–65] Since the perinatal bereavement experience is outside of western cultural and societal norms, healthcare providers often report feeling awkward or uncomfortable acknowledging and addressing grief.[36,44,66,67] However, helpful interventions, which may initially seem too simplistic to be efficacious, can prove to be a challenge for care providers to utilize and assimilate into practice.[11,68–70] Understanding grief theory and the rationale behind what may seem sentimental or domiciliary can undergird a structured and ameliorating approach to the care of families anguished by reproductive loss.[22] Since knowledge alone is typically not an effective method of changing provider practices,[71] active communication practice, role play, and case study simulations are preferable exercises for correcting or enhancing these aspects of professional performance.[72]

## Validating the Loss and Grief Experience

An initial and important facilitator of processing grief is acknowledgement of the loss and validation of the subsequent grief.[73] For instance, some studies revealed that when this task is neglected or ignored, parents reported anger and resentment towards healthcare providers which contributed to a complicated grief trajectory.[48,74,75] Empathetic responses to loss and grief can mitigate the risk for prolonged or maladaptive grief responses (Table 1).[74,76] Additionally, satisfaction in health care services improved when the loss and grief were validated by the care providers.[77]

**Table 1:** Interventions that foster acceptance of reproductive loss

| | |
|---|---|
| Communication strategies | • Empathetically acknowledge the loss[78–80]<br>• Use language free of jargon and medical terminology[63]<br>• Provide handouts[81]<br>• Encourage journaling[25,73] |
| Efficacious resources | • Provide grief education[47,60]<br>• Involve supportive persons[58,59,61,82]<br>• Offer chaplain assistance[83] |
| Fostering remembrance | • Discuss intentional memory making[11,68,84]<br>• Provide keepsake items[68,85]<br>• Inquire about aspects of pregnancy and thoughts about their child that they would cherish and/or like to honor[69,86]<br>• Assess cultural or spiritual preferences[25,87,88] |
| Enduring assistance | • Provide a grief care packet with discharge instructions[47]<br>• Send a sympathy card or letter signed by the staff/provider(s) at the time of the loss[89] |

## Allowing Painful Emotions Associated with the Loss

Another important way to support those grieving a reproductive loss is to allow for open expression of painful emotions and concerns about the loss and grief process (Table 2). Encouragement and provision of an environment conducive to share private feelings should be offered during private discussions about the care of their child or family member.[90] In a 2021 study on early pregnancy loss, one participant with multiple miscarriages wrote, "I feel broken. Like there's something wrong with me or I'm doing something wrong. I feel like a horrible wife because I know my husband shares my dreams of a big family. It hurts me so bad that I can't give him that or give my daughter a sibling. I don't know how to deal with it, because outside of parenting my child, I am no one" (p. 10).[18]

## Fostering the Adjustment for Life After the Loss

After any death, individuals must come to terms with the life adjustments necessary for living without the one lost. There are often multiple life circumstances, activities, roles, dreams, practices, and social contacts that change as a result of each loss and a shift from one's "normal" associations and patterns of living. There are also changes that will occur with one's fundamental beliefs about life and what brings them meaning as described earlier with the concept of ontological death.[34] Often, bereaved individuals will try to maintain balance by clinging to what they have always done only to discover that the way forward has changed and requires multifaceted adjustment[73] (Table 3). Because of the disenfranchised nature and ambiguity associated with reproductive loss, many find the associated grief trajectory to be uniquely challenging. "A miscarriage is hard because there is no funeral. There is no service of remembrance. There is no formal marking of a life passing. To me, that felt like there was no moving forward. I felt torn into pieces. My husband hurt, but no one else missed our baby."[18]

**Table 2:** Interventions that facilitate emotional healing

| | |
|---|---|
| Communication modalities | • Encourage sharing of emotions[90,91]<br>• Affirm uniqueness of reaction[79,80]<br>• Normalize reaction[92]<br>• Allow ample time to process information[63,93]<br>• Encourage journaling[25,73]<br>• Assess desired cultural mourning practices/expression of grief[66,82,94,95] |
| Efficacious resources | • Acknowledge partner's grief reaction[96]<br>• Provide grief education and supportive modalities[92]<br>• Involve interdisciplinary team[97–99] |
| Enduring remembrance | • Discuss symbolism in healing (positive associations)[68,73,100]<br>• Consider resources or referrals for creative or art therapies[73] |
| Follow-up assistance | • Schedule and make follow-up phone calls[101–103]<br>• Reassess grief intensity (PGIS/PGS)[22,39,69] |

**Table 3:** Interventions that help bereaved adjust to the loss

| | |
|---|---|
| Communication modalities | • Offer follow-up visit to discuss diagnosis in-depth (interdisciplinary)[34,69] |
| Efficacious resources | • Suggest joining support group/ blog[69,100,104]<br>• Connect parents with perinatal loss organizations[100,103]<br>• Suggest strategies for communicating the loss to others[105] |
| Enduring remembrance | • Encourage journaling of journey through loss and moving forward without the child[25,106] |
| Follow-up assistance | • Send out sympathy card(s)[89]<br>• Assess and mitigate unhealthy behaviors[107] |

**Table 4:** Interventions that support an enduring connection to the lost child

| | |
|---|---|
| Communication modalities | • Continue using the name/gender of lost child[68,69,76,93,100]<br>• Inquire about strengths and challenges coping[108] |
| Efficacious resources | • Make bereavement counseling options available throughout care interactions[99] |
| Enduring remembrance | • Birthstone/jewelry/dedications[68,89]<br>• Suggest keepsakes (birth announcement, newspaper dedication, hospital blanket, etc.)[20,68,89] |
| Follow-up assistance | • Milestone cards/calls (birthday, key holidays, etc.)[89]<br>• Offer bereavement ceremonies in the community or hospital[86,109,110] |

## Memorializing to Develop an Enduring Connection to the Lost Child

Cultivating meaning and enduring connections to the deceased is an important aspect of adjusting to loss.[73] Those grieving reproductive losses typically find that this task is more challenging because shared memories or items of remembrance may be few or absent.[48] By providing tangible aspects of perinatal experiences, such as sonography photographs, and encouraging discussion about memorable aspects of the pregnancy or infancy, the care provider can facilitate emotional healing (Table 4). Berry and colleagues[34] (p. 6) found that memory making was important and "a majority (92%) of the parents stated they continue to celebrate their [deceased] child's birthday."

## Conclusions

Perinatal loss is a painful reality for those working in the NICU and the parents they interact with daily. Heartrending and emotional conversations when communicating various aspects of loss are vital for supporting the bereaved. The impact of what is said and done has lasting implications for parental well-being. Bereavement education and coaching can improve relational efficacy and

occupational satisfaction. Additionally, it is crucial that care providers seek debriefing and support for a resilient professional trajectory.[12] Utilizing the tasks of grieving[73] for theory-driven interventions that include evidence-based reproductive grief care, communicative modalities, and practical applications, no matter how simplistic they might seem will foster healing and improve parent provider alliances and neonatal bonding experiences.

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REVIEW ARTICLE

# Major Chromosomal Abnormalities and Necrotizing Enterocolitis: Is there a Link?

Akhil Maheshwari[1], Tamas Jilling[2]

## Abstract

Necrotizing enterocolitis (NEC) is a rare but potentially lethal disease of neonates, and several case reports have associated it with chromosomal disorders. As we know, chromosomal disorders affect approximately 0.6% of live births. Many infants with these abnormalities have no or only mild symptoms, but others can have significant morbidity and mortality. In this review, we summarize the available information about the occurrence of NEC in infants with chromosomal abnormalities. An intriguing aspect of these reports is that many infants with chromosomal abnormalities who developed NEC were near term in gestational age, and would not have otherwise been considered to be at particular risk of this disease. Existing reports have associated NEC with abnormalities of chromosomes 21 (Down syndrome), and 1, 6, 15, and 22. The main limitation of these observations is that the cohorts were not numerically adequate in a statistical sense, and hence the possibility of coincidence cannot be excluded with confidence. The impact of comorbidities or other possible confounders is also not clear. We need studies that are designed specifically, with appropriately large cohorts, to determine the frequency of comorbidities such as NEC in infants with chromosomal abnormalities.

**Keywords:** Chromosome, Chromosomal deletion, Chromosomal duplication, Chromosomal translocation, Down syndrome, Genetics, Neonate, Necrotizing enterocolitis, Robertsonian translocation, Trisomy.

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## Introduction

The global incidence of chromosomal abnormalities is estimated to be 1 per 153 live births, posing a significant challenge to the affected individuals, their families, health care systems, and society. Necrotizing enterocolitis is a severe, life-threatening intestinal disorder that is primarily known as one of the dreaded complications of premature birth, and its incidence among prematurely born neonates (GA 24–31 weeks) is overall approximately 5%, with approximately double the incidence at 24–27 weeks gestation (6.6%) as compared to 28–31 weeks gestation (2.6%).[1] This review focuses on a so-far mostly overlooked area of association between major chromosomal disorders and NEC.

### Classification and Epidemiology of Chromosomal Disorders

The two main forms of chromosomal disorders are numerical and structural. Flowchart 1 summarizes the subtypes of these disorders, which are also briefly described below.

*Numerical chromosomal disorders* can be characterized as trisomy, monosomy, or triploidy (an extra set of chromosomes from one of the parents, present in each cell). These abnormalities can involve the (a) Sex chromosomes, such as Klinefelter syndrome and Turner syndrome; or the (b) Somatic chromosomes such as trisomy 13, trisomy 18, or trisomy 21.

*Structural chromosomal disorders* refer to an altered organization of genetic material within individual chromosomes. These include:

*(a) Deletions*: A part of a chromosome is left out during DNA replication.

*(b) Duplications*: A region of DNA containing a gene is duplicated.

*(c) Translocations*: Unusual rearrangement of chromosomes. Translocations can be balanced, with an even exchange of material with no loss or gain of genetic information, or unbalanced, where the unequal exchange of chromosome material results in extra or missing genes. Unbalanced translocations are of two types: (a) Reciprocal, where the chromosome abnormality is caused by exchange of parts between non-homologous chromosomes. The detached fragments of two different chromosomes are switched and (b) Robertsonian; arms of two non-homologous chromosomes break at the centromeres and are exchanged.

*(d) Inversions*: A segment of a chromosome is reversed end to end; can be

(i) *Paracentric:* Includes the centromere; both breaks occur in one arm of the chromosome or

(ii) *Pericentric:* Includes the centromere and has a break-point in each arm.

*(e) Isochromosomes*: Unbalanced abnormality in which the two copies of either the long (q) or the short (p) arm join each other. Isochromosomes form due to simultaneous duplication and deletion of genetic material and, therefore, have partial trisomy of the genes present in the isochromosome and partial monosomy of the genes in the lost arm.

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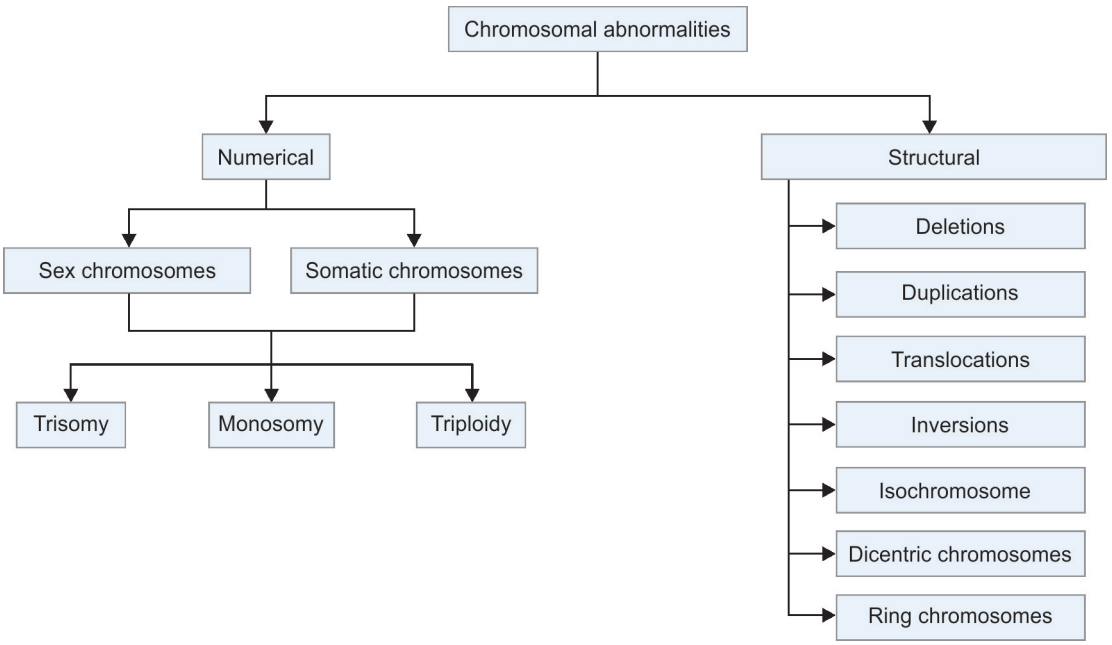
**Flowchart 1:** The types of chromosomal abnormalities we considered in this review




*(f)* *Dicentric chromosomes*: Abnormal chromosomes with two centromeres; formed due to fusion of two chromosome segments, each with a centromere. The acentric fragments lacking a centromere are lost. Dicentric chromosomes may form due to

(i) Robertsonian translocation in acrocentric chromosome pairs (that have one very short arm), namely 13, 14, 15, 21, and 22. The participating chromosomes break at their centromeres and the long arms fuse to form a single, large chromosome with a single centromere and

(ii) Paracentric inversion.

*(g)* *Ring chromosomes*: Aberrant chromosomes with ends fused together to form a ring; both ends of the chromosome are usually missing, enabling the broken ends to fuse together.

## Epidemiology of Chromosomal Disorders

The global epidemiology of chromosomal disorders is summarized in Table 1, adapted from a review by Verma and Puri.[2] The most frequent of all chromosomal disorders, which is also the most common numerical chromosomal abnormality, is trisomy 21 or Down syndrome, which occurs in 1 per 729 live births. The second most common chromosomal disorder overall, and the most common sex chromosome disorder, is the extra-numerary Y-chromosome, which affects 1 infant in 815 live births. The best known and most common extra-numerary Y-chromosome disorder is the Klinefelter syndrome, 47 XXY, which is the most frequent condition with an extra-numerary Y chromosome. Other forms include 47 XYY, 48 XXXY, and 48 XYYY (Table 1).

The most frequent structural chromosomal disorders are balanced chromosomal translocations (1 per 500 live births), which result in no gain or loss of overall genetic material, but show parts of genetic material joined with each other (Robertsonian translocation) or swapping of places within or between chromosomes. Balanced chromosomal translocations commonly have no overt phenotype but may sometimes result in infertility. If fertility remains intact, the offspring may be predisposed to chromosomal disorders such as unbalanced chromosomal translocations. Some of the balanced translocations, particularly when a chromosome breakage point interrupts a gene, may result in a clinically apparent phenotypic change. Unbalanced chromosomal structural abnormalities occur less frequently than balanced translocations (1 per 4500 live births), and they result in the gain or loss of genetic material. One subtype of these abnormalities are the unbalanced translocations, where an extra part of a chromosome is lost or added on another chromosome. Such abnormalities may include the so-called partial trisomies or monosomies, and these are almost always symptomatic.

**Table 1:** Chromosomal abnormalities in liveborn babies ($n$ = 120, 290; 70,115 males and 50,175 females) (Adapted from Verma and Puri, 2015)

| | *Rate* | |
|---|---|---|
| *Abnormality* | *Per 1000* | *1 in* |
| 47 XYY | | |
| 47 XXY, or 48 XXXY, or 48 XYY | 1.23 | 815 |
| 45 X | 0.14 | 7,168 |
| 47 XXX | 0.94 | 1,068 |
| +D | 0.06 | 24.058 |
| +E | 0.21 | 4,812 |
| +G | 1.37 | 729 |
| Balanced Robertsonian translocations | 0.91 | 1,098 |
| Translocations, insertions | 0.97 | 1,028 |
| Unbalanced structural abnormalities | 0.22 | 4,545 |
| Marker chromosomes | 0.41 | 2,455 |
| Total | 6.52 | 153 |

+D = Trisomy 13, +E = Trisomy 18, +G = Trisomy 21

### *Germline vs Somatic Origin of Chromosomal Abnormality*

If a chromosomal defect originates prior to conception during oogenesis or spermatogenesis, it results in germline chromosomal defects that can be seen in all the cells of the neonate, and are likely to be inherited by his/her offsprings. In contrast, chromosomal defects that occur later in one or more of the downstream somatic

cells are likely to affect only the cells downstream from these progenitor(s). Such defects cause mosaicism, where the neonate will have chromosomal defects in some, not all, cells.

## Chromosomal Abnormalities Recorded in Infants with Necrotizing Enterocolitis

The etiology of NEC is considered to be multifactorial, including both host and environmental influences. Key environmental factors include feeding modalities, antibiotic use, transfusions, use of steroids and non-steroidal anti-inflammatory drugs, and perinatal infections. The most important host factor determining the susceptibility to NEC is prematurity, as NEC is almost non-existent in full term neonates and its incidence can be >10% in extremely low birth weight [(ELBW); <1000 gm birth weight] neonates. Additional host factors contributing to predisposition to NEC include various comorbidities, the microbiomes of both the mother and the neonate, and genetics. Of the contributing genetic factors, the association of mutations or single-nucleotide polymorphisms (SNPs) in individual genes with the incidence of NEC have been most investigated; reviewed by Cuna et al.[3] Additionally, association between NEC with genome-wide SNPs and with organ-specific and gene-specific epigenetic changes have been analyzed, which have been summarized in other reviews in this issue.[4–6]

## Numerical Chromosomal Disorders and NEC

*Trisomy 21; Down syndrome*: A large cohort study from the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network (NRN) analyzed morbidity and mortality in neonates with trisomy 13 (T13; $n = 36$), trisomy 18 (T18; $n = 125$), and trisomy 21 (T21; $n = 139$) and compared them to 49,600 neonates without birth defects.[7] Intriguingly, there were 20 (16%) cases of NEC in the T21 cohort, but there were none in the T13 and T18 cohorts among neonates that survived ≥12 h after birth. These differences could be potentially explained by the significantly higher percentage of neonates with gestational ages <26 weeks in the T21 group ($n = 22$, 16%) than in the T18 group ($n = 3$, 3%); T13 was not significantly different ($n = 2$, 6%). However, for instance, the incidence of BPD was not different between T21 ($n = 38$, 39%), T18 ($n = 8$, 62%), and T13 ($n = 3$; 60%) among infants who survived >36 weeks postmenstrual age, and the incidence of respiratory distress syndrome was lower in the T21 ($n = 88$, 67%) than in the T18 ($n = 71$, 82%) and was not significantly different from T13 ($n = 14$; 82%). Furthermore, in a study of 29 term (>37 weeks gestational age) neonates with NEC, there were two cases (6.9%) of T21,[8] and in another full-term NEC study of 39 neonates, there were another two cases (5.1%) of T21,[9] which is significantly higher percentage than the incidence of T21 overall in the general population (13.5/10,000 births).[10] These findings suggest that there may be an association of NEC with Down syndrome that may warrant further exploration.

*Turner syndrome*: The same study that identified two full-term neonates with T21 also found that one of the full-term neonates with NEC had Turner syndrome (2.5%).[9] In another study of 14 neonates with full-term NEC, there was one patient with Turner syndrome (7.1%). Since the incidence of Turner syndrome is 1/2500 female births, their representation in the cohorts can be considered very high. Since full-term NEC is commonly associated with congenital heart defects and Turner syndrome is associated with congenital heart disease in 23–50% of cases,[11] it is plausible that the association is indirect due to cardiovascular causes.

## Relationship between Structural Chromosomal Disorders and NEC

*Chromosome 1 (Chr1)*: A study of identical twins with identical deletions in chromosome 1 (1p32.3-p22.2: 57652246_89311711) identified a number of pathologies in both twins, including NEC that required surgery and resulted in the loss of the majority of small bowel in one of the twins.[12] Necrotizing enterocolitis in both twins was unique, as compared to other described cases of Chr1 deletions.[13–15] The various described Chr1 deletions in the four publications affected different regions of Chr1, suggesting that the particular region deleted in the twins with NEC may harbor genes whose absence may increase susceptibility to NEC. The region deleted in the twins with NEC contains 181 known genes, which were not listed in the publication, but we are providing here. Among the 181 genes, there are several that could be obvious candidates for NEC susceptibility due to their roles in inflammation, cellular differentiation, and cell death pathways, such as jun proto-oncogene (JUN), autophagy-related 4C, cysteine peptidase (ATG4C), forkhead box D3 (FOXD3), and integrin beta 3 binding protein; beta3-endonexin (ITGB3BP). While the reported cases are relatively few, the data are intriguing enough that a follow-up analysis on larger cohorts of chromosome 1 defects appears to be justified.

*Chromosome 6*: NEC-like intestinal necrosis had been described in two infants and in one neonate that was full-term or near full-term born by Esdal et al.[16] All three cases shared a deletion on chromosome 6 in the region 6q25.3 to q26. One of the infants had a deletion on chromosome 6, while one of the infants and the neonate were siblings and had identical unbalanced chromosome 6 to chromosome 18 translocations resulting in the loss of genetic material from chromosome 6. All three patients had severe bowel necrosis with pneumatosis intestinalis and bloody stools that required surgery. The authors noted that there were four other patients reported in the literature with similar chromosome 6 deletions, but without reported intestinal complications.[17]

The deleted chromosomal region of chromosome 6 in the three patients that had bowel necrosis contained a large number of genes (Table 2). Esdal et al. singled out ezrin (EZR), as a potential important gene among the ones that are deleted in these patients, due to its localization in intestinal epithelial cells and due to its significance in intestinal development in murine models.[18,19] Additionally, there are other genes deleted with this portion of chromosome 6, such as superoxide dismutase 2 (SOD2) and NADPH oxidase 3 (NOX3), which are critical antioxidant enzymes

**Table 2:** Genes within the shared deleted region arr [GRCh37] 6q25.3q26(155699183_163554531) × 1 (Adapted from Esdal et al. 2018; Table 1)

| *Gene symbol* |
|---|
| NOX3, tRNA-Pseudo, LOC105378068, MIR1202, SNORD28B, ARID1B, M1R4466, TMEM242, ZDHHC14, MIR3692, AK092386, SNX9, SYNJ2, AK026758, SYNJ2-IT1, SERAC1, GTF2H5, TULP4, SNORA116, MIR7161, TMEM181, DQ586009, DYNLT1, SNORA116, SYTL3, MIR3918, EZR, AX747826, EZR-AS1, OSTCP1, C6orf9, RSPH3, TAGAP, LOC101929122, FNDC1, AK130765, MIR_633, LINC02529, BC016015, SOD2, WTAP, LOC100129519, SOC20T1, AC AT2, TCP1, SNORA20, SNORA29, MRPL18, PNLDC1, MAS1, IGF2R, AIRN, LOC729603, SLC22A1, SLC22A2, SLC22A3, LPAL2, LPA, PLG, MAP3K4, AGPAT4, AGPAT4-IT1, PRKN (PARK2), LOC105378098, PACRG, PACRG-AS2, AK058177 |

*Sources:* RefSeq and UCSC Genome Browser (ucsc.genome browser; hg19), order approximate

and Mitogen-Activated Protein Kinase Kinase Kinase 4 (MAP3K4), which is a central component of the MAPK signaling pathway that may be implicated as well.

It is similar to several other chromosomal abnormalities discussed in this article that bowel necrosis occurred in full-term or near full-term infants and a neonate, which may be viewed as a potentially strong indication that the affected region of chromosome 6 in these infants harbors genes that are relevant to NEC pathogenesis. However, two of the patients had significant cardiac abnormalities, and one of them had intestinal malrotation that had to be surgically repaired, offering the potential explanation that the bowel necrosis was caused indirectly by the chromosomal abnormality by causing conditions that may have served as underlying causes for bowel necrosis.

*Chromosome 15 and Chromosome 22*: There were incidental case reports of NEC being observed in neonates with 15q26.1 deletion and with 22q11 deletion syndrome.[20,21] 22q11 deletion syndrome is one of the more common chromosomal abnormalities with an incidence of ~1:4000 live births. The affected neonate was born at 40 weeks gestation and developed NEC on postnatal day 3. Although there are no other reports focusing on 22q11 deletion syndrome and NEC, it is notable that cases of NEC are sporadic in published cohorts of 22q11 deletion syndrome, which mostly consist of full-term neonates. 22q11 deletion is the most common cause of DiGeorge syndrome. In a cohort of 467 neonates with partial DiGeorge syndrome, there were 460 with 22q11 deletion and 3 cases of NEC.[22] It is unknown what the gestational ages of these neonates were at birth and at what age they were diagnosed with NEC. Deletions of the terminal region of chromosome 15 are rare and have been suggested to be potentially predisposing to congenital diaphragmatic hernia.[23,24] Beyond the single case report of NEC association with 15q26.1 deletion, we could not find additional evidence for a stronger association of chromosome 15 deletions and NEC. Therefore, the associations with 15q26.1 deletion and 22q11 deletion syndromes in these cases could well be incidental.

## Conclusions

The most convincing data regarding association of chromosomal disorders with NEC is available for trisomy 21. This may be due to the fact that a very large cohort of T21 cases was studied, along with cohorts of T13 and T18, allowing a robust analysis. The other chromosomal disorders mentioned have not been studied at the same scale, and based on the sporadic publications, the evidence is intriguing but far from being conclusive. It will be worthwhile to systemically analyze the potential association of NEC with all common chromosomal disorders as such data may provide useful prognostic value.

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REVIEW ARTICLE

# Neurological Abnormalities in Infants of Mothers with Diabetes Mellitus

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## Abstract

Fetal anomalies, neurocognitive disorders, and perinatal mortality rates are higher in infants of diabetic mothers (IDMs) than in infants of mothers without diabetes. The pathology of these defects is significantly influenced by maternal glucose control and the onset of diabetes during pregnancy. Maternal hyperglycemia, abnormal inflammatory response, and fetal oxidative stress contribute to the pathogenesis of neurological deficits in IDMs. Pregestational diabetes mellitus (PGDM) have a higher incidence of congenital neurologic structural anomalies than gestational diabetes mellitus (GDM). The assessment of neurodevelopmental impairment in IDMs is confounded by perinatal factors, including birth asphyxia, acute and chronic metabolic insults, and iron deficiency. The incidence of these defects tends to reduce with appropriate antenatal care and maternal glycemic control. We discuss the structural neurologic malformations, cognitive disorders, motor deficits, and psychosocial disorders in the offspring of diabetic mothers.

**Keywords:** Anencephaly, Attention-deficit hyperactivity disorder, Autism spectrum disorder, Caudal regression syndrome, Cognitive impairment, Encephalocele, Fetal pathology, Infants of diabetic mothers, Myelomeningocele, Neural tube defect, Neurodevelopmental delay, Neurodevelopmental impairment, Oxidative stress, Schizophrenia.

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## Key Points

- IDMs are at an increased risk for central nervous system structural defects and long-term neurodevelopmental impairments.
- The pathogenesis of neurodevelopmental delay could be explained by immunological and inflammatory mechanisms.
- Maternal hyperglycemia and fetal oxidative stress are the major contributors to the neurodevelopmental impairment.
- Neural tube defects (NTDs) and caudal regression syndrome are the most common congenital neurological anomalies in IDMs.
- These anomalies may be detected prenatally and are more common in infants of mothers with PGDM.
- Psychosocial disorders including autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), and schizophrenia are more common in IDMs than in infants of mothers without diabetes.

## Introduction

The World Health Organization (WHO) defines diabetes mellitus (DM) as "a chronic disease that appears when the pancreas does not produce enough insulin or when the body does not effectively use the insulin it produces." There are two types of diabetes in pregnant mothers, depending on the onset time: PGDM, onset before pregnancy; GDM, onset between 24 and 28 weeks of pregnancy. Several studies have demonstrated that fetal anomalies, neurobehavioral abnormalities, and perinatal mortality rates are higher in IDMs than infants of mothers without diabetes.[1,2] Maternal glycemic control during pregnancy has been shown to affect the pathogenesis of these morbidities.[3,4]

Timely diagnosis and adequate control of maternal diabetes during pregnancy could decrease the rates of cognitive and intellectual impairment, motor disorders, and psychosocial disorders.[5] Yamamoto et al., in their meta-analysis, demonstrated that there is adequate evidence to indicate that maternal PGDM leads to adverse cognitive and neurobehavioral outcomes in children.[6] Ornoy et al. showed that school-age children born to mothers with DM during pregnancy had some degree of impairment in their motor function and attention span, which was worse in the case of poor maternal glycemic control.[7,8] The neurological sequelae in terms of cognition, motor function, and psychosocial development have been discussed in separate sections later. In this review, we cover the effects of maternal DM on the neurological development of the offspring. We aim to discuss the short-term and long-term neurodevelopmental impairments in IDMs.

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## Pathogenesis

The pathogenesis of the fetal morbidities in IDMs has been described scrupulously in several animal and human models. Inflammation and oxidative stress have significant implications for placental function and fetal well-being in GDM and PGDM.[9] In a normal pregnancy, the T-cells have a predominant anti-inflammatory response favoring maternal-fetal immune interactions leading to normal fetal outcomes.[10,11] However, conditions such as GDM are associated with an inflammatory response and disproportionate cytokine production that can lead to adverse maternal and fetal outcomes.[12] In maternal DM, there is an increased production of reactive oxygen species and free radicals, which results in mitochondrial dysfunction.[13,14] The decrease in cellular energy production due to mitochondrial dysfunction and oxidative stress could affect neurotrophin levels and impair fetal neurological development.[9]

Neurotrophins are peptides that influence neuronal growth and differentiation during fetal growth.[15] Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophin, neurotrophin-3, and neurotrophin-4/5, regulate the growth and development of the fetal brain and nervous system.[15] These peptides also safeguard the developing neurons by preventing apoptosis and promoting angiogenesis.[15] The normal development of the nervous system depends on a positive feedback loop between antioxidant processes and neurotrophins.[16] In maternal DM, increased oxidative stress decreases the levels of neurotrophins, thereby disturbing the positive feedback loop.[16]

Brain-derived neurotrophic factor (BDNF) and NGF are the most investigated neurotrophins. Animal models have demonstrated that BDNF plays a role in neuronal development and differentiation in the cerebral cortex and hippocampus.[17] In addition, it may also protect fetal neurons by reducing apoptosis and promoting angiogenesis during hypoxic conditions.[17] Similarly, NGF contributes to neuroplasticity during fetal brain development and influences neuronal survival.[18] Briana et al. reported that the levels of BDNF and NGF were low in the cord blood of neonates born to mothers with GDM.[19] Su et al. showed impairment in language development and low BDNF levels at 12 months of age in IDMs, indicating a correlation between BDNF levels and cognitive development.[20] These alterations in neurotrophins in mothers with GDM and PGDM could lead to adverse neurodevelopmental and neurobehavioral outcomes in their offspring.

## Congenital Neurologic Anomalies

Infants of diabetic mothers have an increased incidence of congenital anomalies of the central nervous system, including microcephaly, cysts, hydrocephalus, NTDs, and caudal regression syndrome, compared to the general population. The incidence of congenital anomalies is not increased in the offspring of mothers with GDM. These anomalies are probably induced by chronic hyperglycemia, causing the build-up of advanced glycosylation end-products (AGE) and oxidative stress in early gestation.[21] The risk of diabetic embryopathy rises if maternal glucose control, as estimated by glycosylated hemoglobin levels, is dysregulated in early gestation.[22–24]

Increased oxidative and nitrosative stress in mothers with PGDM plays a role in diabetic embryopathy and abnormal placentation.[25] The mechanism of this embryopathy is poorly understood; few animal studies showed that high oxidative stress in the embryo decreases cell proliferation and increases cell apoptosis.[26] In addition, Salbaum et al.[27] reported that maternal diabetes' teratogenic effect is because it disturbs gene regulation and modifies the epigenome in the embryo. The effects of specific genetic variants associated with maternal glucose metabolism and NTD-related genes such as FT0, TCF7L2, and LEP may underline the molecular pathology of NTDs.[28]

### Neural Tube Defects

The common anomalies of the central nervous system are due to the failure of neural tube closure, including anencephaly (Fig. 1), encephalocele (Fig. 2), and meningomyelocele (Fig. 3). Tinker et al. showed that, compared to offspring of non-diabetic mothers, the risk for holoprosencephaly increases around 13-fold; hydrocephalus, 8-fold; encephalocele, around 5-fold; and anencephaly, 3.5-fold in offspring of PGDM mothers.[29] An extensive study of over 29 million mother-infant pairs in the United States demonstrated that even mothers with GDM had a higher risk of congenital malformations of the neonate, albeit less than PGDM.[30] The likelihood of spina bifida was significantly around 2-fold in PGDM and around 1.25-fold in GDM.[30] A Texas population-based study reported that the rate of NTDs such as anencephaly, spina bifida, and holoprosencephaly (Fig. 4) in IDMs was higher if maternal obesity was present.[31]

### Caudal Regression Syndrome

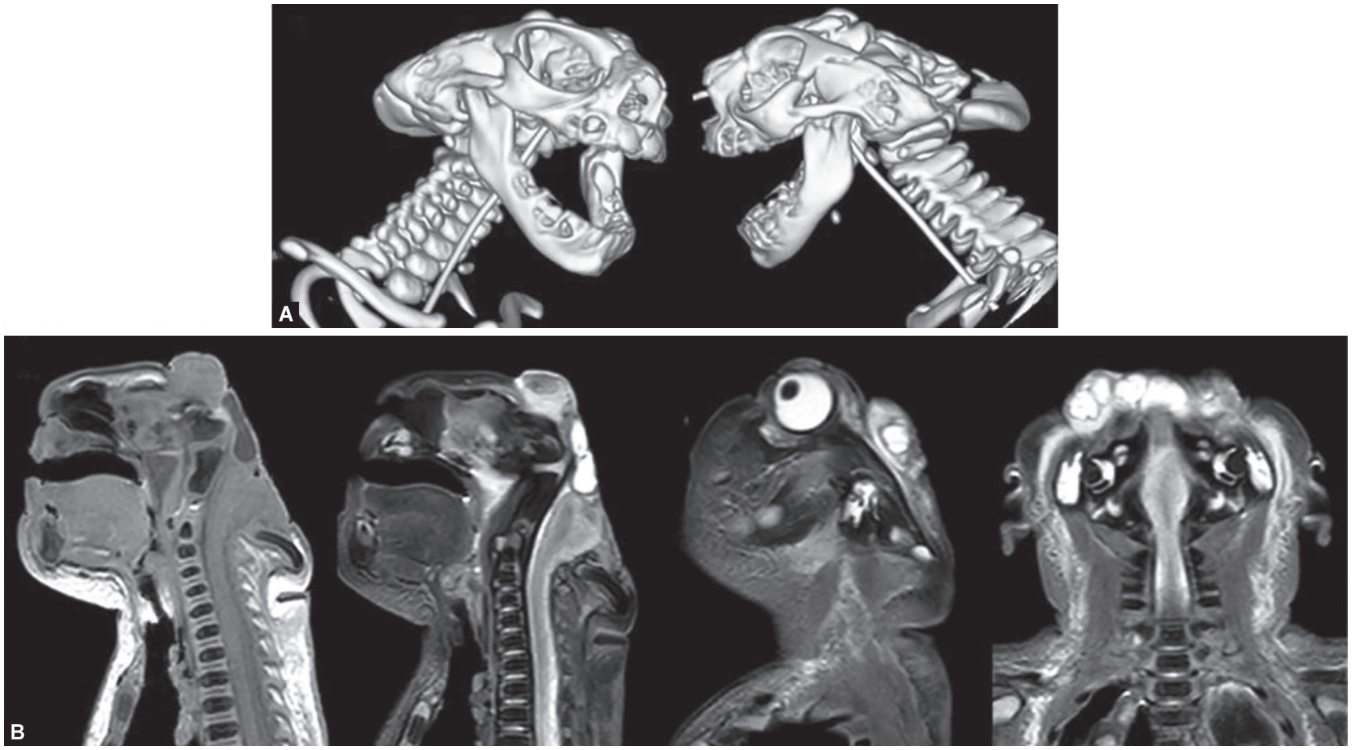
Caudal regression syndrome (Fig. 5) includes a group of anomalies of the lower spine, lower limbs, and genitourinary system, and it is strongly associated with maternal diabetes. The Welch and Alterman classification of congenital sacral malformations includes four subgroups (Table 1). Some reports suggest that this syndrome might be seen more than 200 times more frequently in IDMs than in infants of non-diabetic mothers.[32] Garne et al.[33] studied 18 population-based EUROCAT registries of congenital anomalies from 1990 to 2005 and found that the odds ratio for caudal regression syndrome was 26.4 (95% CI = 8.98–77.64) in IDMs.

Lower spine malformations can lead to spinal cord abnormalities, thereby predisposing to neurological lower limb defects, abnormal bladder and bowel control, and flexion contractures of the knee and hip.[34] In type I caudal regression syndrome, the spinal cord ends abruptly and bluntly, deforming the conus medullaris and cauda equina. Some case reports have shown that in IDMs, caudal regression syndrome can occur with additional malformations such as spinal-pelvic instability, hip dislocation, popliteal webbing, and extrahepatic biliary atresia.[35–37] Severe caudal regression syndrome can be associated with multisystem anomalies, including VACTERL complex (vertebral, anorectal, cardiac, renal, and limb defects).[38]

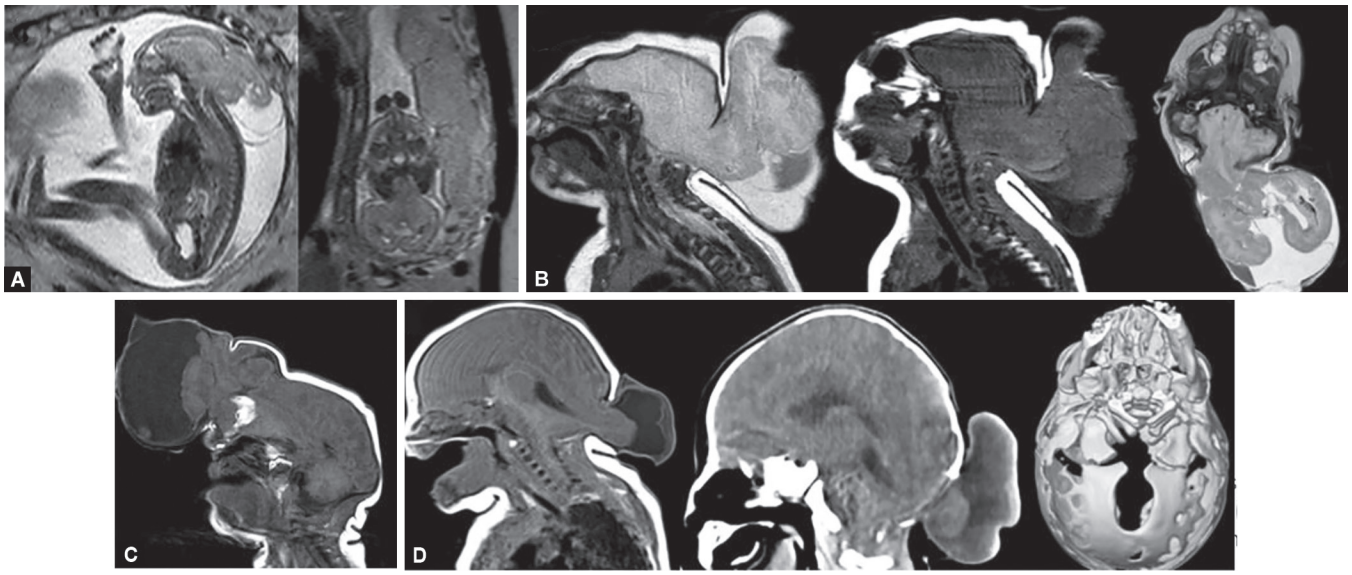
## Neurological Sequelae

### Cognitive Disorders

Cognition is evaluated in terms of intelligence, memory, and attention. These functions are essential for communication, learning, motor coordination, and problem-solving and are controlled by sophisticated coordination among the cerebral cortex, basal ganglia, amygdala, and hippocampus. Several animal models have demonstrated that maternal hyperglycemia adversely affects neurological development and synaptic plasticity in the hippocampus, as this process is regulated by receptors to insulin



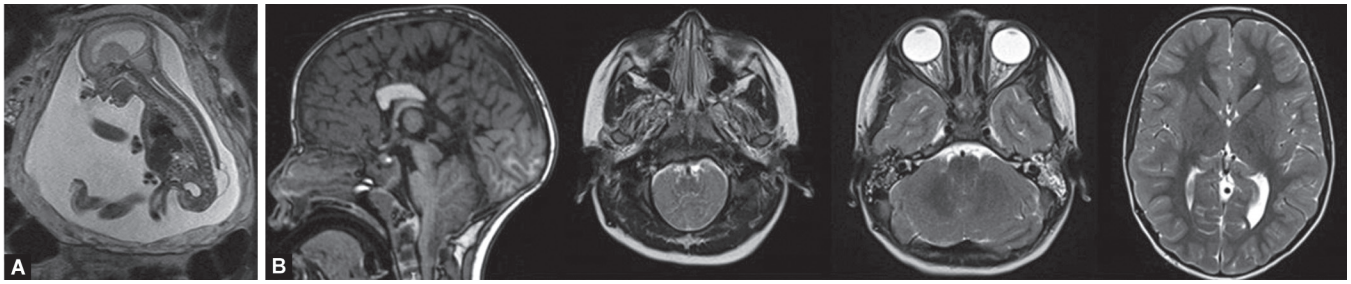

**Figs 1A and B:** Classic severe anencephaly with no skull and severe microcephaly, as seen on (A) Three-dimensional (3D) computed tomography (CT); (B) Matching T1- and T2-weighted magnetic resonance imaging (MRI). A small hypoplastic brain stem and cerebellum are visible, the entire supratentorial brain is lacking with some degenerative T2-hyperintense cysts



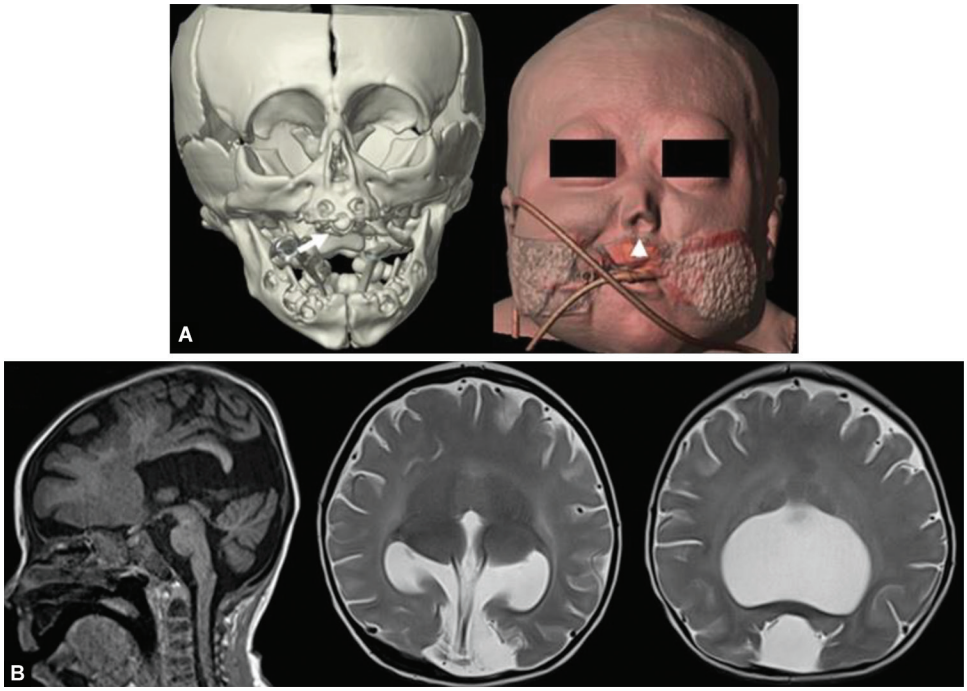

**Figs 2A to D:** Encephaloceles. (A) Sagittal and axial T2-weighted fetal MRI and matching; (B) Postnatal sagittal T2/T1-weighted and axial T2-weighted MRI show a large occipital encephalocele with herniation of large parts of the occipital and parietal lobes including part of the lateral ventricles; (C) Fronto-ethmoidal encephalocele. The T1-hyperintense structure in the brain corresponds to a lipoma and (D) Sagittal T1-weighted brain MRI and matching sagittal CT and 3D skull of a newborn child with a large occipital encephalocele with herniation of the brain and meninges through a focal skull defect which communicates with the foramen magnum

and insulin-like growth factors.[39–41] This hippocampal defect could lead to cognitive and behavioral abnormalities.

A study of school-age children demonstrated that those born to mothers with GDM performed poorly in verbal tasks and motor coordination compared to their peers; however, they did not have reduced cognitive ability.[7,8,42] In another study of school-age children, the GDM group showed mild cognitive impairment and lower scores for general intelligence and working memory.[43] In addition,




**Figs 3A and B:** (A) Fetal T2-weighted MRI with non-skin-covered cystic appearing lumbo-sacral meningomyelocele including an associated Arnold Chiari type-II malformation with a small posterior fossa and herniation of cerebellar tissue into the upper cervical spinal canal and supratentorial hydrocephalus; (B) Sagittal T1-weighted and axial T2-weighted postnatal MRI in a patient with Arnold Chiari type-II secondary to an open meningomyelocele with severe crowding of the posterior fossa. The infants received a ventriculoperitoneal shunt; decompressed supratentorial ventricles and malformed dysplastic corpus callosum are noted



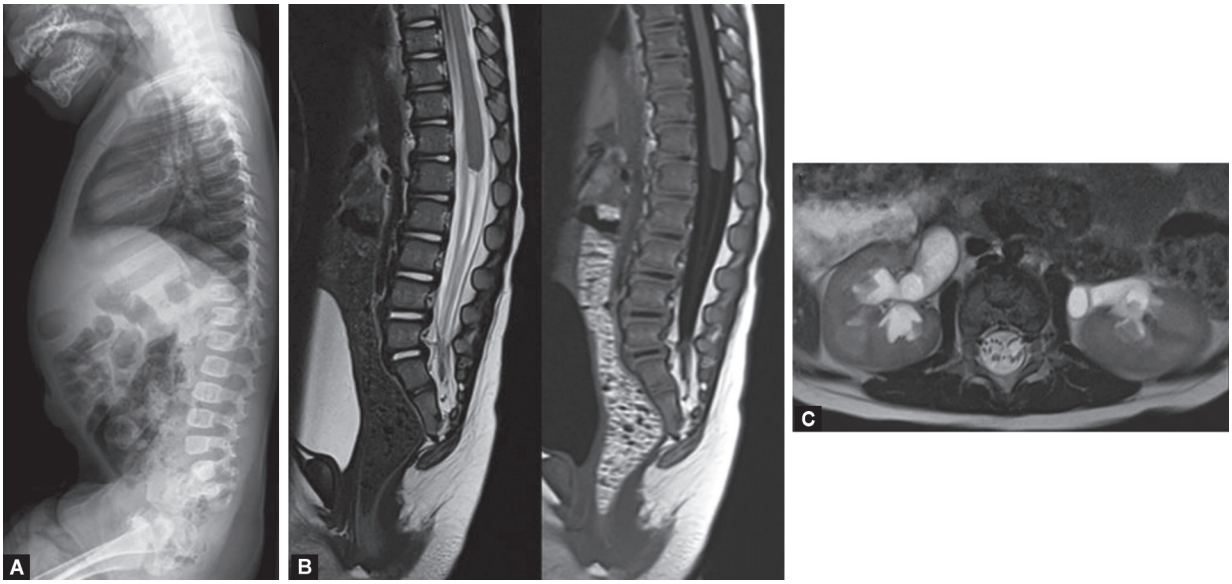

**Figs 4A and B:** Holoprosencephaly. (A) 3D CT bone and soft tissue reconstruction of a neonate with near-complete alobar holoprosencephaly with matching characteristic facial features including a single central incisor, hypotelorism, and proboscis; (B) Sagittal T1-weighted and axial T2-weighted MRI of the same child show the classic non-dived or fused cerebral hemispheres and large monoventricle

18–27-year-old adults born to women with GDM had lower cognitive test scores than their counterparts, and the scores were negatively correlated with maternal blood glucose levels.[44] These observational studies suggest a causal influence of maternal hyperglycemia and insulin resistance on cognitive impairment in the offspring.

## Motor Disorders

Abnormal maternal glucose metabolism is associated with impaired intellectual and psychomotor development in offspring during pregnancy.[45] There was impairment of fine motor and gross motor control in school-age children of mothers with GDM.[8] Experimental studies have shown a decrease in cerebellar size due to reduced density of Purkinje and granular cells in the cerebellar cortex in gestational diabetes rat offspring models.[46,47] These findings suggest possible defects in cerebellar development in offspring of GDM mothers.

Ratzon et al. showed that offspring of diabetic mothers have poorer control in fine and gross motor functions when compared to children of mothers without DM.[48] In addition, the motor control in children of diabetic mothers was negatively correlated to the severity of hyperglycemia assessed by glycosylated hemoglobin levels and acetonuria.[48] A prospective cohort study in Iran reported that the risk of motor developmental delay in IDMs was 1.49 (95% CI = 0.98–1.87).[49] A recent meta-analysis showed that children born to mothers with DM had delayed motor development when compared to children born to mothers without diabetes, and this impairment in motor development was worse in PGDM than GDM.[50]




**Figs 5A to C:** Lateral views in (A) Conventional X-ray; (B) Sagittal T1- and T2-weighted MRI images show classic caudal regression syndrome with blunt ending of the distal spinal cord, and absence of the conus medullaris and terminal ventricle with matching lack of the osseous spinal column and sacrum below $3 (deficient secondary neurulation) and (C) Axial T2-weighted MRI images show notable colonic constriction and functional urinary tract obstruction

**Table 1:** The Welch and Alterman classification of congenital sacral malformations

| *Type* | *Description* |
|---|---|
| I | A non-familial type associated with maternal DM showing complete absence of the sacrum and lower vertebrae with congenital anomalies |
| II | Agenesis of the distal sacral or coccygeal segments |
| III | Hemisacral dysgenesis with presacral teratoma |
| IV | Hemisacral dysgenesis with anterior meningocele |

**Table 2:** Summary of neurologic anomalies and defects in IDMs

| *Category* | *Abnormalities* | *References* |
|---|---|---|
| Congenital anomalies | Neural tube defects—anencephaly, encephalocele, and spina bifida | 25,26 |
| Cognitive impairment | Deficits in intelligence, memory, and verbal tasks | 30–32 |
| Motor disorders | Delayed motor development, poor gross and fine motor control | 36–38 |
| Psychosocial disorders | Autism spectrum disorder, attention-deficit hyperactivity disorder, and schizophrenia | 46–52 |

## Psychosocial Disorders

Maternal DM has been linked to multiple psychosocial disorders in the offspring, including ASD, ADHD, and schizophrenia (Table 2). As stated above, intrauterine hyperglycemia could reduce the size of the fetal cerebellum by reducing the density of Purkinje and granular cells.[46,47] Autism spectrum disorder has also been associated with developmental cerebellar abnormalities, including cerebellar hypoplasia and a decrease in size.[51–53] The increased production of free radicals and oxidative stress, which are present in the cases of PGDM and GDM, have been shown to be associated with ASD and schizophrenia.[54–57]

A large prospective cohort study of 66,445 pregnancies reported a significantly higher risk of ASD in association with GDM (odds ratio = 1.76, 95% CI = 1.34–2.32, $p$ <0.05).[58] However, other studies have contested these findings and rendered this association as controversial.[59,60] Similarly, the link between maternal DM and schizophrenia has also been a matter of debate.

In a case-control study, Hultman et al.[61] noted increased risk of schizophrenia in offspring of mothers with GDM or PGDM but the association did not reach statistical significance. A meta-analysis showed a strong association between diabetes in pregnancy and schizophrenia; however, this was based on only two studies.[62] A Swedish population-based study of 15,615 children born to mothers with type 1 DM showed a significantly higher risk of ADHD (hazard ratio = 1.35, 95% CI = 1.18–1.55).[63] Xiang et al. demonstrated that the risk of ADHD was higher in PGDM and if the GDM required management with antidiabetic medications (Table 2).[64]

## Conclusion

Our review shows that the pathogenesis of congenital malformations and neurodevelopmental impairment in offspring of mothers with DM is complex and poorly understood. The assessment of neurodevelopmental impairment in IDMs is confounded by perinatal factors, including birth asphyxia, acute and chronic metabolic insults, and iron deficiency. The incidence of these defects tends to reduce with appropriate antenatal care and maternal glycemic control.

There are multiple factors, including oxidative stress and inflammation and molecular mechanisms involving neurotrophins interfering with the development of the nervous system. Global research initiatives are needed to elucidate these mechanisms. The review also highlighted that PGDM and GDM are associated with an increased risk of multiple neurobehavioral disorders, including cognitive impairment, motor developmental delay, ASD, schizophrenia, and ADHD. Attention-deficit hyperactivity disorder has the most substantial evidence to support its association with maternal DM among psychiatric disorders.

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REVIEW ARTICLE

# Approach to Neonatal Alloimmune Thrombocytopenia: The Perspective from a Transfusion Medicine Service

Greeshma Sharma[1], Ratti Ram Sharma[2], Akhil Maheshwari[3]

## Abstract

Neonatal alloimmune thrombocytopenia (NAIT) is an important hematological disorder in neonates. The pregnant mother's immune system gets sensitized to antigens expressed on fetal platelets that have been inherited from the father and begins producing specific alloantibodies against these antigens. Some of these antibodies get transported across the placenta into the baby and can damage/destroy platelets to cause fetal/neonatal thrombocytopenia. Many of these fetuses/infants develop major clinical complications such as intracranial hemorrhages. In this article, we describe normal platelet counts in neonates, the pathogenesis and epidemiology of NAIT, specific platelet antigens that have been identified as targets in NAIT, and the approach for laboratory diagnosis of NAIT. From the perspective of a transfusion medicine service, there are two targets as follows: (a) To identify the differences in the antigenic profiles of the platelets of the mother and her fetus/infant and (b) To detect alloantibodies in the maternal serum that may be specifically reactive to these platelet antigens. Early identification of NAIT can help timely institution of appropriate treatment. In this project, we reviewed the laboratory profiles of infants who were diagnosed to have NAIT at our own institution and also mined the literature in the databases EMBASE, PubMed, and Scopus.

**Keywords:** Alloantibdies, Alloantigens, Antigens capture elisa glycoproteins, Newborn, Platelet genotyping, Platelet specific antigens.

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## Key Points

a. In fetal/NAIT, the mother forms antibodies against paternal antigens expressed on the surface of platelets of her fetus/infant. These antibodies cross the placenta and damage the fetal/neonatal platelets.
b. Neonatal alloimmune thrombocytopenia (NAIT) is a major cause of severe, isolated thrombocytopenia in term neonates. The incidence may be as high as 1 in 1,000 live births.
c. Although the term NAIT emphasizes the disease manifestations after birth, the condition can commence *in utero* with serious consequences including intrauterine death or intracerebral hemorrhage during the 20–24 weeks' period of pregnancy.
d. Nearly in 85% of all Caucasian mothers develop some alloimmunization against HPA-1a.
e. We have limited information on the immunogenicity of various platelet antigens in terms of the alloantibody production, the efficacy of various antibodies in terms of transplacental transfer, and the impact of different alloantibodies on platelet function or on the incidence of bleeding complications. Our population data on the distribution of different platelet antigens in various ethnic groups is also limited. Consequently, the development of screening programs for NAIT has been difficult.

## Introduction

Thrombocytopenia is a frequently seen hematological abnormality in neonates.[1,2] Platelet counts reach levels of around $150 \times 10^9$/L by the late second trimester in fetuses and then plateau at these levels until term gestation.[3] Platelets counts between 100–150 $\times$ $10^9$/L have been defined as mild thrombocytopenia, 50–100 $\times$ $10^9$/L as moderate, and counts <50 $\times$ $10^9$/L as severe thrombocytopenia.[4] Mild thrombocytopenia may be seen in up to 25–30% of term infants and is usually self-limiting and of short duration. Moderate/severe thrombocytopenia occurs less frequently and is seen in 5–10% infants.[5–8]

Neonatal thrombocytopenia with platelet counts less than 30–50 $\times$ $10^6$/L has been associated with an increased risk of serious hemorrhages into vital organs.[9,10] There are important associations with intrauterine infections, low Apgar scores, sepsis, and an overall higher acuity of illness even when the etiology is unclear. In premature infants, thrombocytopenia is a stronger predictor of intracranial hemorrhage (ICH) than their birth weight or gestational age.[11]

In this review, we summarized the current definitions of neonatal thrombocytopenia and then focused on NAIT. It is noted that NAIT is an important cause of severe thrombocytopenia in neonates; we present the current evidence on its pathogenesis, clinical manifestations, evaluation, treatment, outcomes, and the

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future directions. This article combines peer-reviewed evidence from our own studies with an extensive literature search in the databases PubMed, EMBASE, and Scopus.

## Normal Platelet Counts in Neonates

Existing studies show that 98% of term neonates have platelet counts at or above $150 \times 10^9$/L, and thrombocytopenia is usually defined as a number of circulating platelets below these levels. Some extremely premature infants born at 22–24 weeks' gestation may have lower platelet counts at less than $100 \times 10^9$/L in the first few days after birth, and most of them are asymptomatic.[3] The timing of presentation of neonatal thrombocytopenia can also be used in diagnostic evaluation. Early-onset thrombocytopenia is noted within the first 72 hours after birth, and it may be caused by intrauterine infections, immune-mediated causes, perinatal asphyxia, and infections. Late-onset thrombocytopenia may be related to more diverse causes including bacterial and viral infections, systemic inflammation, hepatitis, necrotizing enterocolitis, and sometimes, may be iatrogenic due to thrombi in central lines or may develop as adverse drugs of certain drugs.[12–26] Genetic disorders with bone marrow dysfunction are less frequent, but can appear at any age.[27–29]

Baer et al.[23] examined 11281 NICU admissions and identified severe thrombocytopenia in 273 (2.4%). Nearly 30% presented in the first 3 days after birth. Half presented by day 10, 75% by day 27, and 95% by day 100. The prevalence was inversely related to birth weight. Cutaneous bleeding was more common in patients with platelet counts of less than $20 \times 10^9$/L; however, there was no statistically significant correlation between platelet counts and pulmonary, gastrointestinal, or intraventricular bleeding. The most common explanations for severe thrombocytopenia were acquired varieties of consumptive thrombocytopenia. Platelet transfusions (median, 5; range, 0–76) were administered to 86% of the patients. No deaths were ascribed to exsanguination. The mortality rates did not correlate with the lowest platelet counts but were proportionate to the number of platelet transfusions.

Wiedmeier et al.[24] examined platelet counts in neonates between the first and the ninetieth day after birth, from 47, 291 neonates delivered at 22–42 weeks gestation. The platelet counts obtained in the first 3 days of life, increased over the range of 22–42 weeks gestation. In those born in less than or 32 weeks gestation, the lower reference range (fifth percentile) was less than $104 \times 10^9$/L, but it was less than $123 \times 10^9$/L in late-preterm and late-term neonates. Advancing postnatal age affected platelet counts; during the first 9 weeks, the counts showed a sinusoidal pattern with two peaks; one at 2–3 weeks and a second at 6–7 weeks. The upper limit of expected counts (95th percentile) during these peaks were as high as less than $750 \times 10^9$/L.

Christensen et al.[25] examined blood counts from extremely-low-birth-weight (ELBW) infants. Multiple platelet counts were obtained in 284, and 208 (73%) had one or more platelet counts less than $150 \times 10^9$/L. Most were detected during the first days of life; 80% were detected before postnatal day 7. Thrombocytopenia was seen frequently in the smallest infants; 85% incidence among those born with weights less than or 800 gm, 60% among those 801–900 gm, and 53% among those 901–1000 gm. In 48% of cases, the cause of the thrombocytopenia went undiagnosed. The most common explanations were being small-for-gestational-age (SGA) or delivery to a hypertensive mother, disseminated intravascular coagulation, bacterial infection, fungal infection, and necrotizing enterocolitis, respectively.

The same group of scientists[26] studied a large cohort of SGA infants. A total of 31% (905 of 2,891) showed first-week thrombocytopenia compared to the 10% of matched non-SGA controls ($p$ <0.0001). Of the 905, 102 had a recognized cause of thrombocytopenia (disseminated intravascular coagulation, early-onset sepsis, or extracorporeal membrane oxygenation). The remaining 803 did not have an obvious cause for their thrombocytopenia and were grouped as having "thrombocytopenia of SGA." These infants had a mean nadir count on postnatal day 4 of $93 \times 10^9$/L (standard deviation $51.8 \times 10^9$/L, tenth percentile $50 \times 10^9$/L, ninetieth percentile $175 \times 10^9$/L). By postnatal day 14, platelet counts were more than or $150 \times 10^9$/L in more than half of the patients. Severely SGA neonates (less than first percentile) had lower counts and longer duration of thrombocytopenia ($p$ <0.001). Thrombocytopenia was more associated with SGA status than with the diagnosis of maternal preeclampsia.

## Neonatal Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia is a condition in which maternal antibodies are formed against the paternal alloantigen expressed on fetal platelets.[30] The pathogenesis is analogous in some ways to that of the hemolytic disease of the newborn, which affects red blood cells. The fetal platelets carrying paternal antigens cross into the maternal circulation during normal low-grade transplacental cellular exchange or during larger-scale fetal-maternal hemorrhages/transfusions, which may occur during miscarriage or delivery. Antigen-presenting cells in maternal lymph nodes and spleen recognize these fetal antigens and stimulate the production of alloantibodies. The antiplatelet immunoglobulin G (IgG) antibodies are then actively transferred into the fetus and promote phago-immune destruction (Fig. 1).

The fetal platelets express specific human platelet antigens (HPAs) from sixteenth week onward.[31] Platelets carrying HPA epitopes such as HPA-1a present on the glycoprotein (GP) IIIa binding to the syncytiotrophoblasts-derived microparticles (STMPs) increases the likelihood of alloimmunization (Fig. 2). Trophoblasts normally escape allorecognition because of low expression of human leukocyte antigen (HLA) class I and II molecules. There is some expression of HLA-G, which is a non-classical HLA-I molecule and promotes alloantigen tolerance.[32]

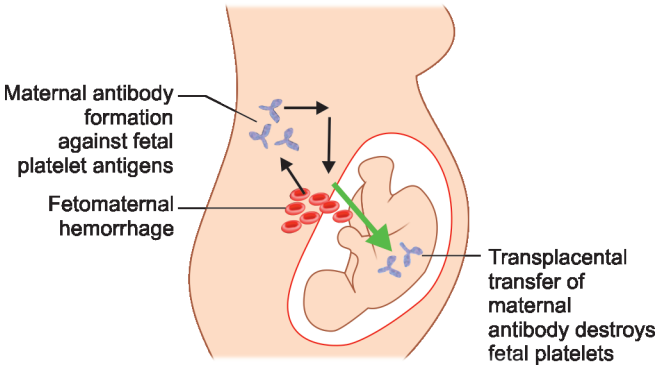


**Fig. 1:** Pathogenesis of NAIT

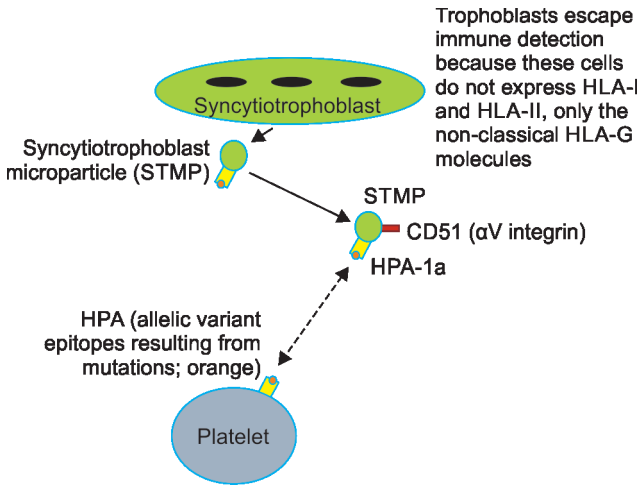


**Fig. 2:** The β3 integrin (platelet GPIIIa); CD61is expressed on the placental syncytiotrophoblast, the syncytiotrophoblast microparticles (SMTPs), and on platelets. Molecular variations are read as HPA-1a antigen, which evoke an antibody response. The STMPs show these antigens complexed with CD51, which potentiates the immune responses and may cause antibody-mediated platelet destruction

Several antigen systems can be seen on the surface of human platelets, including the HPAs, the ABO antigens, and the HLA class I.[33,34] So far, 29 HPA systems have been identified on six platelet membrane GPs (GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa, and CD109); 12 are grouped into 6 biallelic systems (HPA-1, -2, -3, -4, -5, and -15). All but one of these HPAs represents single nucleotide polymorphisms (SNPs) that result in single amino acid substitutions.

Most HPAs are located on the GPIIb/IIIa although the distribution of various HPAs may show some ethnic/geographic variation.[35] Anti-HPA-1a alloantibodies are the major cause of immune mediated thrombocytopenia in Caucasian, whereas the HPA-4 and Naka (anti-CD36) antibodies are the predominant cause in Asian population, especially in the Japanese.

Most infants with NAIT develop mild-moderate thrombocytopenia, although these reductions can add to the morbidity and mortality if these infants become critically-ill.[36] The destruction of platelets by maternal antibodies can increase the risk of bleeding, particularly that of ICH. Alloimmunization has been best studied with the HPA-1a antigen expressed on the β3 integrin (platelet GPIIIa; CD61).[37] This integrin may be intrinsically expressed on placental STMPs or may be acquired from circulating platelets.[38] The syncytiotrophoblasts-derived microparticles show these antigens complexed with CD51, which evokes an immune response. Platelets also express various surface molecules such as the integrin β2, β3, αIIb, CD109, and the complex GPIbα that may carry various HPAs.[39] The syncytiotrophoblasts-derived microparticles can induce variable immune responses, which include fetal alloantigen tolerance or induce immune responses that cause antibody-mediated platelet destruction.[40]

## Epidemiology of NAIT

The incompatibility between fetal and maternal platelet antigens evokes the synthesis of maternal IgG antibodies, which then cross the placenta to induce fetal platelet destruction and cause NAIT.[41] Similar to red cell alloimmunization such as in Rh antigen-mediated hemolysis, most cases of NAIT follow immune sensitization against platelets at the time of delivery in a previous pregnancy. However, many cases are seen in the very first pregnancy.[42]

Human platelet antigens-1a is the best-studied trigger for the production of antiplatelet antibodies and causation of NAIT.[43] In one study, the incidence of thrombocytopenia in incompatible HPA-1a positive infants was 1:1000–2000.[44] The HPA-1bb phenotype in Caucasian population was about 2.5% and out of these one-third expressed the HLA-DR antigen B3*0101.[45] One-third of infants in this subset developed antibodies against HPA-1a and with moderate-to-severe thrombocytopenia.[3]

## Platelet Antigens

### Important HPAs

A system of HPA nomenclature was developed by international consensus following confirmation of polymorphisms in platelet GPs. These antigens were designated as HPA1 and HPA2 in the order of discovery.[46] The suffix "a" or "b" indicated decreasing frequency of expression. The HPA-1a antigen, the first HPA implicated in NAIT, showed a leucine/proline substitution at position 33 of the integrin plexinsemaphorin.[47]

Other antigens implicated in NAIT included the platelet membrane GPs, GPIb-V-IX (von Willebrand receptor), GPIIb/IIIa, GPIa/IIa, and CD109, a glycosylphosphatidylinositol-anchored protein of uncertain function.[48] These platelet GPs and proteins interact with coagulation factors to promote hemostasis. The maternal immunization during pregnancy resulted in NAIT due to polymorphisms from 27 single amino acid substitution present in six different GPs (GPIIb, GPIIIa, GPIba,GPIbb,GP1a, and CD109).[45]

Human platelet antigens-1a contributes to NAIT in up to 85% of all cases with Caucasian and African ancestry. These figures are interesting because only 2% of women in the community are HPA-1a negative and are at risk to develop antibodies against HPA-1a.[34] Most (90%) women who express class II histocompatibility antigen DRB3*0101 produce antibodies against HPA-1a.[46,49]

### Other HPAs

In the Caucasian population, nearly 95% of serologically confirmed cases of NAIT are rooted in alloimmunization against only a few antigen systems (HPA-1,- 2, -3, -5, and -15).[50] In a few cases of apparent NAIT, the maternal antibodies for these antigens were not detected and other mutations were identified. Human platelet antigens-9b has been found to be the most immunogenic, and has been detected in about 1 in 400 normal individuals and is located close to the HPA-3 antigenic site in the calf-2 domain of GPIIb.[51] Human platelet antigens-4b, HPA-6b, and HPA-21b are significantly more prevalent in Asians than in Caucasians.[51] However, the maternal alloimmunization against less frequently seen antigens contribute only a very small fraction of NAIT cases.[52]

### The ABO Antigens

Platelets normally express the A and B antigens in very small concentrations.[53] One study showed that the platelets from only about 5% of normal subjects test positive for A and B blood groups. However, some mothers may express high levels of the antigens A1 and B on platelets and may be at higher risk of thrombocytopenia.

### Glycoprotein IV (CD36, Nak)

Nearly 5% of infants with African and Asian ancestry seem to have lost the expression of CD36 and are at risk to undergo alloimmunization. Originally, the findings were considered to be specific for an alloantigen named Nak, but subsequent studies showed these antibodies to recognize multiple other epitopes on CD36.[43,54]

### Human Leukocyte Antigens

Human leukocyte antigens antibodies account for up to a third of all cases of NAIT. Human platelets express at least 20,000 copies of class I HLA antigens, and contribute to a majority of the HLA antigens present in circulating blood.[55] Anti-HLA antibodies have been documented in nearly 31% of all pregnant women, particularly those who are multiparous.[55] However, very interestingly, the number of infants with NAIT due to these antibodies is much smaller.

Neonates born to mothers sensitized to class I HLA typically have normal platelet counts at birth. The association between the antibody concentrations and platelet concentrations has not been consistent.[55] However, some studies suggest that anti-HLA antibodies developed by the mother may cause NAIT.[56] Further studies are required to determine the impact of antibody titers, specificity, and potency of HPA and HLA antibodies.[56]

Sasaki et al.[57] reporteda neonate with NAIT caused by maternal anti-HLA A24 and B52. Treatment with platelet transfusions was ineffective because of the presence of maternal anti-B61 antibody. In another study, a high prevalence of anti-HLA antibodies was seen in mothers carrying low birth weight infants, who were thrombocytopenic.[57] The incidence of NAIT in these infants was higher than those born at term.

## Antenatal Screening

Neonatal alloimmune thrombocytopenia can be associated with intracranial hemorrhages in fetuses *in utero*. About 40 in 100,000 pregnancies can present with fetal-onset NAIT, with severe bleeding episodes in about three to four of these cases.[58] Most of these bleeds seem to before 36 weeks of gestation. Hence, antenatal screening is justified in pregnancies following one with documented NAIT.

To design and implement an appropriate screening program for NAIT, resources are needed to identify women at a risk for fetal-onset NAIT.[59] We need both experienced personnel and access to cost-effective, continuously-available laboratory protocols. These antenatal screening programs need to include both HLA typing and HPA detection in at-risk pregnancies.[60]

## Laboratory Diagnosis of NAIT

When thrombocytopenia is detected in a newborn, a CBC should be obtained to ascertain whether thrombocytopenia is isolated or is a part of pancytopenia syndrome. Maternal blood counts should be obtained to refute the possibility of autoimmune thrombocytopenia. These should be followed by platelet serological tests on parental blood to confirm NAIT. The diagnostic testing for NAIT has the following two objectives: (a) To determine the incompatibility between the maternal and fetal platelet antigenic profile and (b) The detection of alloantibodies in the mother's serum. Based on the results, the risk to the neonate can be projected.[24]

The assays for detecting antigen are performed on parents' blood, and if an incompatibility is detected, serum samples from the mother are tested to identify antibodies against any antigen(s) that may be detectable on the father's platelets. If there are differences in parental genotypes and there are specific antibodies in the mother's serum against the putative antigen, the diagnosis of NAIT needs consideration. The certainty of NAIT as a diagnosis is higher when an alloantibody against specific paternal antigen(s) identified on neonatal platelets is detectable in the maternal serum.[34,43] The antiplatelet antibodies in maternal serum can be detected by a variety of tests including the platelet suspension immunofluorescence test (PSIFT), monoclonal antibody immobilization of platelet antigens (MAIPA), radioimmunoprecipitation (RIA), and flow cytometry-based assays. These tests are briefly described below:

### Platelet Suspension Immunofluorescence Test (PSIFT)

The intact platelets are incubated with the patient's or the control serum and allowed to bind to the antigenic epitopes. Then, fluorescence-labeled anti-human IgG/IgM are added as the secondary antibody and allowed to bind to the antibody bound to the antigenic epitope. The fluorescence-labeled platelets are then analyzed by fluorescence microscopy or by flow-cytometry.[61]

Flow-cytometry is highly sensitive to detect antibodies against most HPAs except for those against HPA-5 and HPA-15.[62] These two antigens are expressed in lower densities on the platelet surface; only about 3,000–5,000 HPA-5 antigenic sites and only 1,000 HPA-15 sites are expressed on platelets. The binding assays can be confounded due to the simultaneous presence of multiple antibodies, particularly those against the HPA and HLA. To remove reactivity against anti-HLA antibodies, platelets can be pre-treated with chloroquine or acid to destroy the platelet surface β2 microglobulin. However, it might be difficult to completely eliminate this cross-reactivity if the anti-HLA antibodies are present in high titers. To reduce the confounding effect of anti-A and anti-B antibodies, we use blood group O platelets for these assays.

### Antigen Capture Assays

There are three types of antigen capture assay [antigen capture Elisa (ACE)]; the ACE, the modified ACE (MACE), and the MAIPA.[63] These methods differ in the way the GP antigens are captured. The MAIPA is widely used in Europe and other countries, whereas MACE is preferred in the USA.

*(a) Antigen capture Elisa assay:* Platelet lysates containing membrane GPs are placed in the wells of a microtiter plate coated with GPs-specific antibodies, which capture specific GPs. The well is then washed and incubated with the antiplatelet antibody. The antiplatelet antibody bound to the GP is detected by the addition of a peroxidase-labeled anti-human IgG, followed by an appropriate substrate.[64]

*(b) Modified antigen capture Elisa assay:* Platelets are incubated with antiplatelet antibodies, and then lysed. The complex consisting of GPs/antiplatelet antibody is added to the well of a microtiter plate coated with specific monoclonal antibody that capture the complex, and the captured complex is detected by the addition first of a peroxidase-labeled anti-human IgG and then an appropriate substrate.[64]

*(c) Monoclonal antibody-specific immobilization of platelet antigen (MAIPA):* Platelets are exposed to antibodies that can recognize *specific* target GPs, and the lysates are then placed in a microtiter plate coated with capturing antibodies. The antibody complexes can be measured using color- or fluorescence-generating laboratory methods. The antigen capture methods allow the discrimination of HPA and HLA antibodies.[63] It is important to know the strengths/weaknesses of the assays because some antibodies such as those against the Naka antigens may compete with others and may give erroneous results.

Brighton et al.[65] used MAIPA to examine the specificity of antiplatelet antibodies in patients with immune thrombocytopenia. They used direct methods in 40 patients and indirect in 23. The patients with direct positivity showed a trend, which was statistically not significant, toward more antibodies against GPIIb/IIIa. The direct-positive patients showed antibodies against anti-GPIIb/IIIa in 19 (48%), anti-GPIb/IX (21%), and to both in 16 (40%). Those with indirect positivity had anti-GPIIb/IIIa in 7 (30%), anti-GPIb/IX in 7 (30%), and against both in 9 (40%).

## Radioimmunoprecipitation (RIP)

Radioimmunoprecipitation is more sensitive than MAIPA.[66] It utilizes unbound radioisotopes such as Iodine$^{125}$ for tagging surface GPs on platelets. These immunoprecipitated GPs are captured on a solid phase such as protein agarose, where these are recognized by maternal alloantibodies. The immunoprecipitated GPs are first eluted, and then identified using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography. These proteins are identified based on characteristics such as molecular weight. More recently, several sensitive modifications of the RIP using non-fluorescent labeling have also been developed (Fig. 3).[67]

In 2019, Vrbensky et al.[68] evaluated direct and indirect antiplatelet antibody tests for the diagnosis of immune thrombocytopenia (ITP). They concluded that the overall sensitivity of antiplatelet antibody testing was low (53%), but its specificity was high (>90%).

## Newer Laboratory Tests

### *Bead-based Technologies*

Recently, many different bead-based high-throughput techniques have been developed. Considering the relatively higher frequency of alloimmunization against HPA-1a, many of the first bead-based assays have focused on these antibodies. The bead-based technologies have been used for multiplex testing, which has lowered the cost of testing and increased efficiency.

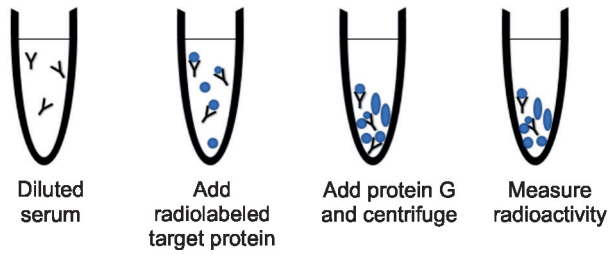


**Fig. 3:** Schematic representation of radioimmunoprecipitation

### *Immune-complex Capture Fluorescence Analysis (ICFA)*

Immune-complex capture fluorescence analysis is a methodology based on antigen capture methods combined with fluorescence measurements.[35] The platelets are first exposed to the patient's serum, which might contain specific antibodies. Then, a small aliquot of the lysate is tested for detection of antibodies against HPA and HLA. The data in this article show that the assays can be used with confidence to detect antibodies against HPA-1a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6b, and the Naka antigens. Anti-HPA-15 antibodies have not been tested extensively. These tests are based on antigen-capture methods and can be false-negative below certain diagnostic thresholds.

### *Fluorescent Bead-based Platelet Antibody Detection Methods*

These assays have been developed using fluorescence beads for the detection of antiplatelet antibodies.[69] Currently available assays can detect antibodies against HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, and the Naka antigens, but not the anti-HPA-15a and -15b antibodies.[57] These tests show high sensitivity and are relatively easy to establish. The training of personnel is relatively simple, HPA-type platelets are not required, and only small amounts of sera are needed. Anti-HPA-15 antibodies can be clinically significant in NAIT, and therefore, specific assays are needed.[43] In addition, the tests are less-sensitive for antibodies such as anti-HPA-3a. In those cases, the methods such as the PIFT and MAIPA using appropriate monoclonal antibodies are needed.[57] In addition, low titers and low-avidity antibodies may be missed.

### *Assays for Platelet Genotyping*

Platelet genotyping requires whole blood samples from both the mother and father. For antibody screening, maternal serum is used.[41,70] The genotypic analysis is done by PCR techniques, and antibody screening can be performed using MAIPA or RIP.[71] However, Elisa can be used for well-characterized antigens such as HPA-1a.[72] Amniocytes obtained by amniocentesis may be useful for confirming the genotype of fetal platelets.[58] When the status of the father is uncertain or the father is heterozygous, amniocentesis becomes important. Amniocytes can be grown in culture to obtain sufficient DNA needed for PCR analysis. Fetal and maternal DNA can be differentiated by using the variable number tandem repeat analysis (VNTR).[41]

In reference laboratories, several high-throughput methods are used for platelet genotyping, including sequence-specific primer-polymerase chain reaction (SSP-PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), and TaqMan real-time PCR.[73]

*(a) Sequence-specific primer-polymerase chain reaction:* This is an allele-specific PCR that uses two reactions, using two sets of primers; one is specific for each allele and the second control primer used to monitor the efficiency of PCR.[74,75] When there is 3'-terminal nucleotide mismatch between the allele-specific primer and the target DNA, there may be some loss of efficiency of Taq polymerase in DNA amplification and this forms the basis of SSP-PCR. The HPA profile is identified by the presence or absence of DNA bands that appear after gel electrophoresis of the products obtained by PCR.[76,77] Sequence-specific primer-polymerase chain reaction is relatively simple and cost-effective for genotyping of HPA.

*(b) Polymerase chain reaction-restriction fragment length polymorphism:* The loss or gain of recognition sites of the restriction enzyme, which is essentially present at the polymorphic site in the target gene, constitutes the basis of PCR-RFLP. There is amplification of the gene that encodes the polymorphism followed by digestion with specific restriction enzyme. The fragments formed after the digestion are then separated according to their lengths by gel electrophoresis. After the separation according to their length, there is visualization of DNA using UV transilluminator, followed by fragment pattern interpretation. The PCR-RFLP is also simple and cost-effective, but requires an extra step of digestion which cannot be automated. One of the disadvantages of PCR-RFLP is the requirement of controlled reaction parameters for the activity of the restriction enzyme in order to avoid incomplete digestion and false results.[74]

*(c) TaqMan real-time PCR:* This molecular technique carries out the quantitative PCR amplification of the target gene in real time. This assay uses a sequence-specific primer (probe), that binds the SNP of interest and carries a reporting fluorophore attached to the 5′-end. The 3′-end of the probe is the quencher. The probe binds the DNA, and the extension is done by Taq polymerase. The 5′-nuclease activity of Taq polymerase displaces the fluorophore from the 5′-end of the probe, when it extends the SSP in the 5′-3′-direction, which will cause the reporter dye to cause florescence, leading to quantification of the amount of the PCR product.[77] This is an automated process and can differentiate between the homozygosity and heterozygosity in biallelic HPA systems using allele-specific probes with different reporter dyes.[74,77]

*(d) High-throughput methods:* The development of rapid high-throughput methods allows the amplification or multiplexing of multiple targets in a single assay, which can be used for screening of pregnant women for HPAs.[60] Because of this automation, there is a decreased risk of human error in both technical aspects and interpretation. However, these high-throughput methods require the use of expensive computer software programs and reagents.

Many bead arrays have been developed; these are useful, multiplex high-throughput methods that can be used for HPA typing. Multiple beads can be used simultaneously, each targeting a different SNP. The assays utilize allele-specific probes attached to beads tagged with florescent dyes. The target fragments of the DNA then anneal to the probes which are elongated using fluorescent labeled nucleotides. The beads are fixed to a chip or flow cytometry, where the fluorescence patterns are analyzed.[78,79]

*(e) Multiplex SNP genotyping:* Another high-throughput method is based on the multiplex SNP genotyping using oligonucleotide extension. This method was first used to carryout genotyping of HPA profile of platelet pheresis donors by Shehata et al.[80] In this assay, primers for multiplex PCR are designed to flank the SNP of HPA, and fragments amplified in the PCR anneal to probes with single base extensions. These probes are hybrid oligonucleotide in which one part is attached to the target that it amplifies and is in immediate proximity to the SNP of interest, and the other part, the tag portion, immobilizes the attached complex to a chip for fluorescence and laser activation.

Identification of HPA systems through high-throughput methods is valuable for blood centers in order to screen the platelet donors.[81] These methods have allowed identification of antigen-negative donors and enabled specific transfusions if needed. Human platelet antigen genotyping also has several other advantages over serological methods. First, genotyping methods do not require fresh platelets, and genomic DNA can be procured from various sources such as leukocytes, amniocytes, and buccal smears. Second, low frequency HPA can be used when serum is not available for typing. Finally, genotyping methods are mostly automated and have lower risks of error and need less time to perform the assays. However, the diagnosis of NAIT is still dependent screening of the maternal serum for antibodies, and subsequent incompatibility testing between the parents for HPA antigen likely to cause alloimmunization and platelet genotyping for HPA typing is considered to a gold standard for investigation NAIT.[76,77]

We still confront many limitations in platelet genotyping.[82] Platelet genotyping requires prior isolation of DNA of high quality and quantity, and no contamination.[75] Differences have also been reported between the genotype and the phenotype of the HPAs including HPA-1.[83,84] Primer annealing can be also be affected due to the presence of polymorphisms near SNPs in the gene of interest, which can sometimes lead to erroneous results.[62,74]

## Treatment Options and Transfusion Practices for NAIT

The treatment of choices for full-term neonates with suspected NAIT, with and without bleeding includes intravenous immunoglobulins (IVIG), corticosteroids, and antigen-negative or irradiated maternal platelets as emergency supportive measures.[30,85,86]

Clinicians usually do not have continuous 24-hour access to maternal HPA-1a negative platelets.[87] Therefore, most physicians choose IVIG at a dose of 1 gm/kg weight for 2 consecutive days to the neonates who had no signs of bleeding but with platelet counts below a pre-decided threshold.[88] Small doses of corticosteroids may help by improving capillary fragility.[30] We will soon describe the current norms and preferences for treatment of NAIT in another review.

## Summary

Neonatal alloimmune thrombocytopenia is an important cause of severe neonatal thrombocytopenia. The clinical presentation may range from incidental, isolated abnormalities in laboratory tests to major clinical hemorrhages with life-threatening sequalae. If the platelet counts are less than $150 \times 10^9$/L with no obvious cause to explain the thrombocytopenia, NAIT should be considered in the differential diagnosis. Although a large number of antigen systems have been clearly associated with NAIT, there are still many infants who have with suggestive clinical/laboratory profiles but unclear molecular diagnosis. These infants may have still-unidentified low-frequency and rare antigens.

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CASE SERIES

# Neonatal Acute Liver Failure

Gurpreet Kaur Grewal[1], Horacio Osiovich[2], Richard Schreiber[3]

## Abstract

Acute liver failure is a rare event in the newborn period yet early recognition in the neonatal intensive care setting is essential for best outcome. Neonatal acute liver failure (NALF) is distinct from acute liver failure in older children and adults having different etiologies, presentation, and unique treatment interventions. There is a paucity of literature regarding NALF and several newly identified conditions merit discussion. Herein we report three cases of liver failure who were admitted to our neonatal intensive care unit and review the diagnostic approach and management of liver failure in this age group.

**Keywords:** Diagnosis, Enterovirus, Gestational alloimmune liver disease, Hemophagocytic lymphohistocytosis, Neonatal acute liver failure, Neonatal disease, Neonate.

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## Introduction

Acute liver failure is a rare event in the newborn period yet early recognition in the neonatal intensive care setting is essential for best outcome. Neonatal acute liver failure is distinct from acute liver failure in older children and adults having different etiologies, presentation, and unique treatment interventions. There is a paucity of literature regarding NALF and several newly identified conditions merit discussion. Herein we report three cases of liver failure who were admitted to our neonatal intensive care unit and review the diagnostic approach and management of liver failure in this age group.

## Case 1

A term female infant was born by spontaneous vaginal delivery to a healthy 33-year-old Caucasian gravida 2 para 1 mother in a community hospital. Mother's antenatal course was unremarkable with normal ultrasounds; GBS and GDM screening were negative, blood O positive, and protective serologies. She went into spontaneous labor with rupture of membranes one hour prior to delivery. The infant did not require any resuscitation; Apgar scores were 9 at 1 minute and 9 at 5 minutes. Infant's birth weight was 3480 gm (50–90th centile), length 50 cm (50–90th centile), and head circumference [34.5 cm (50th centile)]. Infant received IM Vitamin K at birth and was transferred to room in with her mother.

At 4 hours of age, the infant was noted to be pale, apneic, hypothermic (temperature of 35.9°C), and hypoglycemic (0.8 mmol/L). A D10W bolus (2 mL/kg) was given followed by maintenance IV D10 NS at 60 mL/kg/day. Blood culture was drawn and ampicillin and gentamicin were started. Hypoglycemia subsequently improved. Initial investigations were as follows: WBC $19.2 \times 10^9$/L ($9–30 \times 10^9$/L), neutrophils $15.4 \times 10^9$/L ($6–27 \times 10^9$/L), hemoglobin 188 gm/L (135–195 gm/L), hematocrit 0.52, platelets $136 \times 10^9$/L ($150–450 \times 10^9$/L), CRP <5 mg/L.

At 20 hours of age, she had hematemesis with hematochezia. Her vital signs remained stable. A nasogastric tube was inserted, and the infant was made NPO. An abdominal X-ray showed no signs of air fluid levels or obstruction or NEC. Her hemoglobin dropped to 105 gm/L with hematocrit 0.30. She was transfused with 20 mL/kg packed red blood cells, a second dose of vitamin K, and IV pantaprazole bolus was given, and she was transferred to our tertiary NICU.

On arrival, the infant was hemodynamically stable and in no respiratory distress; temperature 36.8°C, HR: 160 bpm, RR: 43 bpm, BP: 66/49 (55). She looked pale but well perfused, good air entry bilaterally, and no murmur. Her abdomen was soft, nontender, without hepatomegaly or splenomegaly. The rest of the physical examination was normal; there were no dysmorphic features or neurological findings. Laboratory results showed: WBC count $15.3 \times 10^9$/L ($9–30 \times 10^9$/L), neutrophils: $9.27 \times 10^9$/L ($6–27 \times 10^9$/L), hemoglobin 112 gm/L (135–195 gm/L), hematocrit: 0.32; platelets: $103 \times 10^9$/L ($150–450 \times 10^9$/L), INR 2.4 (N: 1.2–1.5), APTT 66 seconds (N: 28.0–40.0 seconds), PT 22.4 seconds (N: 9.6–11.8 seconds), fibrinogen <0.7μmol/L (N: 1.70–4.0 gm/L), ALT 40 U/L (6–50 U/L), AST 88 U/L (35–140 U/L), GGT 40 U/L (N: 34–263 U/L), unconjugated bilirubin 113 μmol/L, conjugated bilirubin <2 μmol/L (<5 μmol/L), ferritin 2770 μg/L (6–400 μg/L), serum albumin 18 gm/L (26–36 gm/L), alpha fetoprotein 340,000ng/ml (<20,000 μg/L), serum ammonia 18 μmol/L (<49 μmol/L), and triglycerides 0.94 mmol/L (<1.7 mmol/L). Newborn screen was negative for amino acid, fatty acid oxidation, organic acid disorders, hypothyroidism, hemoglobinopathies, galactosemia, and cystic fibrosis. Blood and urine cultures were negative. Serum HSV 1 and 2 and CMV PCR were negative. An echocardiogram showed normal anatomy and function and the ECG was normal. Abdominal ultrasound showed no focal abnormalities seen in the liver. Gall bladder, intra- and

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extrahepatic biliary ducts, pancreas, spleen, and kidneys were all unremarkable.

She received cryoprecipitate and fresh frozen plasma. Pantaprazole and IV vitamin K were continued. Acyclovir was added to the antibiotic therapy.

On day two of life, with a presumptive diagnosis of gestational alloimmune liver disease (GALD), she received intravenous immunoglobulin (IVIG) at 1 gm/kg and a double volume exchange transfusion, followed by a second dose of IVIG at 1 gm/kg. Magnetic resonance imaging of the abdomen [Fig. 1 (Image 1) and (Image 2)] was consistent with moderate-to-severe iron overload in the liver, mild pancreatic iron overload, and possible iron deposition in the choroid plexus within the brain. Buccal/salivary gland biopsy did not show evidence of iron deposition. Whole exome sequencing showed a heterozygous *de novo* variant of uncertain significance in KRT8, which puts her at risk for a KRT8 "keratin 8"-related liver disease.

Over the ensuing days, the thrombocytopenia, coagulopathy, and IV glucose requirement gradually improved and feeding was initiated without incident. At discharge on day 35 of life, she was exclusively breastfeeding and gaining weight. The hemoglobin was 130 gm/L (135–195 gm/L), platelet count $297 \times 10^9$/L ($150–450 \times 10^9$/L), INR 1.3 (N: 1.2–1.5), APTT 38 seconds (N: 28.0–40.0 seconds), PT 13.2 seconds, fibrinogen 1.4 μmol/L (N: 1.70–4.0 gm/L), albumin 31 gm/L (26–36 gm/L), ALT 38 U/L (6–50 U/L), AST 74 U/L (35–140 U/L), unconjugated bilirubin 57 μmol/L, and conjugated bilirubin 3 μmol/L (<5 μmol/L).

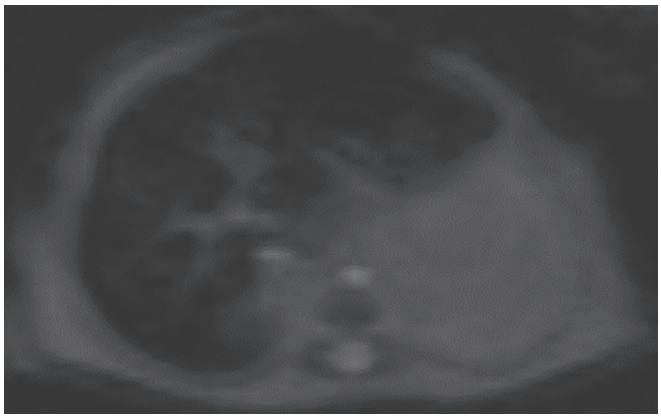


**Fig. 1 (Image 1):** Magnetic resonance imaging of the abdomen on day 3 of life shows moderate-to-severe iron load in the liver with mild pancreatic iron overload. Liver iron time to echo (TE) 2.4 ms. The TE is the time between the delivery of the radiofrequency pulse and the receipt of the echo signal

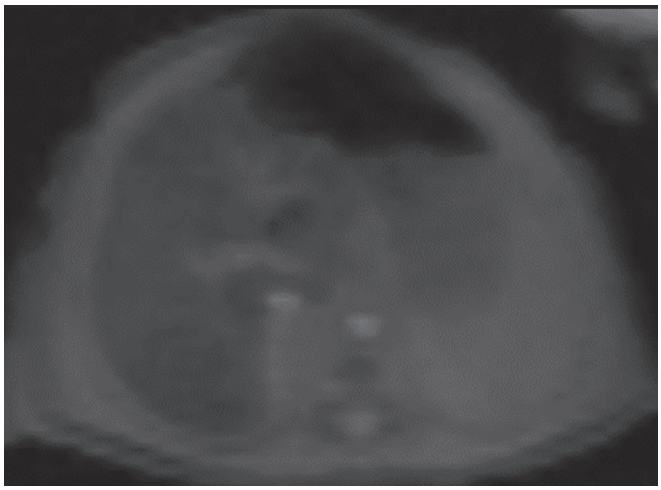


**Fig. 1 (Image 2):** Magnetic resonance imaging of the abdomen on day 3 of life shows moderate-to-severe iron load in the liver with mild pancreatic iron overload. Liver iron TE 12.27 ms

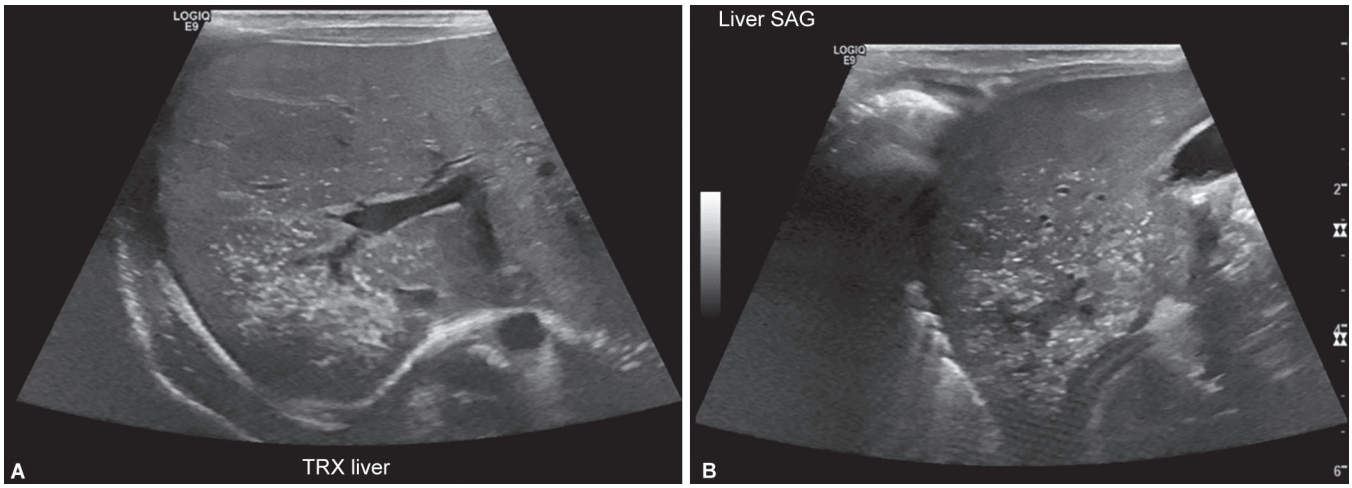
## Case 2

A male infant was born at 38.5/7 weeks by repeated cesarean section to a 33-year-old G3T1A1 mother with a history of migraine. Mother was blood group O positive, serologies protective, GBS negative, and insulin-dependent GDM. Antenatal ultrasounds at 10- and 22-weeks GA were normal. Her pregnancy was complicated with a febrile illness in the third trimester. Meconium-stained amniotic fluid was noted at the time of delivery. Apgar's scores were 2, 5, and 7. Birth weight was 3450 gm (50th centile), length 55 cm (>97th centile), and head circumference 36.5 cm (90th centile). At 1 hour of life, hypoglycemia was noted (1.2 mmol/L) requiring increased rate of glucose delivery up to 18 mg/kg/minute glucose infusion rate via total parenteral nutrition to maintain normal blood glucose levels. Initial investigations showed: WBC: $10.9 \times 10^9$/L ($9–30 \times 10^9$/L), neutrophils $3.5 \times 10^9$/L ($6–27 \times 10^9$/L), hemoglobin 139 gm/L (135–195 gm/L), hematocrit 0.43, platelets $19 \times 10^9$/L ($150–450 \times 10^9$/L), unconjugated bilirubin 139 μmol/L. The infant was on mild respiratory distress requiring CPAP 5 cm $H_2O$ $FiO_2$ 21% with normal blood gases. The infant was transferred to our tertiary level NICU for further management.

At admission, the temperature was 37.6°C, HR 137 bpm, RR 59 bpm, BP 62/34 (mean 44). Infant was well perfused, with normal pulses and no murmurs, abdomen was soft, mildly distended with hepatomegaly at 4 cm below the right costal margin and the tip of the spleen was felt 1 cm below left coastal margin. Scattered petechiae were noted on his back and groin. He had no dysmorphic features or neurological signs. An echocardiogram was normal. Laboratory findings revealed WBC $11.2 \times 10^9$/L, platelets $18 \times 10^9$/L, and hemoglobin 164 gm/L. The INR was high at 3.8 (N: 1.2–1.5), APTT of 51 seconds (N: 28.0–40.0 seconds), PT of 34 seconds (N: 9.6–11.8 seconds), and low fibrinogen <0.7 μmol/L (N: 1.70–4.0 gm/L). He received fresh frozen plasma, cryoprecipitate, platelets, and vitamin K.

Liver function tests showed ALT 49 U/L (6–50 U/L), AST 187 U/L (35–140 U/L); LDH 5191 U/L (140–280 U/L) unconjugated bilirubin 169 μmol/L, conjugated bilirubin 44 μmol/L (<5 μmol/L), GGT 66 U/L (34–263 U/L), albumin 19 gm/L (26–36 gm/L), Ferritin 184 μg/L (6–400 μg/L); alpha fetoprotein 47,000 ng/mL (<20,000 μg/L), serum ammonia 42 μmol/L (<49 μmol/L). The newborn screen for amino acid, fatty acid oxidative, organic acid, thyroid, CAH, hemoglobinopathies, galactosemia, and cystic fibrosis was negative. TSH/FT4, ammonia, and acylcarnitine were normal, and blood, urine, and CSF cultures were negative.

An abdominal ultrasound (Fig. 2) showed abnormal liver parenchyma with multiple echogenic foci, most marked in the right lobe. Calcifications were noted, likely due to remote prenatal insult with small volume ascites. Viral studies for HSV, HIV, Parechovirus, and CMV were negative. Enterovirus by PCR amplification and nuclei acid testing (NAT) was positive in blood. Conjugated bilirubin peaked to 289 μmol/L (<5 μmol/L) on day 10 of life. Jaundice gene chip panels expressing trio whole exome sequencing and




**Figs 2A and B:** Abdominal ultrasonography on day 2 of life shows abnormal liver parenchyma with multiple echogenic foci mostly marked in the right lobe with calcifications also noted

mitochondrial gene sequencing were negative for pathogenic gene mutation.

He failed hearing test twice and ABR showed severe sensorineural hearing loss on the left. The baby received ursodiol, platelet transfusions, and general supportive measures. He was discharged home on day 45 of life on EBM with formula supplement and growing well.

## Case 3

A term male infant was born via urgent C-section due to fetal bradycardia and reduced fetal movements to a healthy 33-year-old Caucasian gravida 2 para 1. Mother's antenatal course was unremarkable. She was blood group B positive with protective serologies; GDM and GBS screening were negative. An ultrasound at 38 weeks showed a fetus with IUGR, hepatosplenomegaly, mild ascites, hydroceles, and liver with starry sky appearance parenchyma with no evidence of anemia/hydrops by normal Doppler. Apgar scores were 8 and 9, birth weight 2600 gm (10th centile), length 50 cm (50th centile), head circumference 34 cm (50th centile). Infant received IM vitamin K in the delivery room and was transferred to the NICU for further evaluation. At admission he had temperature of 37°C, HR 126 bpm, and RR 45 breaths per minute, mean BP 38. He was hemodynamically stable without respiratory distress and no dysmorphic features, and normal neurological examination. Abdomen was soft and mildly distended, liver was palpated 4 cm below left coastal margin, and a tipped spleen. He was noted to have bilateral hydroceles. His first blood glucose was 2.3 mmol/L for which he received a D10W bolus (2 mL/kg).

Initial investigations: CBC: WBC: 20.5 × $10^9$/L (9–30 × $10^9$/L), neutrophils 4.37 × $10^9$/L (6–27 × $10^9$/L), lymphocytes 10.72 × $10^9$/L (2–11 × $10^9$/L), platelets 9 × $10^9$/L (150–450 × $10^9$/L), hemoglobin 240 gm/L (135–195 gm/L), unconjugated Bilirubin 62 μmol/L, Conjugated bilirubin 9 μmol/L, ALT 27 U/L (6–50 μ/L), AST 33 μ/L (35–140 U/L), ALP 44 μ/L (110–300 μ/L), albumin 18 gm/L (26–36 gm/L), triglycerides 0.88 mmol/L (<1.7 mmol/L), ferritin 2100 μg/L (6–400 μg/L), alpha fetoprotein 510 μg/L (<20,000 μg/L), ammonia 19 μmol/L (<49 μmol/L). Coagulation studies: PT: 17.9 seconds (N: 9.6–11.8), INR 1.9 (N: 1.2–1.5), APTT 40 seconds (N: 28.0–40.0 seconds), fibrinogen 0.8 gm/L (N: 1.70–4.0 gm/L). He received a platelet transfusion and 1 mg/kg of IVIG. An abdominal ultrasound showed a liver with smooth margin and homogeneous echotexture and no focal parenchymal abnormalities. Free fluid noted which was anechoic and lacked septation. There was marked periportal echogenicity throughout the liver and especially along the right and left branch portal veins, gallbladder contained sludge-like material, normal pancreas, and spleen. TORCH screen and enterovirus PCR were negative. Metabolic workup for urine organic acids, plasma amino acids, mucopolysaccharides/creatinine ratio, oligosaccharides pattern, plasma, very long fatty acids profile which were all negative.

On day 5 of life, the infant had a rapid deterioration requiring intubation and maximal support to manage significant ascites due to profound liver failure: CBC: WBC: 7.3x$10^9$/L, neutrophils: 1.78x$10^9$/L, Hb: 131 gm/L, platelets 28x$10^9$/L, unconjugated bilirubin 75 μmol/L, conjugated bilirubin 25 μmol/L, albumin 15 gm/L, ALT 23 μ/L, AST 33 μ/L, ALP 114 μ/L, GGT 51 μ/L PT 18.5 seconds, INR 1.9, aPTT 66 seconds, fibrinogen 1 gm/L. He was started on triple antibiotics for possible spontaneous bacterial peritonitis. Double-exchange transfusion was performed followed by a second dose of IVIG for the suspicion of GALD. He had persistently low platelets requiring daily platelet transfusions and FFP and cryoprecipitate for management of a coagulopathy. He received daily vitamin K at 1 mg/kg. A peritoneal drain was inserted to drain the ascites. He received twice a day 25% albumin transfusions at 1 gm/kg and was started on spironolactone, furosemide, acetazolamide to manage the ascites and edema. A repeated abdominal ultrasound showed reversed (hepatofugal) flow in the main portal vein and left branch portal vein (Fig. 3). Increase in splenic size (Fig. 4) and irregular margins of the liver in keeping with the appearance of portal hypertension (Fig. 5). Thrombophilia DNA Factor V Leiden mutation and Prothrombin (Factor II) was done which was normal phenotype.

On day 13 of life, a bone marrow aspiration/biopsy showed presence of megakaryocytes, reversed myeloid: Erythroid ratio suggestive of erythroid hyperplasia. There was no evidence of hemophagocytosis—chromosomal microarray was normal and whole exome sequencing was negative for any genetic cause for HLH. On day 15 of life, the infant was transferred to another level 3 NICU for evaluation of liver transplant due to liver failure with portal hypertension. His ferritin, soluble IL, and CD136 levels were raised, and he was started on high-dose dexamethasone on day 21 of life. Due to suboptimal response to dexamethasone,

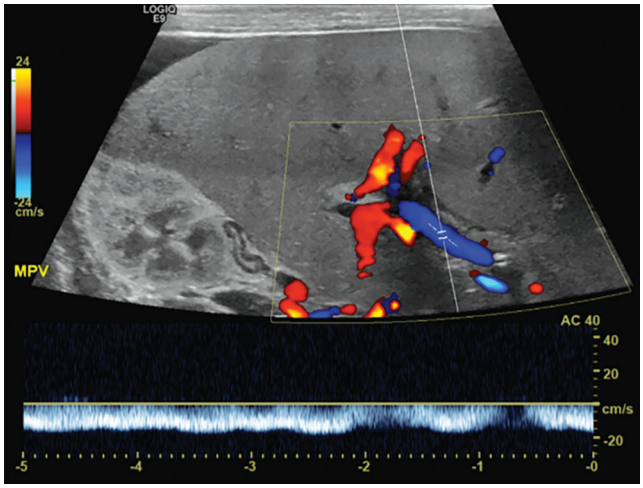



**Fig. 3:** Abdominal Doppler ultrasound showing reversed portal flow

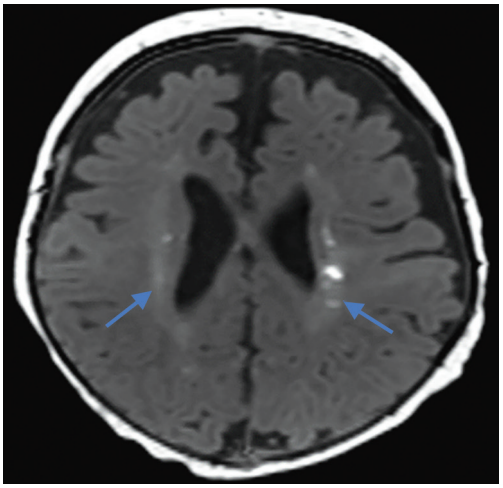


**Fig. 6:** Brain MRI showing bilateral white matter injury

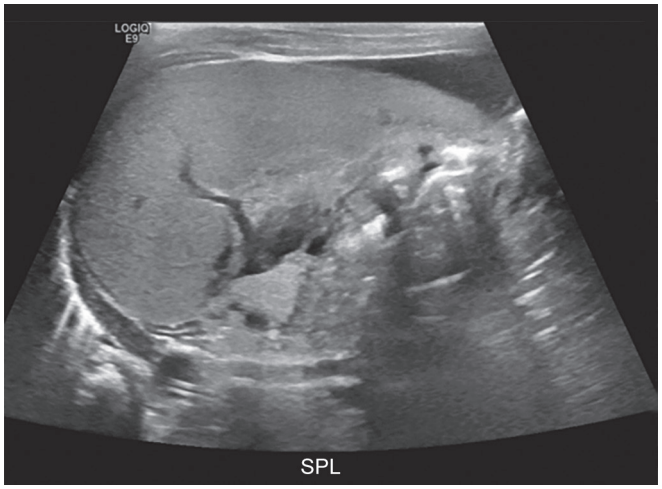



**Fig. 4:** Splenomegaly noted on the abdominal ultrasound

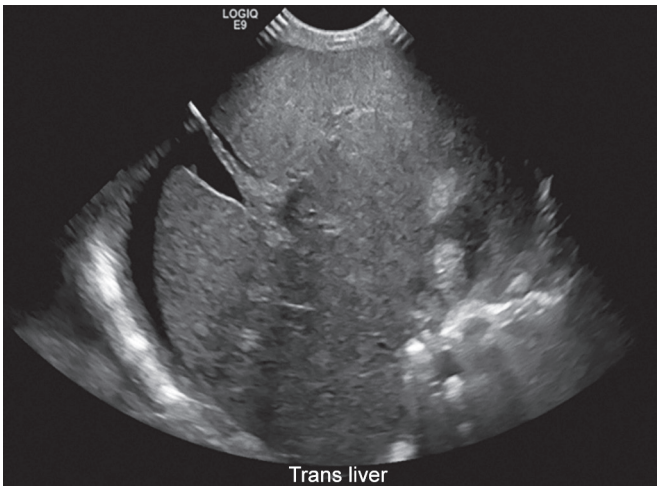



**Fig. 5:** Course liver with ascites noted on abdominal ultrasound

he was started on etoposide and fluconazole at 27 days of life for 8 weeks. A liver biopsy on day 30 of life did not show features of hepatitis. Instead, there was sinusoidal expansion with inspissated histiocytes more prominent in the periportal regions associated with up to 20% surrounding parenchymal loss. There was evidence of hemophagocytosis on both light and electron microscopy. The histological features were highly suggestive of a primary or secondary hemophagocytoic lymphohistiocytosis. There was no ultrastructural evidence supportive of mitochondrial disease or any abnormal storage material. On day 14 of life, he had a brain MRI which showed multifocal periventricular white matter signal abnormalities. A brain MRI on day 75 of life showed multiple T1 hyperintense subcentimeter foci throughout the periventricular white matter bilaterally. There was a focal abnormality in the posterolateral left thalamus (Fig. 6). The brain was otherwise structurally normal. This assessment showed evolution and progression of lesions in the right periventricular white matter which was suspected to be in keeping with CNS HLH. Based on the progression of these lesions, the decision was made to treat for HLH with CNS disease, which included intrathecal methotrexate and hydrocortisone. He continued with chemotherapy until he had received a successful bone marrow transplant at 4 months of age. He has done well post BMT with resolution of the liver disease and is growing well but with gross motor delay secondary to HLH.

## Discussion

Neonatal acute liver failure is defined as the onset of clinical or biochemical features of liver injury in association with severe coagulopathy (INR >2.0) that persists despite the administration of vitamin K, within the first 12 weeks of life.[1] In contrast to acute liver failure in older adolescents and adults, hepatic encephalopathy is not an essential criterion to the diagnosis of liver failure in newborns. The etiologies for NALF include viral infection (15%),[2] metabolic/genetic disease primarily galactosemia, tyrosinemia, and mitochondrial depletion syndromes due to DGUOK (10%),[1,3–5] hematologic disorders (15%),[4,6] and ischemic injury due to poor perfusion with associated congenital heart disease (5%).[4] A newly characterized condition known as GALD, previously referred to as "neonatal hemochromatosis," is now recognized as the leading cause of NALF accounting for almost half of all cases in some centers. In contrast to adults with acute liver failure where acetaminophen is by far the most common cause, this drug toxicity is exceedingly rare in newborns. Whereas up to 50% acute liver failure in older

children and adolescents remain idiopathic, only 5% of neonatal cases are indeterminate.[1]

The typical presenting signs and symptoms of NALF include lethargy, fever, nausea/vomiting, jaundice, hepatomegaly, splenomegaly, and ascites. Neonatal acute liver failure should be considered in newborns with confirmed or presumed sepsis, and persistent coagulopathy despite vitamin K delivery in association with usually abnormal (but occasionally normal) liver function tests (LFTs): AST, ALT, GGT, ALP along with other features of hepatic dysfunction including conjugated hyperbilirubinemia, recurrent or persistent hypoglycemia, hyperammonemia, ascites, gastrointestinal bleeding, and renal dysfunction. Many of the known causes of NALF have disease-specific treatments that can improve outcome and survival. Yet the overall mortality of NALF is 25%.[1,2,4–8]

The immediate assessment for NALF includes a complete hematological profile (CBC, DAT, group, and cross match); coagulation profile; electrolytes, BUN, Cr, LFTs, glucose, blood gas, lactate, ammonia, serum amino acids, acylcarinitnes, cholesterol, triglycerides, alpha-fetoprotein, and ferritin; urine for glucose, ketones, reducing substances, organic acids including succinyl acetone; blood and urine cultures, viral screen and rapid request for your local newborn screening program results. The management of NALF requires the elimination of all feeds, effectively to remove lactose, fructose, and lipid from the diet until rare metabolic-induced NALF is excluded, and to deliver intravenous glucose to support glucose requirements. Given the association of NALF and sepsis, all cases should be promptly treated with antibiotics and acyclovir. Specific treatments for those metabolic diseases that may present as NALF include NTBC [2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione] which is the mainstay treatment in tyrosinemia type I, carnitine, sodium benzoate, and hemofiltration.[9]

## Gestational Alloimmune Liver Disease

Gestational alloimmune liver disease is the most common cause of NALF.[4,6] This disease was first described in 1961 as "neonatal hemochromatosis" (NH) with fewer than 100 cases reported over the next 45 years.[10] "Neonatal hemochromatosis" was the term ascribed to newborns with liver and multiorgan failure who were frequently diagnosed as having sepsis with associated edema with or without ascites, oliguria, and evidence of advanced end-stage liver disease and cirrhosis with portal hypertension that was already present at birth. Typically, LFTs, in particular AST and ALT, were disproportionally low or normal for the degree of liver injury and hepatic dysfunction. Other notable features were the marked coagulopathy (INR >2) with unusually elevated alpha-feto protein and extremely high serum ferritin levels (>800 ng/mL). It was this latter finding in association with the autopsy findings of extrahepatic siderosis that led to the nosology of "neonatal hemochromatosis." Despite the historical treatment of NH with antioxidant cocktail, the mortality rate was upward of 80–90%.[10]

In a careful review of NH cases, an unusual familial pattern of disease penetration was identified that mimicked fetal hydrops rhesus factor incompatibility disease.[11] This led to the more recent characterization of the condition called as GALD. Gestational alloimmune liver disease is now recognized to result from maternal production of immunoglobulin (IgG) against a putative 32 kd fetal liver antigen that remains elusive. In utero, after a first "sensitizing" pregnancy, maternal IgG crosses the placenta to bind with the fetal hepatocyte antigen, causing activation of the compliment cascade that results in the production of membrane attack complex (MAC) with consequent liver cell injury and dysfunction.[7] This hepatic injury involves decreased production of hepcidin and increased transport of placental iron, decreased transferrin production, and increased iron uptake into the liver and extrahepatic tissues.[8] The tissues most affected with siderosis are pancreatic acinar cells, myocardium, thyroid follicular epithelium, adrenal cortex and mucosal glands of oronasopharynx, and respiratory tree. Often a buccal mucosal biopsy is obtained to demonstrate the extrahepatic siderosis. Imaging with T2-weighted MRI can also show evidence of extrahepatic tissue iron overload. Notably, reticuloendothelial cells including Kupffer cells and those in the spleen do not accumulate iron as would be expected in secondary hemochromatosis from multiple blood transfusion.[5] Moreover, the pathogenesis of "NH" or GALD is distinct from adult hemochromatosis which is a genetic disease caused by mutation in the HFE gene.[12]

There is mounting evidence to suggest that the alloimmune injury in GALD begins in midgestation.[8] Ongoing injury to developing hepatocytes during the second half of gestation results in proliferation of progenitor liver cells with production of alpha fetoprotein and extensive parenchymal fibrosis.[13] That there is a fetal onset liver failure which is further evidenced by the associated GALD features of IUGR, decreased fetal movement in the second, and early third trimester of gestation and at birth, the newborn is usually small for gestation age, sick with features of cirrhosis.

The hallmark features for GALD supported the diagnosis in Case 1. The relatively normal liver function tests with signs of hepatic dysfunction (persistent high INR not corrected by vitamin K), elevated ferritin, and high alpha fetoprotein along with the T2-weighted MR evidencing extrahepatic siderosis were in keeping with this newborn having GALD.

Whole exome sequencing found a heterozygous *de novo* variant of uncertain significance in KRT8. KRT8 is a type II keratin that is present in the liver, pancreas, and intestine that is important for the maintenance of the cellular scaffolding and cellular trafficking. KRT8 variants have been associated with liver fibrosis and cirrhosis in patients with cryptogenic and noncryptogenic liver disease. Importantly case 1 did not exhibit neurological disease including nystagmus which can be associated with genetic mitochondrial disorders and mutations in the DGUOK gene, which can be confused with GALD diagnosis.[14]

Gestational alloimmune liver disease is now recognized as a preventable disease. Treatment of future pregnancies with the administration of weekly high-dose IVIG to the mother starting as early as 14 weeks of gestation can abrogate the development of in utero liver injury and GALD. In a recent report of the world experience antenatal administration of IVIG led to significantly increased healthy offspring (94%) compared with untreated gestations (30%).[15] For the untreated GALD cases that present to the NICU, prompt treatment early in the postnatal period with IVIG and double volume exchange transfusion can attenuate the disease progression and improve survival.[16]

## Enterovirus

Enteroviruses, which include the echoviruses, coxsackie A and B viruses, are among the most common viruses causing disease in humans.[17] In contrast to older children and adults for which the hepatotropic viruses A, and rarely B, and EBV are leading causes of acute liver failure; in neonates herpes virus and enteroviruses are the major infectious etiologies for NALF and they are associated with significant morbidity and mortality. Early initiation of intravenous

acyclovir, even before a firm diagnosis is recommended, offers best outcome for newborn herpes disease.[18] Enterovirus infections can be acquired antenatally, intrapartum, and postnatally. In antenatal transmission neutralizing immunoglobulin M (IgM) antibodies can be detected on the first day of life.[19] Studies have reported isolation of enterovirus from amniotic fluid and umbilical cord blood.[20–22] Other modes of transmission include intrapartum exposure to maternal blood, genital secretions, and stool, as well as postnatal exposure to oropharyngeal secretions from the mother and other individuals who have close contact with the baby.[20,23,24] We believe in our case two that the enterovirus infection was acquired antenatally because the infant was presented with acute liver failure at birth.

Newborn enterovirus infection may present with a wide spectrum of clinical manifestations and with a varying degree of severity. A maternal history of recent respiratory disease or diarrhea raises suspicion. In neonates, symptoms may range from nonspecific febrile illness to fatal multisystem disease, frequently referred to as "neonatal enterovirus sepsis" or "enteroviral sepsis syndrome." The most common presenting features include fever, irritability, poor feeding, and lethargy.[20,25–27] A nonspecific macular or maculo-papular rash is observed in many cases during the illness.[25,26] Patients may develop respiratory symptoms, including nasal discharge, cough, apnea, tachypnea, recessions, grunting, and nasal flaring. Gastrointestinal symptoms such as vomiting, abdominal distension, and diarrhea are less common, reported in 20% of cases.[26] Other potential manifestations include pancreatitis, adrenal hemorrhage, and necrotizing enterocolitis.[20] Approximately half of the neonates with enterovirus infection have evidence of acute hepatitis evidenced by a marked rise in ALT, often 10 times the upper limit of normal. This is an important distinguishing feature from GALD where the ALT is low or normal and there is advanced end-stage cirrhotic liver disease. The hepatic inflammation may progress to acute liver failure evidenced by marked coagulopathy not correctable with vitamin K.[26,27] Jaundice with conjugated hyperbilirubinemia may develop during the illness and hepatomegaly may be detected in 20%.[20,25] Splenomegaly is a relatively uncommon feature.[26] Central nervous system disease may manifest as meningitis or encephalitis. Some neonates develop cardiac complication including myocarditis, cardiac arrhythmias, cardiomegaly, poor ventricular function, systemic hypotension, congestive heart failure, pulmonary edema, and myocardial ischemia. Many of these neurologic or cardiac features may also present in genetic mitochondrial disease.[20,26–29]

The diagnosis of an enteroviral infection can be by PCR from blood, CSF, and stool. Though case two had an atypical presentation of enterovirus NALF (normal AST/ALT), PCR and NAT for enterovirus were positive, establishing the diagnosis.

Treatment of neonatal enterovirus infection is supportive and expectant care. Although there is no FDA-approved therapy for enteroviral infection, there is some experience for using IVIG albeit its effectiveness is controversial.[28,30–32] There have also been reports and clinical trials on the use of pleconaril though it is not available for systemic administration. In a RCT of neonates with suspected enetrovirus disease assigned to seven days of pleconaril or placebo, there was more rapid viral clearance and lower mortality among pleconaril-treated infants (23 vs 44% with placebo).[33] Other clinical trials with pleconaril included two combined studies of approximately 200 patients with enterovirus meningitis who were randomly assigned to pleconaril or placebo, patients who received pleconaril had shorter clinical course with modest benefit.[34]

## Hemophagocytosis Lymphohistiocytosis (HLH)

The first report of familial HLH (F-HLH) in 1952 described two siblings who developed fever and hepatosplenomegaly at 9 weeks of age.[35] Subsequently, familial clusters of children with similar phenotypes were observed, as sporadic cases of a similar syndrome were seen in the context of severe infection, rheumatologic disorders, or malignancy.[36–40] In 1999, defects of PRF1 (encoding perforin) were discovered as the first inherited gene defect underlying F-HLH.[41] Before this discovery, a committee from the Histiocyte Society developed consensus enrollment criteria for the HLH-94 study to capture the HLH disease phenotype: Fever, splenomegaly, cytopenias, increased triglycerides/decreased fibrinogen, and hemophagocytosis.[42] Enrollment criteria for the subsequent HLH2004 trial were revised to incorporate ongoing discovery of the genetic basis of F-HLH and development of specialized immune studies.[43] Over the last decade, this criterion has become an effective criterion for defining/diagnosing HLH, though their sensitivity and specificity remain unknown.

Hemophagocytic lymphohistiocytosis (HLH) is an aggressive and life-threatening syndrome of excessive immune activation. It most frequently affects infants from birth to 18 months of age, with highest incidence reported in those <3 months,[44] but it is also observed in children and adults of all ages. Hemophagocytic lymphohistiocytosis can occur as a familial or sporadic disorder, and it can be triggered by a variety of events that disrupt immune homeostasis. Infection is a common trigger both in those with a genetic predisposition and in sporadic cases. It is critical to make an early diagnosis and start treatment, but the greatest barrier to a successful outcome is often a delay in diagnosis due to the rarity of this syndrome, variable clinical presentation, and lack of specificity of the clinical and laboratory findings.

The diagnosis of HLH is often challenging because it mimics severe sepsis and there are several biochemical features that overlap with other diseases causing NALF, in particular GALD and tyrosinemia. While clinical and laboratory criteria for the diagnosis of HLH have been established, HLH therapy is often initiated in suspected patients who deteriorate despite supportive care, regardless of whether they fulfill stringent criteria.[45,46] The diagnostic criteria derived from a 2004 HLH study trial are as follows:

Five of the following eight findings: Fever ≥38.5°C, splenomegaly; pancytopenia, with at least two of the following: hemoglobin <9 gm/dL (for infants <4 weeks, hemoglobin <10 gm/dL); platelets <100,000/μL; absolute neutrophil count <1000/μL, hypertriglyceridemia (fasting triglycerides >265 mg/dL), and/or hypofibrinogenemia (fibrinogen <150 mg/dL); hemophagocytosis in bone marrow, spleen, lymph node, or liver; low or absent NK cell activity; ferritin >500 ng/mL, elevated soluble CD25 [soluble IL-2 receptor alpha (sIL-2R)].

The goal of therapy for patients with HLH is to suppress life-threatening inflammation by destroying immune cells. Induction therapy based on the HLH-94 protocol consists of a series of weekly treatments with dexamethasone and etoposide (VP-16). Intrathecal methotrexate and hydrocortisone are given to those with central nervous system disease. After induction, patients who are recovering are weaned off therapy, while those who are not improving are continued on therapy as a bridge to allogeneic hematopoietic cell transplantation (HCT). Hematopoietic cell transplantation will be required in those with an HLH gene mutation, central nervous system disease, or disease relapse (Table 1).[47]

**Table 1:** Summary on causes of NALF: incidence, mechanism, clinical features, diagnosis, and management

| | *Gestational alloimmune liver disease (GALD)* | *Hemophagocytic lymphohistiocytosis (HLH)* | *Mitochondrial diseases* | *Metabolic* | *Ischemic injury* |
|---|---|---|---|---|---|
| Incidence | 60–70% of NALF | 3–10% of NALF | 2–12% of NALF | Rare | 4% of NALF |
| Mechanism | Is a result of maternal alloimmune injury caused by transplacental passage of specific reactive maternal immunoglobulin G (IgG)<br>The maternal alloantibody activates fetal complement cascade to produce a membrane attack complex and fetal liver injury | Syndrome of excessive immune activation.<br>May be primary (gene mutations affecting the cytotoxic function of NK cells and T cells, in particular perforin) or secondary to viral infection | Broad classification of gene-based diseases includes: 1. Respiratory chain defects, e.g., BCS1L mutation (GRACILE syndrome);<br>2. Errors in fatty acid oxidation, e.g., carnitine palmitoyltransferase II (CPTII) deficiency and<br>3. Mitochondrial DNA depletion syndromes e.g., deoxyguanosine kinase (DGUOK) deficiency, MPV17 mutations | Three autosomal recessive inherited diseases have the potential for producing NALF:<br>1. Hereditary tyrosinemia type I,<br>2. Galactosemia and<br>3. Hereditary fructose intolerance | Decreased blood flow to the liver can result in increased transaminase levels and coagulopathy |
| Clinical features | IUGR<br>Hypoglycemia<br>Coagulopathy<br>Hypoalbuminemia<br>Ascites<br>Hyperbilirubinemia both unconjugated and conjugated<br>Normal or minimally abnormal AST, ALT | Fever, hepatosplenomegaly<br>Normal or abnormal LFTs | Hypotonia<br>Seizures<br>Failure to thrive<br>Poor suck<br>Hypoglycemia | Jaundice, hepatomegaly, ascites, failure to thrive, hypoglycemia, coagulopathy<br>**Tyrosinemia type I** Acute gastrointestinal bleeding<br>**Galactosemia:** *Escherichia coli* sepsis, Cataracts<br>**HFI:** Postprandial hypoglycemia<br>Lactic acidosis, renal failure, seizures, coma | Risk factors for hypoxic liver injury include: Perinatal asphyxia, hypovolemic or cardiogenic shock, and right-sided heart failure |
| Diagnosis/lab findings | T2 weighted MRI: Extrahepatic or extrareticuloendothelial iron overload in pancreatic acinar cells, myocardium, thyroid follicular epithelium, adrenal cortex and mucosal glands;<br>High levels of ferritin (>800 ng/mL and <7000 ng/mL)<br>High alpha-feto protein levels >300,000 ng/mL<br>Salivary gland biopsy confirming siderosis | Pancytopenia; Hypertriglyceridemia and/or hypofibrinogenemia; Elevated ferritin >20,000 ng/mL low/absent NK cell activity; elevated soluble CD25 (soluble interleukin 2 receptor); and evidence of hemophagocytosis in bone marrow, ascites, spleen, lymph node, or liver | Metabolic distress, such as hypoketotic hypoglycemia with lactic acidosis—which may worsen with glucose provision.<br>ALT-typically high and often 100–500 IU/L<br>INR-moderate/significant increase | **Tyrosinemia type I** increased levels of succinylacetone in the urine.<br>**Galactosemia:** Newborn screening test<br>Urine for reducing substances (positive if baby has been fed for 24–48 hours)<br>**HFI:** Molecular testing of the ALDOB gene | Significant increase in transaminases (1,000–6,000 IU/L)<br>INR—moderate/significant increase<br>Hypoglycemia-variable<br>Ferritin level—variable depending on the underlying cause |

| | | | | | |
|---|---|---|---|---|---|
| Management | IV immunoglobulins (IVIG)<br>Exchange transfusion with repeated dose of IVIG.<br>Supportive care | Chemotherapy<br>HCT | Depends on the specific mitochondrial diagnosis | **Tyrosinemia:** Dietary restriction of tyrosine and phenylalanine as well as use of 2 (2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTSB)<br>**Galactosemia:** Elimination of lactose and galactose from the diet<br>**HFI:** Strict avoidance of fructose-containing and sucrose-containing food and products such as sorbitol | Supportive care<br>Severe, non-responsive cases investigate for other causes of NALF<br>Consider Liver transplant |

## Learning Points

### What We Learnt from these Three Cases of NALF

- High index of suspicion for liver failure even in newborns with sepsis.
- Start empiric antibiotics and acyclovir therapy until HSV is excluded.
- History (especially maternal pregnancy history and consanguinity) and physical examination. Initiate diagnostic evaluation immediately, including cultures and viral PCRs (especially for HSV and enterovirus), obtain CBC, liver transaminases, INR, ferritin, alpha-fetoprotein, lactate levels, urinary succinyl acetone, and urinalysis as a minimum to focus on the differential diagnosis since most causes of NALF can be differentiated into four categories based on tests (GALD, viral infections, metabolic disorders, HLH, and ischemic injury).
- Provide supportive care to all neonates while determining underlying cause for NALF, maintain euglycemia, provide adequate nutrition (only glucose).
- Coagulation studies should be performed in all infants with biochemical evidence of abnormal liver function tests (ALT, AST, GGT, ALP).
- Medical management of GALD includes intravenous immunoglobulin (1 gm/kg) and double volume exchange transfusion, which can lead to favorable outcome.
- Liver transplant may be considered in neonates not responding to supportive care or specific therapy. Early consultation with a liver transplant center is encouraged.
- Mothers of infants with GALD should be referred for specialist assessment prior to a next pregnancy. Management with IVIG starting at gestational week 14 should be initiated.
- In case of enterovirus causing NALF, therapy is usually supportive care; however IVIG can be considered.
- Making an early diagnosis and initiation of appropriate HLH treatment with the view toward bone marrow transplant is critical for best outcome.

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