January–March 2023 Volume 2 Issue 1

newborn Official Journal of the Global Newborn Society

ISSN: 2769-514X

January–March 2023
Volume 2
Issue 1
ISSN: 2769-514X

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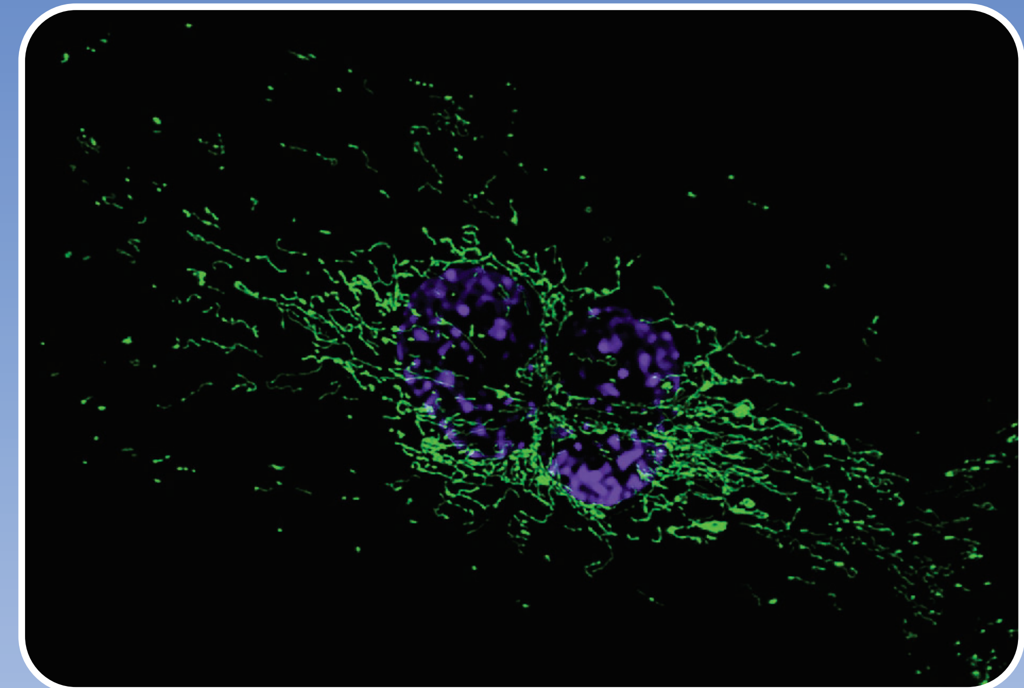

Mitochondrial Dynamics during Development. Mitochondria (green) in a dividing endothelial cell. Nuclei (blue/purple) were stained with DAPI.

Other highlighted articles:

Innate Immune Memory in Macrophages
Pathophysiology of Enteropathogenic Escherichia coli-induced Diarrhea
Congenital Chikungunya Virus Infections

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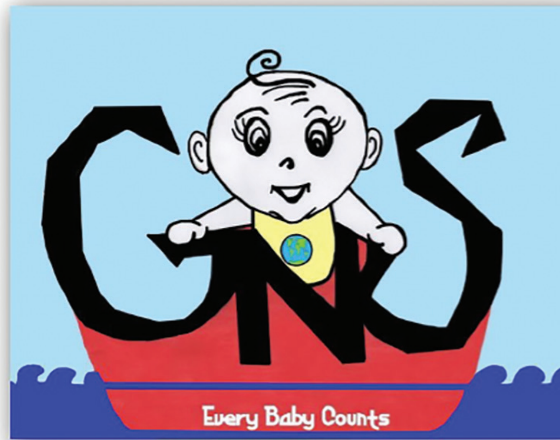

Global Newborn Society

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 cover shows pictures and titles from articles chosen by the editors to be specifically highlighted.

Instructions to Authors

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

Issue Information

Volume 2, Issue 1; January-March 2023
ISSN: 2769-514X
Copyrights: GNS, LLC.
Published: GNS, LLC; 6114 Lily Garden, Clarksville, MD, USA; Ph +1 708 910 8729
Printed: Jaypee Brothers Medical Publishers
4838/24, Ansari Road, Darya Ganj, Delhi - 110 002
Phone: +91 11 4357 4357, Fax: +91 11 4357 4314

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Editorial

10.5005/newborn-2-1-iv

Neonates are not adults; there are unusual pathogens, limitations in immunity, need for new ways to treat, and to monitor for adverse effects

Fetuses, newborns, and young infants are often highly susceptible to unusual pathogens that may be relatively benign in adults.[1–10] The debate continues on whether these epidemiological differences arise in the unique strains of pathogens,[1,11–15] immaturity of the immune system,[16–18] environmental factors in intensive care units[19–21] or in specific climatic conditions that allow these pathogens to reach larger numbers and/or densities.[22–24] These conditions have been difficult to treat as the infectious agents affect the fetus/neonate *in utero* and cannot be treated in a timely fashion, the candidate medications have limited efficacy, or the drugs have had unacceptable short- and long-term adverse effects.[22] Now, the possibility of developing effective vaccines has rekindled hope of finding new solutions.[25] Many of the unusual fetal/neonatal pathogens are transmitted by specific animal/insect vectors,[26,27] and the possibility of preventing insects from becoming long-term carriers through community-wide organizational and genetics-based efforts may bring new solutions.[28] Finally, as we understand epigenetics better, our improved understanding of immunity in fetuses and infants may help in finding new solutions.[29,30] Fetal/neonatal susceptibility reflects an age-related manifestation; we know that these changes in gene expression are epigenetic alterations.[31] If we can understand these changes, we might be able to make a difference. Finally, we still need new treatments and monitoring paradigms.[32] Not every treatment is uniformly available or affordable in different parts of the world.[33] Computational systems to assess, monitor, and treat these highly susceptible patients can be one way to make a difference.[34,35] If we know the possibilities, we can educate and motivate our care-providers to acquire and learn these tools.[36]

Our journal, the *Newborn* aims to cover fetal/neonatal problems that begin during pregnancy or occur after birth during the first 1000 days after birth. In this 1st issue of the second volume, we present 8 important articles (**Figure 1**). In an original study, Motta and his team[37] have evaluated the impact of a web-based software specifically designed for neonatal parenteral nutrition (PN) prescription on extrauterine growth restriction (EUGR) in a cohort of very-low-birth-weight (VLBW) infants. This article is very important because EUGR, a multifactorial condition, is increasingly recognized to be a risk factor for adverse long-term consequences for preterm infants.[38,39] They present a retrospective analysis of serial anthropometric measurements and comorbidities in a cohort of 119 VLBW infants treated with parenteral nutrition for at least 5 consecutive days. They show that a web-based system for prescription of neonatal PN may be useful for ensuring adequate intake of nutrients in preterm infants. These findings need further evaluation, both in terms of short- and long-term outcomes in a larger cohort.

There are 3 important reviews focused on the impact of infectious agents on the developing immunity. Kaur and Dudeja[40] present a scholarly review of the pathogenesis of Enteropathogenic *Escherichia coli* (EPEC) infections in infants. EPEC are an important cause of diarrhea in infants and young children all over the world.[41] Newer molecular diagnostic methods have identified typical and atypical strains of EPEC, and epidemiological studies show that atypical strains might be a more important cause of both endemic diarrhea and outbreaks of diarrhea.[42] The virulence mechanisms and physiopathology of the attaching and effacing lesion (A/E) and the type-three-secretion-system (T3SS) are complex.[43,44] A/E strains use a pool of locus of enterocyte effacement (LEE)-encoded and non-LEE-encoded effector proteins to subvert cellular and barrier properties of the intestinal epithelium.[45] More work is needed to understand the mechanisms of diarrhea in EPEC infections.

In two separate articles, Singh *et al.*[46] and Ethawi *et al.*[47] have reviewed perinatal infections by Chikungunya and Zika viruses. Chikungunya virus is widely transmitted in tropical and subtropical areas by Aedes mosquito vectors.[48] Perinatal/neonatal infections are fortunately not seen very frequently, but some infants can develop fever, thrombocytopenia, lymphopenia, pigmentary changes, and a maculopapular rash.[49] A small subgroup of these infants can develop encephalopathy and have poor neurocognitive outcomes.[50,51] There is no specific treatment, but some candidate vaccines are under evaluation.[52,53] Zika virus (ZIKV) is another viral disease transmitted by Aedes mosquitoes.[54] Infected mothers can vertically transmit ZV to their fetuses, particularly during the first and second trimesters.

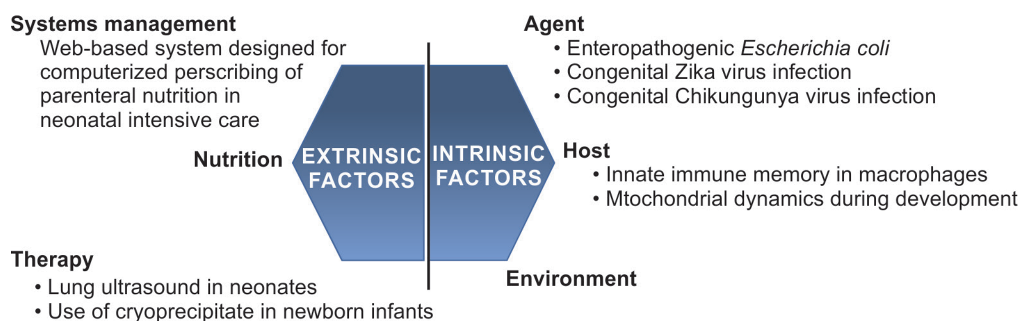

Fig. 1: **Areas of focus in the *Newborn*, volume 2, issue 1.** The *Newborn* has expanded the traditional agent-host-environment trinodal disease model to a hexagonal system. The three additional foci represent extrinsic factors that can affect health; these originate in therapy, nutrition, and systems management. In volume 2, issue 1, we cover 4 of these foci, namely infectious diseases, host factors, therapy, and systems management.

These early-gestation infections can manifest with structural abnormalities of the central nervous system.[55] Unfortunately, we do not have any specific treatment. Evaluation of candidate vaccines is still in an early phase.[56]

There are two extensive reviews focused on macrophages[57] and mitochondria,[58] respectively. In fetuses, neonates, and young infants, macrophages serve a very important role in immune defenses.[59] These cells have thus far been recognized as the primary mediators of innate immunity starting early during development.[60,61] Unlike adaptive immunity, macrophage-mediated defenses have traditionally not been recognized as antigen-specific.[62] However, increasing information suggests that macrophage responses do strengthen with repeated immunological triggers. This concept of innate memory in macrophages has been described as “trained immunity” or “innate immune memory (IIM).” This cellular memory is rooted in epigenetic and metabolic reprogramming.[63]

The second review is focused on mitochondria. As we recognize, mitochondria are dynamic membrane-bound organelles in eukaryotic cells.[64] These are important for the generation of chemical energy needed to power various cellular functions, and also support metabolic, energetic, and epigenetic regulation in various cells. These organelles most likely evolved about 2 billion years ago from α-proteobacteria, a subgroup of the purple non-sulfur bacteria, which most likely belonged to the order *Rickettsiales*.[65] This article provides extensive information about the ontogeny, ultrastructure, structure-function correlation, and biogenesis of mitochondria, and clinical manifestations of mitochondrial dysfunction.

Verma *et al*. have provided a very important review of point-of-care (POC) lung ultrasound, a new modality for assessing the severity of lung disease.[66,67] In the first part of the article, they have defined and discussed various findings in POC lung ultrasound. The second part describes the detection and serial evolution of diagnostic findings in conditions such as respiratory distress syndrome, transient tachypnea of newborn, atelectasis, pneumonia, air-leaks, and bronchopulmonary dysplasia/chronic lung disease of the newborn. These bedside assessments can help in timely evaluation and clinical management of these conditions.[68]

Finally, we have a review article focused on cryoprecipitate, a transfusion blood product that can be useful in critically ill neonates with coagulopathy.[69] Admittedly, it is now being used more often in situations when specific recombinant clotting factors are not available, but it can be life-saving in resource-limited regions of the world.[70,71] Cryoprecipitate is derived from fresh-frozen plasma, and is highly enriched in coagulation factors I (fibrinogen), VIII, and XIII; von Willebrand factor (vWF); and fibronectin.[72] The review presents current information on the preparation, properties, and the clinical importance of cryoprecipitate in treating critically ill neonates.

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Akhil Maheshwari, MD
Kei Lui, MD
Mario Motta, MD

ORIGINAL RESEARCH

Extrauterine Growth Restriction in Preterm Very Low Birth Weight Infants: The Use of a Web-based System Designed for Computerized Prescribing of Parenteral Nutrition in Neonatal Intensive Care

Mario Motta[1], Salvatore Aversa[2], Morotti Francesco[3], Akhil Maheshwari[4], Cesare Tomasi[5], Francesco Maria Risso[6]

Received on: 15 January 2023; *Accepted on*: 28 February 2023; *Published on*: 06 April 2023

Abstract

Aim: Extrauterine growth restriction (EUGR) is a multifactorial condition that may lead to long-term consequences for preterm infants. Providing adequate nutrition is one of the keys to ameliorating growth. Technology can help clinicians with powerful tools. We evaluate the impact of a web-based software specifically designed for neonatal parenteral nutrition (PN) prescription on EUGR in a cohort of very low birth weight (VLBW) infants.

Materials and methods: We retrospectively analyzed anthropometric measurements (AMs) and comorbidities in a cohort of 119 VLBW infants treated with PN for at least 5 consecutive days. International Fetal and Newborn Growth Consortium for the 21st Century (INTERGROWTH-21st) standards were used to identify small for gestational age (SGA, birth weight < 10th centile) infants and to define EUGR. EUGR was defined as "cross-sectional" (AMs < 10th percentile at discharge) and "longitudinal" (loss in AMs *Z*-score from birth to discharge > 1 standard deviation [SD]).

Results: Nutritional intakes were consistent with current available nutritional guidelines. There were significant differences in the measured incidence of EUGR depending on the adopted definition. The longitudinal definition appeared to be the most appropriate than the cross-sectional one for identifying postnatal growth failure in preterm infants. Lower lipid intake and longer durations of PN were risk factors for poor growth in weight and head circumference (HC). Metabolic disorders, such as cholestasis, hyperglycemia, and hypertriglyceridemia, had stronger links with lower AMs and longer PN needs than just the nutritional intakes. No relationships were observed between the most of comorbidities associated with prematurity and EUGR.

Conclusion: A web-based system for the prescription of neonatal PN seems to be useful for ensuring adequate intakes in preterm infants. Further studies with larger sample sizes could be designed for evaluating the application of this software within a neonatal network and its effect on postnatal growth.

Clinical significance: The use of an electronic prescribing system designed for neonatal care can help neonatologists in giving VLBW infants the correct intake of nutrients.

Keywords: Computerized prescribing, Extrauterine growth restriction, Newborn, Parenteral nutrition.

Newborn (2023): 10.5005/jp-journals-11002-0052

Introduction

Preterm very low birth weight (VLBW) infants are at risk of extrauterine growth restriction (EUGR), which has a potential impact on neurodevelopmental outcomes.[1–5] Several factors have been associated with EUGR, including male sex, small for gestational age (SGA) infants, comorbidities of prematurity, and nutritional intakes.[6,7] Guaranteeing optimal nutrition is one of the vital aspects in preterm VLBW infants to ensure adequate postnatal growth and organ development.[8,9] Because of the immaturity of the gastrointestinal system and the limited stores of nutrients,[10] preterm VLBW infants usually need parenteral nutrition (PN) in the first few weeks after birth life to achieve their nutritional requirements of energy, proteins, and lipids, until those can be achieved by full enteral feedings (FEF).[11–13]

In this setting, the use of a specific software dedicated to PN ordering could help to improve the prescription and the final product in many ways.[11,14,15] It could potentially reduce prescribing errors and compatibility and stability of the PN solutions.[16] These software tools could also guide prescribers to order appropriate nutrients and energy in relation to gestational and postnatal age.[14,17] This study aimed to evaluate the following: (a) The use of

[1–3,6]Neonatal Intensive Care Unit, Children's Hospital, ASST Spedali Civili, Brescia, Italy

[4]Weatherby Healthcare, Fort Lauderdale, Florida, United States of America

[5]Department of Experimental and Applied Medicine, ASST Spedali Civili, University of Brescia, Brescia, Italy

Corresponding Authors: Mario Motta, Neonatal Intensive Care Unit, Children's Hospital, ASST Spedali Civili, Brescia, Italy, Phone: +390303995219, e-mail: mario.motta@asst-spedalicivili.it; Salvatore Aversa, Neonatal Intensive Care Unit, Children's Hospital, ASST Spedali Civili, Brescia, Italy, Phone: +390303995219, e-mail: salvatore.aversa@asst-spedalicivili.it.

How to cite this article: Motta M, Aversa S, Francesco M, *et al*. Extrauterine Growth Restriction in Preterm Very Low Birth Weight Infants: The Use of a Web-based System Designed for Computerized Prescribing of Parenteral Nutrition in Neonatal Intensive Care. Newborn 2023;2(1):1–10.

Source of support: Nil

Conflict of interest: Dr. Akhil Maheshwari is associated as Editor-in-Chief of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of the Editor-in-Chief and his research group.

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the web-based system,[18] Par/Ent®, for computerized prescription of PN in preterm VLBW infants; (b) the occurrence of EUGR in relation to demographic characteristics at birth, PN intakes and common co-morbidities of prematurity;[19] and (c) the frequency of complications in relation to PN intakes.[20]

Materials and Methods

This is a population-based study of preterm VLBW infants who were admitted to the Neonatal Intensive Care Unit of Children's Hospital, ASST Spedali Civili of Brescia, Italy, and received PN from the day of birth for at least five consecutive days. Data were retrospectively collected from March 2019 to October 2020. Exclusion criteria were death in the first 15 days of life, major congenital malformations, genetic syndromes, and delayed admission after the first day following birth.

PN was prescribed using the computerized web-based system, Par/Ent®, which was designed specifically for neonatal intensive care by Link Up, S.r.l of Italy, on behalf of the Italian Society of Neonatology. After entering the gestational age and weight of the infant into the program, the software suggests nutritional intakes per current guidelines.[21,22] It also provides functionalities for the following: (a) Integration between enteral and parenteral supplies through an algorithm that avoids the dispersion of nutrients and ensures correct overall intakes, (b) counting and appropriate scaling of drugs, (c) the use of separate lines for PN and for infusing blood components, and (d) evaluating the compatibility of nutrients and drugs with the PN bag. Once data are entered for enteral feeding, intravenously administered drugs, and transfusions of blood components, the system recalculates the doses of all components so that the total amount of liquids and nutrients remains as prescribed. Moreover, by choosing the prescription method called "recommended calculation," the system takes coefficients of intestinal absorption of the nutrients into account.[23] It also has a monitoring system that produces alerts in case of lipid emulsion instability, the enhanced risk for insoluble salt precipitates, and excessive osmolarity of the peripheral infusion.

Clinical and laboratory data were collected using the electronic healthcare applications, Milos 1.0 (Gruppo Finmatica, Italy) and Fenix OE (EL.CO. Italy), while daily intake and PN solutions data were collected using Par/Ent® database. Percentile and *Z*-scores for weight, length and head circumference (HC) were calculated using the International Fetal and Newborn Growth Consortium for the 21st Century (INTERGROWTH-21st) international growth standards for newborn size and postnatal growth of preterm infants, at birth and at discharge, respectively.[24–26]

Small for gestational age was defined as a birth weight below the 10th percentile for gestational age.[27] To define EUGR, we used the following two definitions: (a) Weight, length, and HC below the 10th percentile at discharge, the "cross-sectional EUGR"[28] and (b) *Z*-scores[29] for weight, length, and HC loss between birth and discharge below 1 standard deviation (SD), the "longitudinal EUGR."[19,30] Infants were classified according to these definitions to be with- or without-EUGR for each measure. The following definitions for comorbidities of prematurity and PN complications were used: cholestasis was defined as a conjugated when the direct bilirubin levels were above 2 mg/dL when the total bilirubin was below 5 mg/dL, or if these levels were above 20% of total bilirubin levels higher than 5 mg/dL[31]; hypertriglyceridemia as serum triglycerides >250 mg/dL;[32] hyperglycemia as repeated blood glucose levels above 180 mg/dL that were treated with continuous insulin infusions;[33] hypophosphatemia as a serum phosphate level below 5 mg/dL; severe hypophosphatemia as serum phosphate levels below 3.1 mM; and hypercalcemia as serum calcium levels above 11 mg/dL.[34] Neonatal sepsis (symptomatic infants with a pathogen isolated from the blood culture) was classified as early-onset (EOS; <72 hours of life) or late-onset (LOS; >72 hours of life).[35] Necrotizing enterocolitis (NEC) was defined according to the Vermont–Oxford Network (VON) criteria (clinical and radiographic gastrointestinal signs).[36] We defined bronchopulmonary dysplasia (BPD; any severity) according to Jobe and Bancalari's original definition,[37] retinopathy of prematurity (ROP) using the International Classification of ROP guidelines,[38] and intra-ventricular hemorrhage (IVH) according to the criteria of Papile et al.[39] Patent ductus arteriosus (PDA) was diagnosed when the echocardiography showed findings consistent with at least one of the following: Left to right shunt, bidirectional shunt, and systolic or continuous murmur, and these were detected in the contextual presence of above or equal to 2 of the clinical signs such as hyperdynamic precordium, palpitations, systemic arterial hypertension, pulmonary vascular congestion, and cardiomegaly.[40,41]

Demographic and clinical information data and nutritional intakes were analyzed using descriptive statistics.[42] Continuous variables were presented as the median and interquartile range (IQ) and were compared using the Mann–Whitney U test.[43] Categorical variables were presented as the frequency and percentage and were compared using Fisher's exact test.[44] To identify the association between multiple variables, logistic regression was used.[45] Statistical tests were considered significant for $p < 0.05$.[46] We used the software programs MedCalc for Windows (MedCalc Software, Mariakerke, Belgium) and Statistica for Windows (StatSoft, Inc., Tulsa, OK, USA) to analyze these data.[47]

Results

During our study period, we reviewed the case records of 133 VLBW infants who were treated with PN for at least 5 days at the NICU of Spedali Civili, Italy. Fourteen infants were excluded from the study cohort; the reasons were death during the first 15 days after birth ($n = 3$), congenital malformations ($n = 9$), transfer to another hospital within a few days after hospitalization ($n = 1$), and admission after the first day following birth ($n = 1$). A total of 119 infants were included in the study. Demographic characteristics of infants per the INTRERGROWTH-21st standards[24,25] are presented in Table 1.

Male sex was not associated with either cross-sectional or longitudinal EUGR for all definitions. The occurrence of EUGR for weight, length, and HC and its relationship with the demographic characteristics at birth are shown in Table 2. The cross-sectional definitions for weight and HC showed significantly higher frequencies for EUGR than the longitudinal definition. However, the occurrence of EUGR for length was similar between the two definitions. According to the cross-sectional definition, infants with EUGR for weight had a higher gestational age at birth than those without postnatal growth retardation. Extrauterine growth restriction infants according to the longitudinal definition for weight had a lower gestational age. Cross-sectional EUGR for length infants had birth weights and lengths lower than those without EUGR. Infants with longitudinal EUGR for length had lower gestational ages, birth weights, and HCs than those without EUGR. Cross-sectional EUGR for HC infants had lower birth weights, lengths,

Table 1: Demographic characteristics of infants according to INTERGROWTH-21st standards

Gestational age (weeks)	29.7	(27.8–31.7)
Female, number (%)	63	(53)
Birth weight (gm)	1129	(922–1335)
Birth weight *Z*-score	–0.82	(−1.70 to −0.08)
Birth weight percentile	20.5	(4.4–46.7)
SGA, number (%)	42	(35)
Birth lengths (cm)	37.5	(35.0–39.2)
Birth lengths *Z*-score,	–0.83	(−1.40–0.01)
Birth lengths percentile	20.3	(7.9–48.5)
Birth head circumference (cm)	26.0	(25.0–27.9)
Birth head *Z*-score	–0.86	(−1.65 to −0.32)
Birth head percentile	19.4	(4.9–37.1)
Lengths of stay (days)	54.0	(38.0–75.0)
Discharge PMA (weeks)	37.6	(36.2–39.5)
Discharge weight (gm)	2190	(2001–2703)
Discharge weight *Z*-score	–1.07	(−2.00 to −0.44)
Discharge weight percentile	13.5	(2.2–31.1)
Discharge length (cm)	45.0	(43.0–47.0)
Discharge length *Z*-score	–1.46	(−2.60 to −0.57)
Discharge length percentile	7.2	(0.5–28.2)
Discharge head circumference (cm)	32.0	(31.0–33.5)
Discharge HC *Z*-score	–0.77	(−1.99–0.10)
Discharge HC percentile	22.0	(2.3–54.0)

Values are expressed as number (%) and median (IQ). PMA, postmenstrual age

and HCs than those without EUGR. Infants with longitudinal EUGR for HC had lower gestational ages and birth weights than those without EUGR. The relationship between nutritional characteristics, including nutrients intake, and the occurrence of EUGR for weight, length, and HC are summarized in Tables 3 to 5.

Infants with postnatal growth retardation per the cross-sectional definition of EUGR had lower 7 days calcium/phosphate (C/P) ratios than the subgroup who did not have EUGR. Length- and HC-restricted infants had lower parenteral lipid intakes. When the longitudinal EUGR definition was applied, weight- and length-restricted infants showed longer durations of PN than those without EUGR, with consequent delay in the achievement of FEF. Length- and HC-restricted infants were also more likely to have received less parenteral lipids.

The relationship between comorbidities of prematurity and the occurrence of EUGR for weight, length, and HC is presented in Table 6. With the cross-sectional definition, SGA infants developed EUGR more frequently for all three anthropometric parameters. In contrast, they developed EUGR less frequently for weight and HC per the longitudinal definitions. According to these findings, SGA infants showed significantly higher Δ*Z*-scores for weight and HC (see Supplementary Material). Late–onset sepsis was significantly associated with EUGR for weight according to the longitudinal definition. Other evaluated comorbidities (EOS, IVH, NEC, PDA, BPD, and ROP) were not associated with EUGR. Infants who developed LOS showed significantly lower Δ*Z*-scores of lengths (Supplementary Material). Similarly, infants with NEC showed a significantly lower Δ*Z*-scores for lengths and HC (see Supplementary Material).

The complications related to PN showed associations with the demographic characteristics of the patients at birth, the duration of PN, and the parenterally administered nutrients (Table 7). Cholestasis was significantly associated with lower birth weight,

Table 2: Comparison between cross-sectional (below 10th percentile) and longitudinal (*Z*-score loss > 1) EUGR definitions among different sizes, and comparison of demographic characteristics between infants with or without EUGR for different definitions of the three anthropometric parameters

	EUGR–weight (below 10th percentile)			
	No, n = 66 (55)		*Yes, n = 53 (45)****	
Gestational age (weeks)	28.8***	(27.5–30.5)	31.5***	(29.0–32.7)
Birth weight (gm)	1145	(948–1339)	1043	(905–1305)
Birth length (cm)	38.0	(35.0–39.0)	36.7	(34.5–40.0)
Birth head circumference (cm)	26.2	(25.0–27.5)	26.0	(24.7–28.0)
	EUGR–weight (Z-score loss > 1); no (0) yes (1)			
	No, n = 95 (80)		*Yes, n = 24 (20)****	
Gestational age (weeks)	30.50***	(28.0–32.0)	28.43***	(26.8–29.1)
Birth weight (gm)	1137	(932–1335)	1040	(667–1329)
Birth length (cm)	37.5	(35.0–40.0)	37.0	(34.6–39.0)
Birth head circumference (cm)	26.5	(25.0–28.0)	25.0	(23.1–27.0)
	EUGR–length (below 10th percentile); no (0) yes (1)			
	No, n = 51 (43)		*Yes, n = 68 (57)*	
Gestational age (weeks)	29.7	(28.0–30.8)	29.7	(27.5–32.3)
Birth weight (gm)	1221**	(998–1380)	997**	(734–1270)
Birth lengths (cm)	38.5**	(37.0–40.0)	36.0**	(34.5–38.5)
Birth head circumference (cm)	26.5	(25.0–28.0)	26.0	(24.0–27.6)

(Contd)

Table 2: *(Contd...)*

	EUGR–length (Z-score loss > 1); no (0) yes (1)			
	No, n = 65 (55)		*Yes, n = 54 (45)*	
Gestational age (weeks)	30.6**	(28.8–31.9)	28.43**	(27.2–31.2)
Birth weight (gm)	1215**	(977–361)	996**	(707–1225)
Birth length (cm)	38.0	(35.0–39.0)	36.7	(35.0–9.7)
Birth head circumference (cm)	27.0*	(25.1–28.0)	25.5*	(23.6–27.0)
	EUGR–HC (below 10th percentile); no (0) yes (1)			
	No, n = 75 (63)		*Yes, n = 44 (37)***	
Gestational age (weeks)	29.9	(28.0–31.6)	29.0	(27.5–32.5)
Birth weight (gm)	1200**	(972–1366)	991.0*	(669–1211)
Birth length (cm)	38.0***	(36.0–40.0)	35.7***	(33.0–38.0)
Birth head circumference (cm)	27.0**	(25.0–28.0)	25.5**	(22.5–27.0)
	EUGR–HC (Z-score loss > 1); no (0) yes (1)			
	No, n = 97 (81.5)		*Yes, n = 22 (18.5)***	
Gestational age (weeks)	30.0**	(28.0–31.8)	28.1**	(26.0–29.6)
Birth weight (gm)	1171*	(948–1340)	978*	(721–1187)
Birth length (cm)	37.7	(35.0–40.0)	36.0	(34.7–38.2)
Birth head circumference (cm)	26.0	(25.0–28.0)	26.2	(23.2–27.0)

Values are expressed as number (%) and median (IQ). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Fisher's exact test or Mann–Whitney *U* test.

Table 3: Comparison of nutrition between infants with or without EUGR for weight according to cross-sectional (below 10th percentile) and longitudinal (*Z*-score loss > 1) definitions

	EUGR–weight (below 10th percentile)			
	No, n = 64		*Yes, n = 46*	
Duration of PN (days)	17	(12–25)	15	(11–29)
First day of MEF	1	(1–2)	2	(1–2)
First day of FEF	27	(18–37)	23	17–39
PN kcal	73.0	(69.6–77.5)	74.7	(69.2–79.4)
Total kcal	103.7	(99.2–108.2)	102.0	98.2–108.8
PN carbohydrate (gm)	10.2	9.6–11.1	10.7	9.5–11.4
Total carbohydrate (gm)	13.3	12.8–14.0	13.5	12.7–14.3
PN protein (gm)	3.1	2.9–3.2	3.1	3–3.2
Total protein (gm)	3.7	3.6–3.8	3.7	3.6–3.8
PN lipid (gm)	2.2	1.8–2.4	2.0	1.8–2.3
Total lipid (gm)	3.5	3.3–3.9	3.6	3.2–3.9
PN calcium 7 days (mg)	62.8	57.8–68.5	62.8	57.6–68.5
PN phosphorus 7 days (mg)	49.8	45.7–55.0	51.4	47.8–55.7
Ca/P ratio 7 days	0.98*	(0.94–1.02)	0.95*	(0.91–0.98)
	EUGR–weight (Z-score loss > 1)			
	No, n = 88		*Yes, n = 20*	
Duration of PN (days)	14.5*	(11.0–22.0)	25.0*	(19.2–43.7)
First day of MEF	1.0	(1.0–2.0)	1.0	(1.0–2.0)
First day of FEF	23.0*	(17.0–30.0)	38.5*	(27.0–49.5)
PN kcal	74.5	(69.6–78.0)	71.2	(67.6–80.2)
Total kcal	102.5	(98.5–108.1)	105.1	(100.9–109.2)
PN carbohydrates (gm)	10.4	(9.6–11.2)	10.4	(9.2–11.5)
Total carbohydrates (gm)	13.3	(12.7–14.0)	13.4	(13.0–14.3)
PN protein (gm)	3.1	(2.9–3.2)	3.1	(2.9–3.2)
Total protein (gm)	3.7	(3.5–3.8)	3.7	(3.7–3.8)
PN lipid (gm)	2.2	(1.9–2.3)	2.0	(1.8–2.4)

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Total lipid (gm)	3.5	(3.2–3.9)	3.8	(3.4–4.1)
PN calcium 7 days (mg)	62.8	(58.6–68.6)	62.8	(56.4–68.4)
PN phosphorus 7 days (mg)	50.8	(46.4–55.7)	50.0	(45.9–54.4)
Ca/P ratio 7 days	0.97	(0.94–1.01)	0.95	(0.9–1.0)

Values are expressed as median (IQ); *$p < 0.01$ by Mann–Whitney *U* test. FEF, full enteral feeding; MEF, minimal enteral feeding

Table 4: Comparison of nutrition between infants with or without EUGR for length according to cross-sectional (below 10th percentile) and longitudinal (*Z*-score loss > 1) definitions.

	EUGR–length (below 10th percentile)			
	No, n = 48		*Yes, n = 62*	
Duration of PN (days)	16	(11–22)	18	(12–35)
First day of MEF	1	(1–2)	2	(1–2)
First day of FEF	26	(17–30)	25	(19–45)
PN kcal	75.0	(69.6–78.7)	73.4	(68.9–78.2)
Total kcal	104.1	(99.8–108.8)	102.9	(98.7–108.2)
PN carbohydrate (gm)	10.3	(9.6–11.1)	10.5	(9.5–11.3)
Total carbohydrate (gm)	13.3	(12.6–14.0)	13.4	(12.9–14.2)
PN protein (gm)	3.1	(2.9–3.2)	3.1	(2.9–3.2)
Total protein (gm)	3.7	(3.6–3.8)	3.7	(3.6–3.8)
PN lipid (gm)	2.2**	(1.9–2.4)	2.0**	(1.7–2.2)
Total lipid (gm)	3.7	(3.4–3.9)	3.5	(3.2–3.9)
PN calcium 7 days (mg)	61.4	(57.1–69.3)	63.6	(58.6–68.6)
PN phosphorus 7 days (mg)	49.3	(45.7–55.7)	51.2	(48.0–55.3)
Ca/P ratio 7 days	0.97*	(0.94–1.02)	0.96*	(0.91–1.00)
	EUGR–length (Z-score loss > 1)			
	No, n = 61		*Yes, n = 69*	
Duration of PN (days)	14.0*	(11.0–23.0)	19.0*	(13.0–36.5)
First day of MEF	1.0	(1.0–2.0)	2.0	(1.0–2.0)
First day of FEF	22.0**	(17.0–30.0)	29.0**	(19.0–49.0)
PN kcal	73.4	(70.3–78.0)	73.5	(67.1–78.2)
Total kcal	102.7	(99.3–107.8)	103.4	(98.5–108.8)
PN carbohydrate (gm)	10.4	(9.6–11.1)	10.3	(9.5–11.3)
Total carbohydrate (gm)	13.4	(12.724–14.0)	13.4	(12.9–14.3)
PN protein (gm)	3.1	(2.9–3.2)	3.1	(3.0–3.2)
Total proein (gm)	3.7	(3.6–3.8)	3.7	(3.6–3.8)
PN lipid (gm)	2.2**	(1.9–2.4)	2.0**	(1.7–2.2)
Total lipid (gm)	3.6	(3.3–3.9)	3.5	(3.3–4.0)
PN calcium 7 days (mg)	62.1	(57.3–69.1)	63.9	(57.8–68.2)
PN phosphorus 7 days (mg)	50.0	(45.7–55.9)	50.7	(47.8–54.7)
Ca/P ratio 7 days	0.97	(0.94–1.0)	0.95	(0.92–1.0)

Values are expressed as median (IQ); *$p < 0.05$; **$p < 0.01$ by Mann–Whitney *U* test. FEF, full enteral feeding; MEF, minimal enteral feeding

but not with PN intakes. Hyperglycemia requiring insulin treatment was seen more frequently in infants with lower gestational age and lower birth weight, but not with carbohydrate intakes. The occurrence of hypertriglyceridemia was associated with lower gestational age and birth weight, but not with the total lipid intake. Cholestasis, hyperglycemia requiring insulin treatment, and hypertriglyceridemia were significantly associated with longer PN duration. Logistic regression confirmed these associations, but with a very low magnitude (see Supplementary Material). We did not find an association between calcium and phosphorus metabolism

Table 5: Comparison of nutrition between infants with or without EUGR for HC according to cross-sectional (below 10th percentile) and longitudinal (*Z*-score loss > 1) definitions

	EUGR–HC (below 10th percentile)			
	No, n = 71		*Yes, n = 39*	
Duration of PN (days)	16	(12–20)	19	(11–36)
First day of MEF	1	(1–2)	2	(1–2)
First day of FEF	24	(17–34)	28	(17–45)
PN kcal	75.4	(69.6–79.1)	72.5	(68.3–76.8)
Total kcal	103.8	(99.8–108.5)	101.7	(98.2–108.0)
PN carbohydrate (gm)	10.5	(9.6–11.2)	10.4	(9.4–11.3)
Total carbohydrate (gm)	13.4	(12.8–14.0)	13.4	(12.5–14.2)
PN protein (gm)	3.1	(2.9–3.2)	3.1	(2.9–3.2)
Total protein (gm)	3.7	(3.6–3.8)	3.7	(3.6–3.8)
PN lipid (gm)	2.2*	(1.9–2.3)	2.0*	(1.7–2.2)
Total lipid (gm)	3.6	(3.3–3.9)	3.6	(3.2–3.8)
PN calcium 7 days (mg)	63.5	(57.8–70.7)	62.5	(56.4–67.5)
PN phosphorus 7 days (mg)	51.3	(45.7–56.4)	50.5	(46.9–54.7)
Ca/P ratio 7 days	0.97	(0.93–1.0)	0.95	(0.91–0.99)
	EUGR–HC (Z-score loss >1)			
	No, n = 93		*Yes, n = 17*	
Duration of PN (days)	16	(12–26)	19	(13–33)
First day of MEF	1	(1–2)	2	(1–2)
First day of FEF	24	(17–38)	30	(19–38)
PN kcal	74.2	(69.5–78)	71.4	(68.7–76.1)
Total kcal	103.6	(98.9–108.6)	102.9	(97.6–105.6)
PN carbohydrate (gm)	10.4	(9.6–11.2)	10.2	(9.5–11.4)
Total carbohydrate (gm)	13.4	(12.7–14.2)	13.4	(12.9–13.9)
PN protein (gm)	3.1	(2.9–3.2)	3.1	(3.0–3.2)
Total protein (gm)	3.7	(3.6–3.8)	3.7	(3.6–3.8)
PN lipid (gm)	2.2*	(1.9–2.4)	2.0*	(1.6–2.2)
Total lipid (gm)	3.6	(3.3–3.9)	3.4	(2.9–3.8)
PN calcium 7 days (mg)	62.8	(57.8–68.5)	63.6	(57.5–67.8)
PN phosphorus 7 days (mg)	50.9	(45.9–55.7)	49.6	(46.6–52.6)
Ca/P ratio 7 days	0.96	(0.93–1.0)	1.0	(0.94–1.0)

Values are expressed as median (IQ); *$p < 0.05$ by Mann–Whitney *U* test. FEF, full enteral feeding; MEF, minimal enteral feeding

disorders with gestational age, birth weight, parenteral calcium and phosphorus intakes, and Ca/P ratio at 7 days of life.[48] Being SGA was also not associated with metabolism disorders of calcium and phosphate (Table 8).

Based on logistic regression, infants who developed EUGR for weight according to the cross-sectional definition were more significantly SGA at birth [logit: 3.7; OR: 42.5 (95% CI: 12.4–142.1); $p < 0.001$)]. Other demographic characteristics at birth, the length of PN dependence, and the occurrence of both LOS and NEC were not different from those who did not have EUGR for weight. Appling the longitudinal definition of EUGR for weight, no differences were observed using logistic regression analysis.

Infants with EUGR for length, according to cross-sectional definition, showed significantly lower gestational age, birth weight, and parenteral lipid intake compared to those who did not develop EUGR [logit -1.8; OR 0.16 (95% CI 0.04–0.68); $p = 0.013$]. The demographic characteristics at birth, duration of PN, and the occurrence of SGA, LOS, and NEC were similar between the same two groups. Similarly, applying the longitudinal definition of EUGR for length, infants with EUGR received lower parenteral lipid intakes [logit: –1.4; OR: 0.25 (95% CI: 0.07–0.85); $p = 0.026$] and had lower birth length; no other differences were observed using logistic regression analysis.

Logistic regression showed that infants who had EUGR for HC according to the cross-sectional definition, were more frequently SGA [logit: –1.5; OR: 4.6 (95% CI: 1.82–11.48); $p = 0.001$], whereas the other demographic characteristics at birth, the number of PN days, the parenteral lipid intake and the occurrence of both LOS and NEC were similar to infants who did not have EUGR for HC. When the longitudinal definition was used for EUGR for HC, infants with EUGR had lower parenteral lipid intakes [logit: –1.45; OR: 0.23 (95% CI: 0.06–0.91); $p = 0.037$]. No other differences were noted in logistic regression.

Discussion

We present an observational study of 119 preterm VLBW infants who were treated with PN using a specifically designed web-based

Table 6: Comparison between comorbidities of prematurity and EUGR occurrence

	SGA		EOS		LOS		NEC		BPD		IVH		ROP		PDA	
	No (N = 77)	Yes (N = 42)	No (N = 115)	Yes (N = 4)	No (N = 95)	Yes (N = 24)	No (N = 117)	Yes (N = 2)	No (N = 77)	Yes (N = 42)	No (N = 113)	Yes (N = 6)	No (N = 115)	Yes (N = 4)	No (N = 95)	Yes (N = 24)
EUGR–weight < 10th percentile	16*** (20)	37888 (88)	52 (45.2)	1 (25)	45 (47.3)	8 (33.3)	52 (45.2)	1 (50)	38 (49.3)	15 (35.7)	51 (45.1)	2 (33.3)	52 (45.2)	1 (25)	43 (45.2)	10 (41.6)
EUGR–weight loss Z > 1	20** (26)	3** (7)	21 (18,2)	2 (50)	16 (16.8)	7 (29.1)	22 (18.8)	1 (50)	14 (18.1)	9 (21.4)	21 (18.5)	2 (33.3)	23 (20)	0	17 (17.8)	6 (25)
EUGR–length < 10th percentile	36** (46)	32** (76)	66 (57.3)	2 (50)	5 (57.8)	13 (54.1)	67 (57.2)	1 (50)	42 (54.5)	26 (61.9)	65 (57.5)	3 (50)	66 (57.3)	2 (50)	54 (56.8)	14 (58.3)
EUGR–length loss Z > 1	36 (46)	16 (38)	51 (44.3)	1 (25)	36* (37.8)	16* (66.6)	51 (43.5)	1 (50)	30 (38.9)	22 (52.3)	50 (44.2)	2 (33.3)	51 (44.3)	1 (25)	39 (41)	13 (54.1)
EUGR–HC < 10th percentile	22** (28)	22** (52)	42 (36.5)	2 (50)	34 (35.7)	10 (41.6)	43 (36.7)	1 (50)	29 (37.6)	15 (35.7)	42 (37.1)	2 (33.3)	42 (36.5)	2 (50)	35 (36.8)	9 (37.5)
EUGR–HC loss Z > 1	18* (23)	2* (5)	18 (15.6)	2 (50)	15 (15.7)	5 (20.8)	19 (16.2)	1 (50)	12 (15.5)	8 (19)	20 (17.6)	0	0	0	15 (15.7)	5 (20.8)

Values are expressed as number (%). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Fisher's exact test. BPD, bronchopulmonary dysplasia; EOS, early onset sepsis; IVH, intraventricular hemorrhage; LOS, late-onset sepsis; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus, ROP, retinopathy of prematurity; SGA, small for gestational age

electronic prescription system. As shown in Table 3, all nutritional intakes, including that of carbohydrates, amino acids, lipids, calcium, and phosphorus, were consistent with the currently available guidelines.[24,25] Comparing nutritional intakes, we found that infants with EUGR for length and HC had received lower parenteral lipid intake than the non-EUGR infants (see Tables 4 and 5). These findings can be explained by considering that infants with EUGR for length and HC had significantly lower birth weights and may have had lower parenteral lipid tolerance. In addition, multivariate analysis controlling for the effect of other risk factors, such as prematurity and birth weight, showed a significant association between parenteral lipid intake and occurrence of cross-sectional EUGR for length and longitudinal EUGR for length and HC. These results suggest that lower parenteral lipid intake may be an independent risk factor for inferior growth outcomes for weight and HC in preterm VLBW infants.

In our study, among the comorbidities of prematurity, LOS was most frequently associated with longitudinal EUGR for length (Table 6). However, multivariate analysis did not confirm this association (see Supplementary Material). SGA infants were significantly more prone to be classified as EUGR when the cross-sectional definition was used. Interestingly, the longitudinal definition showed lower frequencies of EUGR than the cross-sectional definition. These findings could be explained by considering that the two different definitions of EUGR are based on arbitrary cut-off values that differ from each other. Our study showed that the incidence of EUGR was significantly different depending on the adopted definition (see Table 2).

SGA infants, who had poor intrauterine growth,[49] had a higher probability of having EUGR per the cross-sectional definition with a discharge weight <10th percentile for gestational age. In contrast, these infants were at lower risk of poor postnatal growth and EUGR per the longitudinal definition. These findings were confirmed in logistic regression; in our cohort, cross-sectional EUGR for weight and HC were associated with lower birth weights than longitudinal EUGR (see Supplementary Material). The results of this study suggest that the longitudinal EUGR definition using the INTERGROWTH-21st standards could be more appropriate to identify the risk of postnatal growth failure in preterm VLBW infants.[50] However, these findings need confirmation; one important limitation of our study is that there were only a few preterm births with the lowest gestational ages in our cohort.[24] The construction of standard charts for extremely preterm infants is problematic because there is no definitive information on the nutritional requirements of this relatively-limited population.[51] During the first postnatal weeks, monitoring of growth should be performed only to trace a growth trajectory rather than used as a tool to identify EUGR.

Regarding the safety of PN, it is already known that electronic prescribing systems can decrease the risk of errors and the use of a standardized electronic tool for PN prescription is recommended.[11,52] In our study, the use of Par/Ent®, a specifically-designed web-based system for neonatal care, allowed us to identify any association(s) between the nutritional intakes and the complications secondary to PN administration (Tables 7 and 8). No significant associations were found for cholestasis, hyperglycemia with the need for insulin treatment, hypertriglyceridemia, and disorders of calcium/phosphorus homeostasis. However, once again, the number of infants observed in our cohort might not be sufficient to rule out such associations.[53] Similarly, the absence of associations between EUGR and the most of comorbidities of prematurity might depend on the limited number of observations.[54]

Table 7: Relationship between PN complications, demographic characteristics at birth, duration of PN or parenteral nutrients intake

	Cholestasis			
	No, n = 96		*Yes, n = 23*	
Gestational age (weeks)	29.9	(28.0–31.9)	29.0	(27.8–30.7)
Birth weight (gm)	1165*	(940–1343)	982*	(695–1173)
Duration of PN (days)	14.5***	(11.0–21.0)	32.5***	(19.0–49.0)
PN kcal	73.6	(69.4–78.3)	73.6	(69.6–80.9)
PN carbohydrate (gm)	10.3	(9.5–11.1)	11.0	(9.9–11.5)
PN protein (gm)	3.1	(2.9–3.2)	3.0	(2.9–3.2)
PN lipid (gm)	2.2	(1.9–2.3)	2.0	(1.8–2.3)
SGA	33	(34)	9	(39)
	Insulin treatment for hyperglycemia			
	No, n = 108		*Yes, n = 11*	
Gestational age (weeks)	30.0***	(28.0–31.9)	24.8***	(24.5–28.3)
Birth weight (gm)	1171.5***	(967–1339.5)	658***	(577.2–706.2)
Duration of PN (days)	16**	(11–23)	46**	(23–53)
PN kcal	74.6	(69.4–78.6)	70.988	(69.8–71.9)
PN carbohydrate (gm)	10.4	(9.6–11.2)	10.229	(9.6–10.8)
SGA	38	(35)	4	(36)
	Hypertriglyceridemia			
	No, n = 91		*Yes, n = 27*	
Gestational age (weeks)	30.3***	(28.4–32.0)	26.6***	(24.7–29.0)
Birth weight (gm)	1180***	(987–1345.2)	695***	(593–1023.2)
Duration of PN (days)	15***	(11–20)	36***	(19–47)
PN kcal	75.0	(69.5–79.0)	72.0	(69.8–77.3)
PN lipid (gm)	2.2	(1.9–2.4)	2.0	(1.5–2.3)
SGA	33	(36)	9	(33)

Values are expressed as number (%) and median (IQ); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Fisher's exact test or Mann–Whitney U test.

Table 8: Relationship between metabolism disorders of calcium and phosphorus, demographic characteristics at birth and parenteral intakes of calcium and phosphorus.

	Hypophosphatemia 7 days			
	No, n = 61		*Yes, n = 58*	
Gestational age (weeks)	29.2	(27.8–31.3)	30.1	(28–32)
Birth weight (gm)	1187.5	(965.5–1339.5)	998	(920–1302)
PN calcium 7 days (mg)	62.5	(56.6–68.6)	64.3	(58.6–69.3)
PN phosphorus 7 days (mg)	49.6	(45.7–55.0)	52.1	(48.6–57.1)
Ca/P ratio 7 days	0.97	(0.94–1.0)	0.95	(0.93–1.0)
SGA	16	(26.2)	26	(44.8)
	Hypophosphatemia > 7 days			
	No, n = 101		*Yes, n = 18*	
Gestational age (weeks)	30	(28–32)	28	(25.4–29.6)
Birth weight (gm)	1165	(967–1339.5)	781	(658–1100)
PN calcium 7 days (mg)	62.8	(57.8–68.5)	63.2	(59–68.6)
PN phosphorus 7 days (mg)	50.7	(46.2–55.3)	51.1	(48.6–55.7)
Ca/P ratio 7 days	0.97	(0.93–1.0)	0.9	(0.91–1.0)
SGA	36	(35.6)	6	(33.3)
	Severe hypophosphatemia 7 days			
	No, n = 110		*Yes, n = 9*	
Gestational age (weeks)	29.7	(27.9–31.8)	28.4	(27.9–30.9)
Birth weight (gm)	1140	(931–1338)	943	(631.7–979)
PN calcium 7 days (mg)	62.8	(57.8–68.5)	64.3	(62.3–67.7)
PN phosphorus 7 days (mg)	50.3	(45.9–55)	56.4	(52.3–61.2)

Ca/P ratio 7 days	0.97	(0.93–1.0)	0.90	(0.87–0.95)
SGA	36	(32.7)	6	(66.6)
	Hypercalcemia			
	No, n = 106		*Yes, n = 13*	
Gestational age (weeks)	30	(28–31)	27.8	(24.8–29.0)
Birth weight (gm)	1135	(946.7–1320)	780.000	(637–1339)
PN calcium 7 days (mg)	62.8	(57.8–68.6)	66.429	(61.1–69.3)
PN phosphorus 7 days (mg)	50.7	(46.3–55.0)	55.000	(49.2–56.6)
Ca/P ratio 7 days	0.97	(0.93–1.0)	0.966	(0.93–1.0)
SGA	39	(36.7)	3	(23.1)

Values are expressed as number (%) and median (IQ); No difference by Fisher's exact test or by Mann–Whitney U test.

Conclusion

Even with the declared limitations, this study has highlighted the possible benefits of using a computerized web-based system for prescribing PN in the NICU setting. Our experience could be useful in designing a larger project to be applied within a neonatal network to evaluate the effects of PN on postnatal growth.

Clinical Significance

The use of a web-based system for the electronic prescribing of PN in neonatal care could help neonatologists in ensuring the correct intake of nutrients in preterm VLBW infants.

Author Contributions

Mario Motta and Salvatore Aversa contributed equally to the manuscript. All authors contributed to the manuscript revision, review, and approved the submitted version.

Supplementary Material

The supplementary material is available online on the website of https://www.newbornjournal.org/

Orcid

Mario Motta https://orcid.org/ 0000-0002-9579-2455

Salvatore Aversa https://orcid.org/0000-0003-2634-1498

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

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REVIEW ARTICLE

Use of Cryoprecipitate in Newborn Infants

Manvi Tyagi[1], Brunetta Guaragni[2], Alvaro Dendi[3], Atnafu Mekonnen Tekleab[4], Mario Motta[5], Akhil Maheshwari[6]

Received on: 09 November 2022; *Accepted on:* 30 November 2022; *Published on:* 06 April 2023

Abstract

Cryoprecipitate is a transfusion blood product derived from fresh–frozen plasma (FFP), comprised mainly of the insoluble precipitate that gravitates to the bottom of the container when plasma is thawed and refrozen. It is highly enriched in coagulation factors I (fibrinogen), VIII, and XIII; von Willebrand factor (vWF); and fibronectin. In this article, we have reviewed currently available information on the preparation, properties, and clinical importance of cryoprecipitate in treating critically ill neonates. We have searched extensively in the databases PubMed, Embase, and Scopus after short-listing keywords to describe the current relevance of cryoprecipitate.

Keywords: Cryoprecipitate, Cryoprecipitated antihemophilic factor, Factor I, Factor VIII, Factor XII, Fibrinogen, Newborn neonate infant, Transfusion product.

Newborn (2023): 10.5005/jp-journals-11002-0045

Highlights

- Cryoprecipitate is a transfusion product comprised of the insoluble precipitate that gravitates to the bottom of the container when fresh frozen plasma is thawed and refrozen. It contains physiologically relevant amounts of factors I (fibrinogen), VIII, XIII, vWF, and fibronectin.
- Cryoprecipitate is typically administered in a dose of 1 unit (40 mL) per 10 kg of body weight; this may raise the fibrinogen level by 50 mg/dL. In most neonates, the administration of 5–10 mL/kg is sufficient.
- For inherited coagulopathies such as hemophilia A, deficiency of factor XIII, hypofibrinogenemia, and vWD, cryoprecipitate transfusions are no longer recommended unless specific factor replacement products are not available.
- For treatment of acquired fibrinogen deficiency due to disseminated intravascular coagulation (DIC), severe liver failure, and consumptive coagulopathy, cryoprecipitate is primarily used in the presence of bleeding and fibrinogen levels less than 1 gm/L.

[1]Department of Pediatrics, Augusta University, Georgia, USA

[2,5]Department of Neonatology and Neonatal Intensive Care, Children's Hospital, ASST-Spedali Civili, Brescia, Italy

[3]Department of Neonatology, Centro Hospitalario Pereira Rossell, Universidad de la República, Montevideo, Uruguay

[4]Department of Pediatrics, St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia

[6]Global Newborn Society, Clarksville, Maryland, USA

Corresponding Author: Mario Motta, Department of Neonatology and Neonatal Intensive Care, Children's Hospital, ASST-Spedali Civili, Brescia, Italy, Phone: +1 030-3995219, e-mail: mario.motta@asst-spedalicivili.it

How to cite this article: Tyagi M, Guaragni B, Dendi A, *et al*. Use of Cryoprecipitate in Newborn Infants. Newborn 2023;2(1):11–18.

Source of support: Parts supported by the NIH grant R01HL133022.

Conflict of interest: Dr. Alvaro Dendi and Dr. Akhil Maheshwari are associated as the Editorial Board Members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial Board Members and their research group.

Introduction

Cryoprecipitate (cryo; cryoprecipitated antihemophilic factor) is a transfusion product derived from plasma, enriched in factors I (fibrinogen), VIII, XIII, vWF, and fibronectin.[1–5] It was historically labeled as the cryoprecipitated antihemophilic factor in view of the high concentrations of factor VIII and its hemostatic efficiency in patients with hemophilia A.[6–8]

Guidelines for the use of cryoprecipitate in neonatal medicine are limited to a few conditions. In the setting of inherited disorders of hemostasis, cryoprecipitate should be used as replacement therapy only if specific factor concentrate is not available while in the setting of acquired hypofibrinogenemia during DIC or liver failure, its use is considered standard therapy despite the lack of evidence.[9]

In this article, we aimed to review current information on the preparation, properties, and clinical importance of cryoprecipitate in critically ill neonates. We have extensively searched the databases PubMed, Embase, and Scopus after short-listing the keywords to describe the current relevance of cryoprecipitate. Furthermore, we reviewed the last 10 years of practice in a tertiary neonatal unit in Italy with the aim to describe the common clinical use of cryoprecipitate in critically ill neonates.

Preparation and Storage of Cryoprecipitate

Cryoprecipitate is prepared from whole blood (Flowchart 1).[10] First, freshly isolated whole blood of a specific ABO group is processed to isolate platelet-depleted plasma; it is subjected to a "heavy" spin (5000 *g*) for 10 minutes at 4°C in a refrigerated centrifuge.[11–14] This plasma supernatant is frozen at −65°C ideally within 1 hour, but possibly up until 8 hours after isolation, and is then stored at or below −18°C.[1] The duration prior to its being frozen is important because many coagulation factors get degraded over time. Next, this FFP is used to prepare cryoprecipitate. The FFP is thawed either over 18–24 hours at 1–6°C, or more rapidly in a circulating water bath.[15] A slushy cryoprecipitate layer seen at the bottom of the bag has a thick, white to semi-opaque appearance, and can be separated

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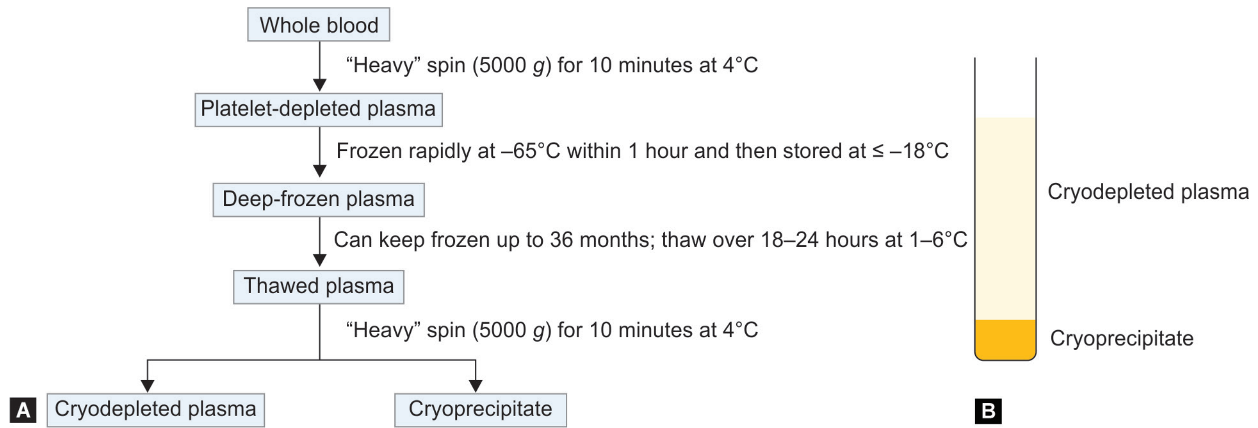
Flowchart 1A and B: (A) Flowchart explaining whole blood is processed to prepare cryoprecipitate in multiple steps; (B) After processing, two distinct layers of cryoprecipitate and cryosupernatant (cryodepleted plasma) can be seen



using another "heavy" spin.[16–19] After complete processing, the cryoprecipitate is deep frozen until needed for clinical use.[20] Most of the supernatant, except for about 10–15 mL, is removed by gravity drainage or a plasma expressor.[21] This clear layer of plasma above this precipitate is known as the cryosupernatant or cryodepleted plasma.[10,22–26]

The American Association of Blood Banks (AABB) recommends that frozen cryoprecipitate should be thawed prior to use in a protective plastic overwrap in a water bath at 30–37°C.[27,28] Once thawed, cryoprecipitate can be stored at 20–24°C for up to 4–6 hours.[29–32] We typically pool thawed preparations from 5 donors before use, and use these units within 4 hours. Fibrinogen and factor VIII in cryoprecipitate are labile proteins and are lost over time.[4,33] Cryoprecipitate unit prepared from a standard 450–500 mL whole blood anticoagulated with citrate–phosphate–dextrose–adenine should contain at least 150 mg of fibrinogen and a minimum of 80 international units (IU) of factor VIII.[4,34,35] This contains approximately 30–70% of factor VIII/vWF and fibrinogen content of the original preparation.[3,36,37]

Cryoprecipitate can also be prepared from plasma frozen within 24 hours of collection [frozen plasma–24 hours (FP24)].[1] It is usually prepared by pooling plasma from multiple donors rather than a single unit. Pooling is performed either before freezing by the central blood bank or after thawing by licensed centers. The freezing and thawing of plasma generate platelet membrane microparticles, and these are further concentrated by cyoprecipitation; the microparticle concentration of cryoprecipitate is 250-fold higher than the source plasma.[38] These microparticles contain glycoproteins that interact with fibrinogen, vWF, and platelets, and these interactions may be enhanced by cryoprecipitation.[1] The role of these microparticles in hemostasis, vascular function, inflammation, or alloimmunoreactivity is unknown.[39] The effect of processing and freezing of cryoprecipitate on these microparticles is also not known. Cryoprecipitates produced by pathogen-reduced apheresis using amotosalen and ultraviolet light A can be useful.[40] Amotosalen hydrochloride (HCl) is a photoactive psoralen compound with a characteristic three-ring structure.[41] It blocks the proliferation of pathogens by non-specific inhibition of DNA and RNA replication in the presence of ultraviolet A, and it can be reliably removed to trace levels prior to transfusions.[42]

Cryoprecipitate can be stored for a maximum of 36 months.[43]After thawing, the product should be visually examined to ensure that there are no insoluble fractions and that the container is intact.[44,45] The cryoprecipitate should be used immediately, ideally within 4 hours of its being thawed and received from the blood bank, and should never be refrozen.[27,46–48] The shelf life of thawed cryoprecipitate is short due to the loss of clotting factor activity, particularly that of factor VIII.

A single unit of cryoprecipitate received from the blood bank is made by thawing and pooling material from several donors.[49] The British Committee for Standards in Haematology recommends that cryoprecipitate should be administered in doses of 5–10 mL/kg, using higher volumes in bleeding neonates. The recipients should be monitored for clinical outcome and fibrinogen levels.[50] One unit (40 mL) of cryoprecipitate per 10-kg body weight may raise the plasma fibrinogen concentrations by up to 50 mg/dL in the absence of continued consumption or massive bleeding.[51] Although cryoprecipitate transfusions do not always need to be ABO compatible due to the small volumes of plasma in the units, neonates should still be given ABO-compatible units whenever possible due to their small body volumes.[52]

Clinical Use

Cryoprecipitate was routinely administered from the 1970s to the 1990s to treat hemophilia A and various factor deficiencies. Today, due to the availability of recombinant or highly purified virus-inactivated plasma-derived concentrates the use of cryoprecipitate is no longer considered the first-choice treatment for inherited coagulopathies such as hemophilia A, deficiency of factor XIII, hypofibrinogenemia, and vWD.[53] Furthermore, clinical guidelines have recommended against cryoprecipitate for these conditions unless specific factor replacement products are not available. The preference for specific factors concentrates is because of less frequent transfusion reactions, transfusion-related acute lung injury, and the risk of infections.[53] In neonates, cryoprecipitate is administered primarily to correct acquired fibrinogen deficiency such as in DIC, liver failure and consumptive hypofibrinogenemia as might be seen in infants with multiple thromboses.

Over the years, increasing experience with viscoelastic tests in neonates has enhanced our confidence in the management of acquired coagulopathies.[54] Viscoelastic tests of coagulation such

as thromboelastography and rotational thromboelastometry analyze the viscoelastic properties of the clot and evaluate the entire hemostatic process from initial formation of the clot to the polymerization of fibrin.[55,56] These tests can help measure the availability of functional fibrinogen (Fig. 1).[57]

To determine the need for cryoprecipitate transfusions in level III neonatal intensive care unit, we reviewed data from the Children's Hospital of Brescia from the last 10 years. Nineteen infants received 26 cryoprecipitate transfusions for hypofibrinogenemia (Table 1). The main cause of hypofibrinogenemia was DIC in 16 cases (84%) secondary to severe infections, Necrotizing enterocolitis (NEC), birth asphyxia, and congenital sacrococcygeal teratoma. Three cases (16%) of liver failure received cryoprecipitate for hypofibrinogenemia. Prior to transfusion, the median (interquartile range) level of fibrinogen was 77 (35–94) mg/dL.

Use of Cryoprecipitate in Inherited Coagulation Disorders

Hereditary fibrinogen abnormalities are rare bleeding abnormalities and can be divided into types I and II disorders. Type I disorders, including afibrinogenemia and hypofibrinogenemia, are quantitative fibrinogen deficiencies. Type II disorders affect the structure/function of circulating fibrinogen.[58] These diseases result from a variety of inherited genetic defects.[59] Most patients are asymptomatic, although some may have bleeding from the umbilical cord, mucosal surfaces, and intracerebral or intra-abdominal bleeding.[58] Tests show prolonged PT, Partial thromboplastin time (PTT), bleeding time and very low fibrinogen levels.[36] These clotting derangements may present in the neonatal period due to trauma of delivery. Fibrinogen concentrates are emerging as an important, safe option as a replacement therapy in congenital fibrinogen disorders. In addition, more accurate dosing can be achieved with fibrinogen concentrates because their potency is known, unlike FFP or cryoprecipitates. However, these products are still useful when fibrinogen concentrates are not available.[58]

von Willebrand disease is an inherited bleeding disorder that manifests clinically with bleeding in approximately 1:10,000 individuals. It is caused by deficiency and/or defect in vWF.[60] The most common symptoms are mucocutaneous bleeding, hematomas and bleeding after trauma or surgery.[61] Cryoprecipitate transfusions containing vWF are administered in patients who do not respond to desmopressin or for patients with type II or III vWD for treating bleeding episodes and for surgical procedures.[62–64] It should be restricted to emergency therapy where factor VIII/vWF concentrates are not immediately available and bleeding is sufficiently severe to warrant the risks associated with cryoprecipitate.[3,4,65] Therefore, it is strictly used as a second line therapy, only when desmopressin is not available.

Hereditary deficiency of factor XIII is an extremely rare condition; the Canadian hemophilia registry identified only 41 cases in 2006.[66] Compared to other factors, factor XIII is more stable with a longer half-life of 9–10 days.[3,4,67] Umbilical bleeding is a frequently-seen finding in neonates, occurring in nearly 80% of cases. Intracranial hemorrhage has been reported in 25–30% cases and is the main cause of death or disability in these patients. Because of the rarity of factor XIII deficiency, specific factor concentrate is usually not readily available in emergent situation, and hence cryoprecipitate can be a useful remedy.[3,4,68] It can be administered in a dose of 1 bag per 10–20 kg every 3–4 weeks.[3,8]

Use of Cryoprecipitate in Acquired Coagulation Disorders

Disseminated intravascular coagulation is an acquired, life-threatening condition that can occur in infants with conditions such as sepsis, respiratory distress syndrome, acidosis, NEC, birth asphyxia, and congenital sacrococcygeal teratoma.[37,69–71] These disorders are marked by systemic activation of anticoagulation pathways. The management of DIC includes identification and treatment of the underlying condition and restoration of the hemostasis by transfusion of platelets, FFP, and cryoprecipitate. Cryoprecipitate has been used at a dose of 5–10 mL/kg in infants

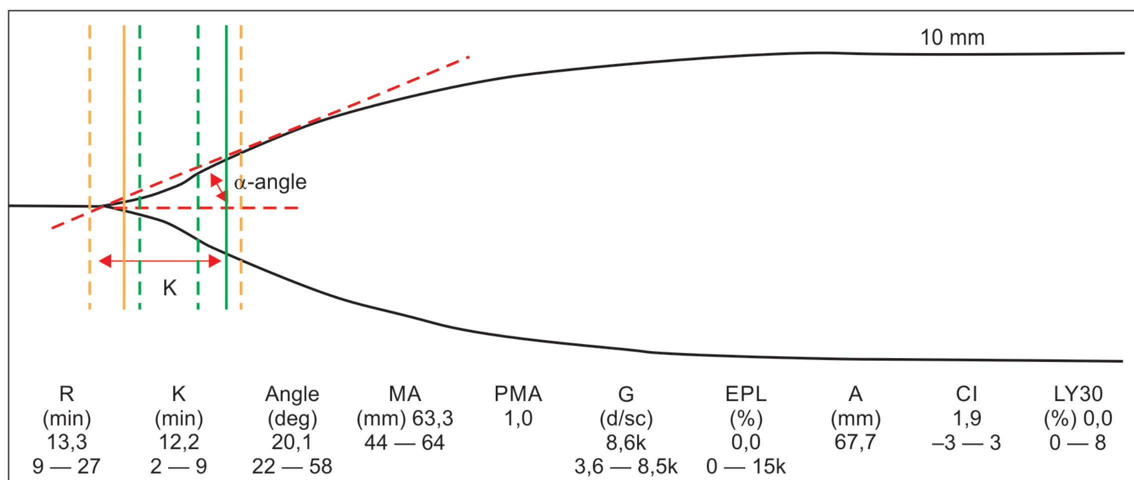


Fig. 1: Graphical representation of a thromboelastography test showing a prolonged K-time (clot kinetics) and a decreased α-angle (rate of clot formation) suggestive of hypofibrinogenemia. All labels are shown in deep red. K is the time taken to achieve a certain level of clot strength (amplitude of 20 mm); α-angle (degrees) measures speed at which fibrin build up and cross-linking takes place, rate of clot formation. R, reaction time; MA, maximum amplitude; PMA, projected MA; G, gear (shear elastic clot strength); EPL, estimated percentage of lysis; A, amplitude (at the latest time point); CI, coagulation index; LY, fibrinolysis

Table 1: Cases of hypofibrinogenemia receiving cryoprecipitate transfusions

Case	*Year*	*GA (weeks)*	*BW (gm)*	*Diagnosis*	*Setting*	*Transfusion (n)*	*Other complications*
1	2012	40	3,350	Galactosemia	Liver failure	1	
2	2012	23	378	Late-onset sepsis	DIC	2	
3	2012	29	1,390	Early-onset sepsis	DIC	3	
4	2013	24	650	Late-onset sepsis	DIC	1	
5	2013	28	700	Late-onset sepsis	DIC	1	
6	2013	30	885	Necrotizing enterocolitis	DIC	2	
7	2014	27	890	Necrotizing enterocolitis	DIC	1	
8	2014	33	4,280	Sacrococcygeal teratoma	DIC	1	
9	2014	37	1,920	Birth asphyxia	DIC	1	
10	2014	33	1,650	Late-onset sepsis	DIC	1	Thrombosis
11	2016	27	439	Late-onset sepsis	DIC	2	
12	2016	26	890	Birth asphyxia	DIC	1	
13	2017	38	2,950	Late-onset sepsis	DIC	1	Thrombosis
14	2018	24	800	Late-onset sepsis	DIC	1	Thrombosis
15	2020	31	1,801	Pneumonia	DIC	1	
16	2020	39	3,130	Mitochondrial disease	Liver failure	1	
17	2020	27	658	Early-onset sepsis	DIC	1	
18	2022	30	1,350	UVC malposition	Liver failure	2	
19	2022	32	1,470	Necrotizing enterocolitis	DIC	2	

BW, birth weight; DIC, disseminated intravascular coagulation; GA, gestational age; UVC, umbilical venous catheter

with DIC and active bleeding if the fibrinogen values fall below 1 gm/L.[50] The British Committee for Standards in Haematology, Blood Transfusion Task Force published guidelines for the use of cryoprecipitate in 2004,[24] and supported the consideration of cryoprecipitate as a therapeutic modality at fibrinogen values below 1 gm/L in infants with active bleeding. However, admittedly, even though all guidelines for cryoprecipitate administration reiterate a therapeutic threshold of fibrinogen levels 1 gm/L, this cutoff is not based on strong clinical evidence.[72] The thresholds for supplementation may have to be tailored based on the gestational and postnatal age of the patient, severity of illness, and the risk of mortality.[73]

Sacrococcygeal teratomas are the most common congenital tumors associated with hemorrhagic complications.[74,75] The coagulopathy that develops in these infants may be due to the consumption of clotting factors as a result of bleeding *in utero* or during labor and delivery. The etiology of the clotting abnormalities is multifactorial and leading to DIC.[75] The tumor may also have endothelial abnormalities and the microvascular disruptions during labor and delivery may trigger DIC. Trauma to the teratomas during delivery may release tissue thromboplastins into the bloodstream, resulting in activation of the coagulation cascade.[75] The blood loss is often difficult to assess as there might be concealed losses inside the necrotic tissues inside the tumors. In one study, the surgeons estimated blood losses of around 300 mL. The average transfusion volumes included packed red blood cell transfusions of 320 Ml; FFP, 43 mL; platelets, 40 mL; cryoprecipitate, 20 mL; and crystalloids 90 mL.[76]

Many infants who have undergone major surgical procedures or have sustained trauma develop large hemorrhages. These hemorrhages can accentuate fibrinolysis and induce hypo-/dysfibrinogenemia.[77–79] Platelets and cryoprecipitate must be considered as therapeutic options if active bleeding persists after initial resuscitation as fibrinogen levels can drop drastically in these patients.[80] Cryoprecipitate is usually given in a dose of 10 mL/kg. Tama et al.[81] reviewed Pediatric Trauma Quality Improvement Program data and evaluated the mortality benefit from early administration of cryoprecipitate. They showed that patients who received cryoprecipitate had lower 24-hour mortality. The benefits were even more prominent in infants and children who needed transfusions more than 100 mL/kg.

Many infants undergoing surgical procedures such as cardiac surgery with cardiopulmonary bypass (CPB) are at risk of life-threatening hemorrhages.[82,83] Usually, pre-operative hemostasis is optimized using steps such as adequate vitamin K replacement. Pre-operative prophylactic transfusion with FFP or cryoprecipitate is not indicated for patients with minor coagulation abnormalities, particularly in those who have been anticoagulated prior to CPB. However, if there is post-operative bleeding and APTT is prolonged it is important to ensure that heparin has been adequately reversed. CPB in neonates may cause marked reduction in clotting factors including fibrinogen, due to hemodilution, loss from the circuit and consumption.[36] A fibrinogen level of 1.5 gm/L is aimed for, and used as a transfusion threshold for cryoprecipitate.[36] There have been some studies to compare the efficacy of cryoprecipitate and fibrinogen concentrates, but the number of subjects has not been statistically adequate.[84]

Extracorporeal membrane oxygenation (ECMO) is increasingly used in critically ill infants to provide life-saving cardiopulmonary

support. As ECMO circuits expose circulating blood to artificial and non-endothelial surfaces, there is fibrinogen adsorption, contact pathway activation, coagulation activation, thrombin generation, and fibrinolysis. Many infants with these hemorrhagic complications are treated with FFP and/or cryoprecipitate.[85–89] Neonates undergoing treatment with ECMO have had a higher frequency of intracranial hemorrhage when they had low fibrinogen levels.[90,91] The ELSO guidelines advise for transfusion of plasma or cryoprecipitate to maintain fibrinogen levels above 150 mg/dL.[92]

Severe liver disease in newborns is relatively rare but can occur due to viral infections, hereditary metabolic diseases, neoplasia, and vascular problems.[93] Liver diseases are frequently associated with low fibrinogen levels and can be treated with cryoprecipitate.[94] The evidence of benefit still needs to be proven as the sample sizes in published studies are small.[95] Cryoprecipitate may sometimes also be inadequate because of the deficiency of multiple coagulation factors.[94]

Some rare but potentially life-threatening causes of acquired hypofibrinogenemia include purpura fulminans due to congenital deficiency of protein C or S. Other cases may have the Kasabach–Merit phenomenon, an acute consumptive coagulopathy that is specifically associated with vascular tumors.[96] These infants with rapidly growing tumors develop platelet sequestration with consequent thrombocytopenia and fibrinogen consumption.[97,98] Cryoprecipitate can be used if fibrinogen levels are <100 mg/dL, particularly if there is clinically-evident bleeding.[97,99]

Adverse Effects

Cryoprecipitate can have adverse effects such as infections, transfusion-associated circulatory overload, transfusion-related acute lung injury, and other transfusion reactions.[100] There have also been reports implicating cryoprecipitate as a cause of anaphylactic shock, intravascular hemolysis, and biliary complications.[101] The risk of infections, such as with bacteria, human immunodeficiency virus, and hepatitis viruses B and C, is similar to other transfusion units.[102–111] The risk of infections with cryoprecipitate might be higher than with fibrinogen concentrate as the latter involves more stringent steps including pasteurization, adsorption, and precipitation, which remove or inactivate a wide range of enveloped and non-enveloped viruses.[112] The risk of acquiring HIV from contaminated blood varies widely among countries and varies with the background incidence rate of HIV among donors, quality of screening assays, access to laboratories, the total number of transfusions, or exposures to the recipient.[112] The risk of transmission may be higher in developing countries as the cryoprecipitates are made from locally supplied blood and as compared to developed countries where the product is virus inactivated.[112] Cryoprecipitate is less likely to cause transfusion-related volume overload as compared to FFP. It has also a lower risk of causing hemolytic transfusion reaction than the plasma and this risk can be further reduced if ABO compatibility can be assured.

Contraindications

Cryoprecipitate may not be adequate as replacement therapy for isolated factor deficiencies of fibrinogen, factors VIII and XIII, or vWF if the appropriate factor concentrates are available.[3,113] It cannot also be used for replacement therapy for other factors.[114] FDA has approved the use of recombinant coagulation factor therapy as individual factor concentrates are now available for replacement therapies for hemophilia, factor XIII deficiency, hypofibrinogenemia, and vWD.[115] Moreover, clinical guidelines have recommended against cryoprecipitate for these conditions unless specific factor replacement products are unavailable because of fewer adverse events.[114,116] It has been withdrawn from many European countries because of safety concerns such as the transmission of pathogens. Nevertheless, cryoprecipitate is still available for hemostatic therapy in several countries, including the USA and Canada.[1] Although fibrinogen concentrate is licensed in the USA for use for congenital deficiencies, cryoprecipitate is still used to treat acquired fibrinogen deficiencies.[114] Considering the variable need for cryoprecipitates versus other blood products, one possible solution may be the development of computational monitoring systems for the utilization of blood products.[117]

Orcid

Atnafu Tekleab Mekonnen https://orcid.org/0000-0001-8263-6915

Mario Motta https://orcid.org/0000-0002-9579-2455

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

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REVIEW ARTICLE

Mitochondrial Dynamics during Development

Ling He[1], Karl Johan Tronstad[2], Akhil Maheshwari[3]

Received on: 02 January 2023; *Accepted on:* 08 February 2023; *Published on:* 06 April 2023

Abstract

Mitochondria are dynamic membrane-bound organelles in eukaryotic cells. These are important for the generation of chemical energy needed to power various cellular functions and also support metabolic, energetic, and epigenetic regulation in various cells. These organelles are also important for communication with the nucleus and other cellular structures, to maintain developmental sequences and somatic homeostasis, and for cellular adaptation to stress. Increasing information shows mitochondrial defects as an important cause of inherited disorders in different organ systems. In this article, we provide an extensive review of ontogeny, ultrastructural morphology, biogenesis, functional dynamics, important clinical manifestations of mitochondrial dysfunction, and possibilities for clinical intervention. We present information from our own clinical and laboratory research in conjunction with information collected from an extensive search in the databases PubMed, EMBASE, and Scopus.

Keywords: Archezoan, Inner membrane, Intermembrane space, Matrix, Mitochondrial DNA, Mitophagy, Neonate, Ontogeny, Outer membrane, Parkin.

Newborn (2023): 10.5005/jp-journals-11002-0053

Key Points

- Mitochondria are highly-dynamic, membrane-bound organelles that generate most of the chemical energy in eukaryotic cells.
- These organelles most likely evolved about 2 billion years ago from α-proteobacteria, a subgroup of the purple non-sulfur bacteria. These precursors of mitochondria likely belong to the order *Rickettsiales*.
- Besides the primary role in energy generation, mitochondria also perform numerous other cellular functions to support metabolism, epigenetic regulation, and cell cycle.
- In this article, we have summarized the ontogeny, ultrastructure, structure-function correlation, biogenesis, and clinical manifestations of mitochondrial dysfunction.

Introduction

Mitochondria are membrane-bound organelles that orchestrate cellular energy production in almost all eukaryotic cells.[1,2] These organelles generate adenosine triphosphate (ATP) through oxidative phosphorylation, the components of which are partially encoded in their own genome.[3] Mitochondria are not only the cellular 'powerhouses', but also play critical roles in supplying intermediary metabolites, temperature maintenance, regulation of Ca^{2+} homeostasis, determination of cellular life-span, and integration of various signaling pathways.[4–11] The physiological importance of mitochondria becomes evident early, and can be seen in the developing oocyte and the embryo, in the fetus, and throughout infancy.[12–15] After birth, the increased energy requirements are associated with a significant increase in the mitochondrial number and function.[16–19]

In this review, we have summarized recent advances in our understanding of mitochondrial dynamics, the importance of the cytoskeleton, cellular signaling, cellular and organ differentiation, regulation of the function of other organelles, and cellular lifespan. The importance of mitochondria as mediators of epigenetic regulation and metabolic processes during development has been explored. The critical role of mitochondria in cellular homeostasis is evidenced by the wide range of clinical manifestations involving multiple organ systems seen in mitochondrial diseases. We present our own clinical and laboratory research, combined with an extensive search in the databases PubMed, EMBASE, and Scopus. To avoid bias, keywords were identified from discussions in our own group and from PubMed's Medical Subject Heading (MeSH) thesaurus.[20]

[1]Department of Pediatrics and Pharmacology, Johns Hopkins University, Baltimore, United States of America

[2]Department of Biomedicine, University of Bergen, Bergen, Norway

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

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

How to cite this article: He L, Tronstad KJ, Maheshwari A. Mitochondrial Dynamics during Development. Newborn 2023;2(1):19–44.

Source of support: This study was supported by DK120309, DK107641.

Conflict of interest: Dr. Akhil Maheshwari and Dr. Ling He are associated as the Editorial Board Members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial Board Members and their research group.

Mitochondrial Ultrastructure

Mitochondria are dynamic intracellular organelles seen in all eukaryotic organisms; one exception might be the oxymonad monocercomonoides, which are obligate animal symbionts that live in the intestinal tracts of vertebrates.[21] Human tissues contain mitochondria with a high degree of numerical heterogeneity; erythrocytes do not contain any, whereas hepatocytes and muscle cells may contain hundreds to thousands per cell.[5,22,23] These numbers vary not only across various tissues, but also during development, cell cycle, and stress.[24]

Mitochondria have been traditionally viewed as 0.5–1 μm ovoids, where the number per unit volume seems to be inversely related to

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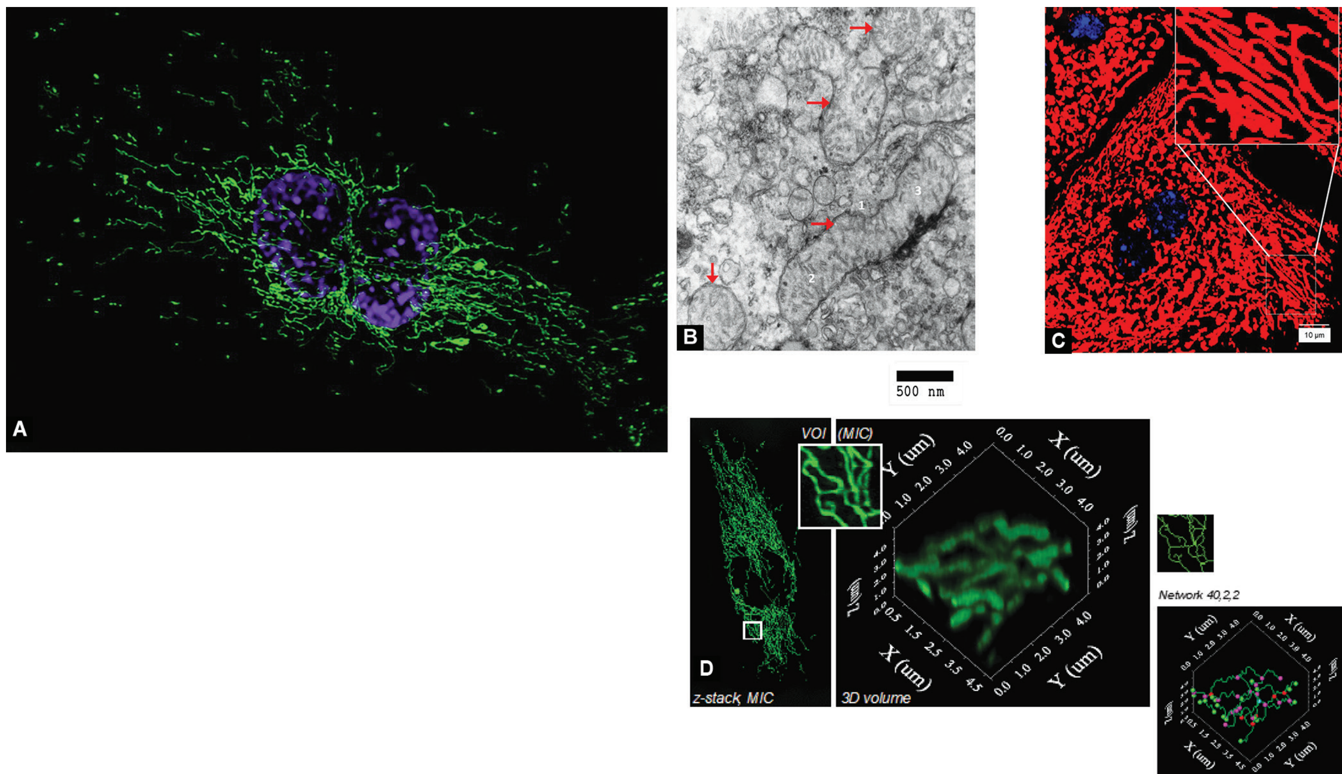
size.[1,25–27] However, the mitochondrial morphology varies between different cell types; cultured endothelial cells contain a mitochondrial reticulum around the nucleus (Fig. 1A). Similar to prokaryotes, mitochondria are uniquely covered in bilayered membranes. These organelles multiply by binary fission and consistently, electron micrographs show many mitochondria as a dumbbell or racket-shaped: Two larger halves with a narrow bridging tube prior to the separation of the daughter organelles.[28–31] Electron micrographs show the mitochondrial membranes, cristae, and matrix (Fig. 1B).

Advanced cellular imaging shows foci where mitochondria appear tubular and as forming a network (Fig. 1C) with active division and fusion.[32,33] The mitochondrial content and morphology are altered by cellular stress. In living cells, the net mitochondrial content or the mitochondrial mass depends on the balance between mitochondrial biogenesis and degradation. Changes in spatiotemporal positioning, which have been described as the mitochondrial dynamics, and mitochondrial morphology are governed by mitochondrial fusion, fission, and motility.[34–36] The dynamics can be seen in sub-nanometer resolution with cryo-electron tomography.[32,33] There may be unique orientations and distribution in different types of cells, and a close association with microtubules in some regions.[37,38] In sites where the energy requirements are relatively high, mitochondria may appear more static and provide ATP directly on-site.[39] However, in other regions, there may be prominent motility either in surveillance for foci with high energy needs or in actual energy production once those are found.[36] Fluorescence microscopy combined with quantitative image analysis is a useful method to study the amount and morphology of mitochondrial organelles within cells (Fig. 1D).[40] Dynamin-related guanosine triphosphate hydrolases (GTPases) may play an important role in mitochondrial motility.[41] Even if the mitochondrial network gets damaged during isolation from cells, some fragments may continue to show respiration and ATP synthesis.[3] The mitochondrial membrane protects the structure and electrical potential of these organelles.[42]

Structural Models

The following section summarizes current information on various compartments in mitochondria. The location of these key elements is shown in Figure 2:

- An outer mitochondrial membrane (OMM) that is freely traversed by ions and small molecules.[3] It is highly porous, and hence no electrical potential difference is detectible across this membrane layer.[3] There are nearly 200 proteins, but the following are some of the best characterized:[43]



Figs 1A to D: Panoramic view of mitochondria. (A) Mitochondria in a cultured dividing endothelial cell. The image shows a cultured mitotic human vascular endothelial cell (HUVEC) expressing mitochondrial green fluorescent protein (GFP). Nuclei (blue/purple) were stained with DAPI. Mitochondrial morphology varies between different cell types; this dividing endothelial cell displays a mitochondrial reticulum around recently-duplicated nucleus; (B) Transmission electron microscopy of mouse liver cells show mitochondria (indicated with red arrow). Mitochondrial membranes, 1; cristae, 2; and the matrix, 3 can be seen; (C) Mouse primary hepatocytes stained with MitoTracker Red, a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. The dye is well-retained after aldehyde fixation; (D) Quantitative image analysis of mitochondria in a human vascular endothelial cell. VOI, Volume-of-interest; MIC, 3-dimensional light microscopic image (described in Nikolaisen et al, DOI: 10.1371/journal.pone.0101365)

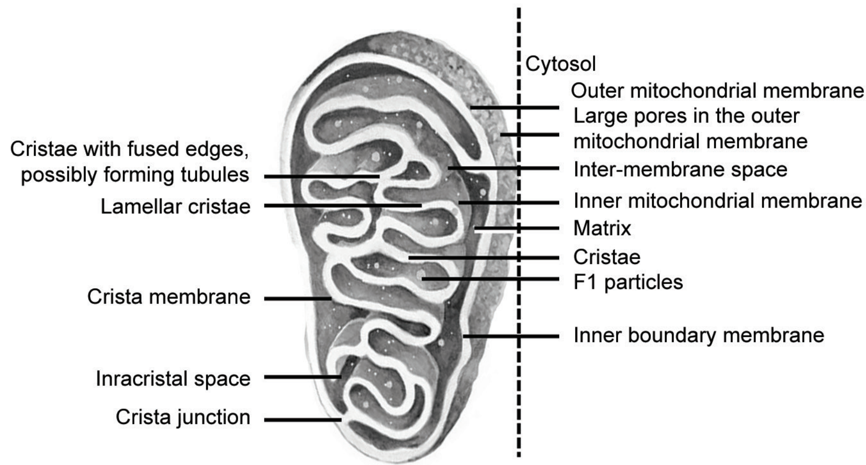


Fig. 2: Graphical depiction of mitochondrial infrastructure showing the location of the most important/better-known elements

- Translocase of the outer mitochondrial membrane (TOM):[44] The TOM complex is comprised of a large number of subunits, and it recognizes, segregates, and translocates precursor proteins to different sites within the mitochondria (Fig. 3);
- Topogenesis of mitochondrial outer membrane β-barrel proteins/sorting and assembly machinery (TOB/SAM): proteins such as Tom40, Tob55/Sam50; and mitochondrial distribution and morphology protein 10 (Mdm10) form channels in OMM;[45,46]
- Mitochondrial import complex (MIM): it inserts α-helical proteins in the OMM independently of the TOM complex;[47] and
- Endoplasmic reticulum-mitochondria encounter structure (ERMES).[48]
- Adenine nucleotide translocator (ANT), has been referred to by various other terms such as the ADP/ATP translocase, ADP/ATP carrier protein, or mitochondrial ADP/ATP carrier. It exchanges free ATP with free ADP across the inner mitochondrial membrane (IMM). Adenine nucleotide translocator is the most abundant carrier protein in the IMM;
- Mitochondrial porins, which are also referred to as the voltage-dependent anion channels (VDACs)–1, –2, and –3, are located on the OMMs and a class of porin ion channels;[49,50] and
- Apoptosis regulators bax and bak. These play an important role in maintaining the cell cycle and in the formation of mitochondrial pores.[51] Many other enzymatic systems are being identified.[52]

- An inter membrane space (IMS) between the inner and outer membranes contains about 5% of the mitochondrial proteins in an aqueous medium (3.8 µL/mg protein).[3,53,54] The large physical size of the OMM pores makes the IMS largely continuous with the cytosol;[55,56] Proteins synthesized on cytosolic ribosomes traverse these pores and bind carriers.[57–61]
  The IMS may contain many pro-apoptotic factors such as the cytochrome c.[62] Other proteins may display CX_3C or CX_9C motifs.[63] The machinery for import and assembly of IMS proteins mitochondrial intermembrane space assembly (MIA) can bring in large proteins that may be up to 11 kDa in size.[59,64]
- An IMM separates the IMS from the mitochondrial matrix. It is very selectively permeable to most molecules, and therefore, carries many specialized transporters.[3,65,66] An electrochemical membrane potential of about 180 mV has been documented across the inner membrane.[67] The membrane is also a site for oxidative phosphorylation, which is used to create electrochemical gradients for ATP synthesis.[68] The IMM is extensively folded, where numerous invaginations called cristae increase its total surface area.[3] These cristae are separated from inner boundary membranes by junctions, and the ends are partially closed by transmembrane proteins that bind opposing membranes.[3,69–71] Cristae also affect the overall chemiosmotic function of mitochondria.[65]

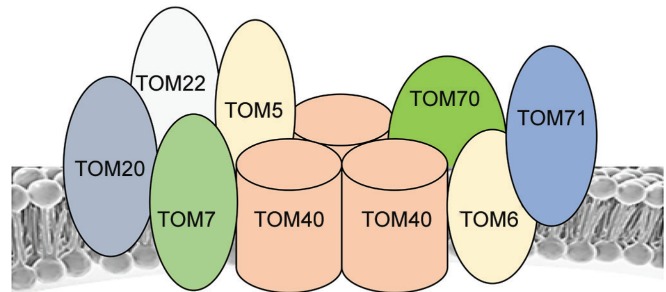


Fig. 3: Translocases of the outer mitochondrial membrane (TOM): The TOM complex is comprised of many subunits; It recognizes, segregates, and translocates precursor proteins to various sites within the mitochondria

- The major role of the IMM is to facilitate molecular transport and signaling for oxidative phosphorylation and ATP synthesis.[69,71] A junctional protein, the inner mitochondrial membrane translocase protein (IMMT), is expressed in the nucleus and is transported to the IMM, where it maintains the electrical potential and the structural invaginations seen in the inner membrane.[72–74] On the matrix side, the crystal membranes are studded with small proteinaceous F_1 particles, which promote proton-gradient-driven ATP synthesis.[3] The electron transport chain on the cristae includes 5 complexes: complex I nicotinamide adenine dinucleotide, hydrogenated (NADH) (NADH: coenzyme Q oxidoreductase), complex II (succinate: coenzyme Q oxidoreductase), complex III (coenzyme Q: cytochrome c oxidoreductase), complex IV (cytochrome c

oxidase) and ATP synthase.[62,75,76] Overall, the cristae membranes are dynamic and can reshape in seconds. Cristae membrane remodeling is regulated by the mitochondrial contact site and cristae organizing system (MICOS) complex, optic atrophy-1 (OPA1), F_1F_0 ATP synthase, and the lipid microenvironment.[77–81]

- The matrix in the core of these organelles is enclosed within the IMM. This gel-like material contains DNA, ribosomes, soluble enzymes, small organic molecules, nucleotide cofactors, and inorganic ions.[3] The pH of 7.8 in the matrix is higher than the 7–7.4 seen in the IMS.[82,83] The water content, about 0.8 µL/mg protein, is lower than that in the IMS.[84] The restricted permeability of the IMM may regulate the osmotic balance.[85] The aquaporin conduits in the membrane may also play a role.[86] The matrix is the site for the tricarboxylic acid (TCA) cycle (citric acid cycle, Krebs cycle) metabolism for ATP production (Fig. 4).[87] It contains the key regulators, including citrate synthase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, fumarase, and malate dehydrogenase; succinate dehydrogenase, located on the IMM, is an exception.[88,89]
- Mitochondrial DNA (mtDNA) is comprised of one or more double-stranded, mainly circular DNA in the matrix. Mitochondrial DNA (mtDNA) encodes for 2 ribosomal ribonucleic acids (rRNAs), 22 transfer RNAs (tRNAs), and 13 proteins involved in the mitochondrial respiratory chain.[90] It is rich in guanine and cytosine and contains 37 genes with about 17,000 base pairs.[91]

The mtDNA accounts for about 1% of the total DNA in a cell. In humans, only 13 proteins are encoded in mtDNA; all are central, hydrophobic subunits of the respiratory chain complexes/ATP synthase.[92] In total, there are 1,500 estimated different mitochondrial proteins; >99% of these proteins are likely encoded in the nucleus, synthesized in the cytosol, and imported into the mitochondria.[93,94]

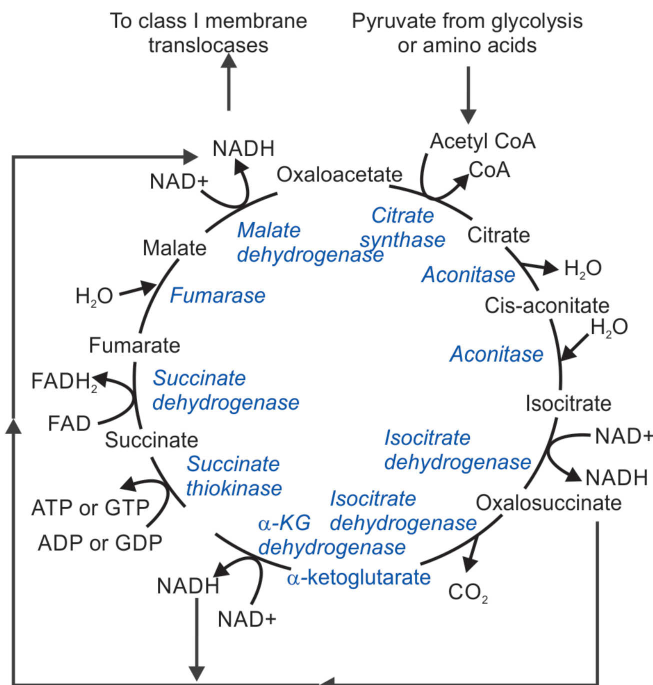


Fig. 4: Graphical depiction of the TCA cycle. Metabolites enter the TCA cycle as acetyl-CoA, and progress to form α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. Nicotinamide adenine dinucleotide (NAD), a coenzyme central to metabolism is hydrogenated to form NADH; KG = ketoglutarate. Enzymes are depicted in blue font

Human mitochondria contain a unique protein translation machinery with ribosomes, transfer-RNAs (tRNAs), and associated protein factors that resemble those seen in bacteria.[95,96] However, mitochondria make surprisingly little use of their specialized protein production machinery. Most of the mitochondrial proteins are synthesized in the cytoplasm and then imported into the organelle by protein translocases.[57] Most of the mitochondrial proteins are transcribed in the nucleus, synthesized in the cytosol, and then imported back into the organelle.[94] More than 3,000 mitochondrial proteins have been estimated in vertebrate animals.[97] During evolution mitochondrial genes have relocated to the nucleus, whereas the translated proteins are imported back into the mitochondria to perform their function.[97–99]

Evolutionary Perspective

There are three models for mitochondrial development, where an existing cellular organism accepted proto-mitochondria.[100] There are two endosymbiotic models, and a third where mitochondria could have evolved from related predecessor organelles (Fig. 5):

- Archezoan scenario: A hypothetical primitive a mitochondrial eukaryote, termed archezoan, accepted a proto-mitochondrial endosymbiont.[97,101,102] Rigorous studies have detected artifacts and raised doubts about the validity of these hypotheses.[103]
- Symbiogenesis scenario: An archaeal cell underwent a single endosymbiotic event with an α-proteobacterium, which generated mitochondria.[104] This event was followed by the evolution of the nucleus and compartmentalization of the eukaryotic cell.[102]
- Evolution from mitochondrion-related organelles (MROs).[105] These models envisage three possible double-membrane mitochondrial precursors, which contained minimal or no DNA:
  - Hydrogenosomes: These lack a genome but may have a few incomplete elements of the TCA cycle and the electron transport chain.[106] The anaerobic metabolism seen in hydrogenosomes suggests that these might have originated through endosymbiosis with an anaerobic bacteria such as *Clostridium*.[107] However, later studies showed that the hydrogenosomes in *Trichomonas vaginalis* may also contain several proteins resembling those in mitochondria, including chaperonins, the NADH dehydrogenase module of electron transport complex I, and components of the mitochondrial machinery for synthesis of iron-sulfur (Fe-S) clusters.[108,109] Hydrogenosomes could very well be relict mitochondria;[110]
  - Mitosomes: These have been seen in anaerobic, parasitic protists such as the amoebozoons *Entamoeba histolytica* and *Mastigamoeba balamuthi*. These organisms were initially considered to be amitochondriate and unable to generate ATP.[105,111] However, these contain several proteins similar to those seen in mitochondria and hence could be evolutionarily related to conventional mitochondria.[97,109] Compared to mitochondria, the metabolic capacity of mitosomes is relatively limited;[112]
  - Transitional MROs: These are seen in anaerobic ciliates *Nyctotherus ovalis*, and *Blastocystis* spp., which are related to the brown algae, diatoms.[97] There is a possibility of a shared origin between mitochondria, hydrogenosomes,

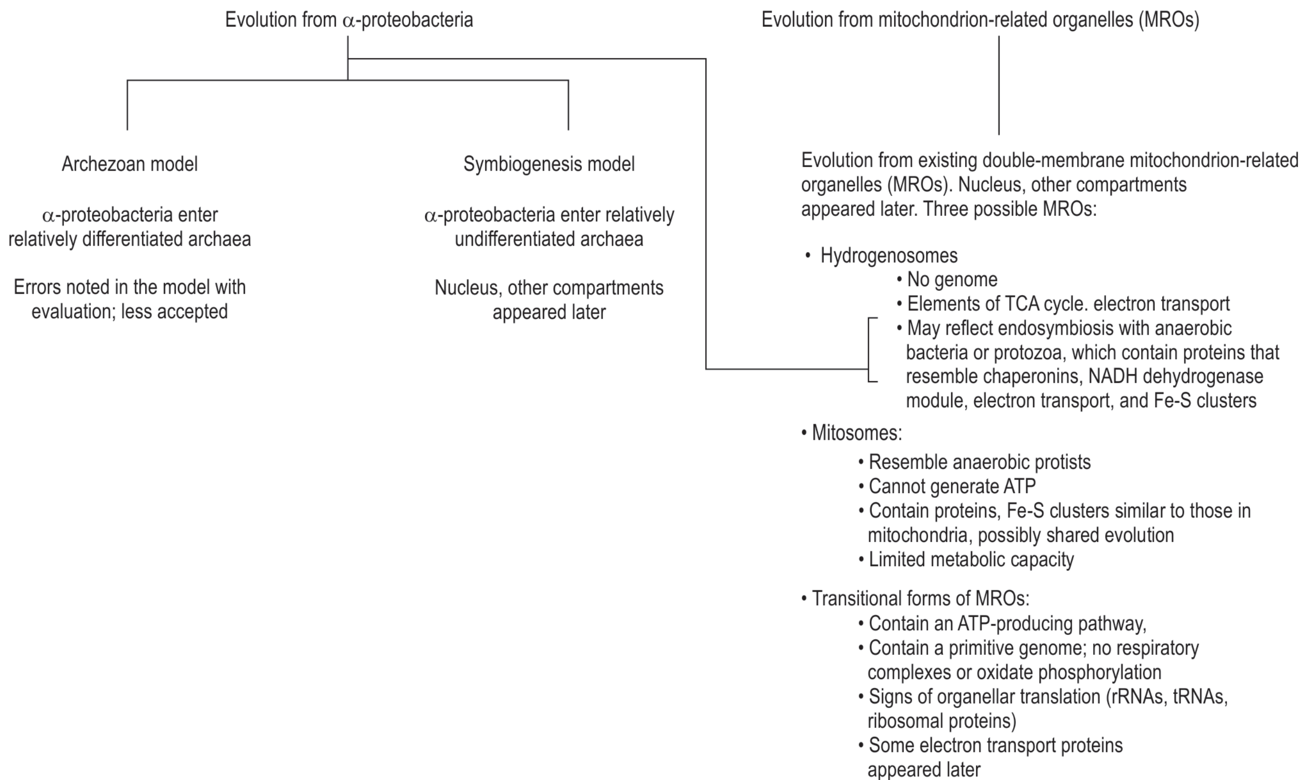


Fig. 5: Existing models for mitochondrial development. Two endosymbiotic models suggest that proto-mitochondria entered an existing cellular organism. The third suggests that mitochondria evolved from related organelles that already existed in host cells

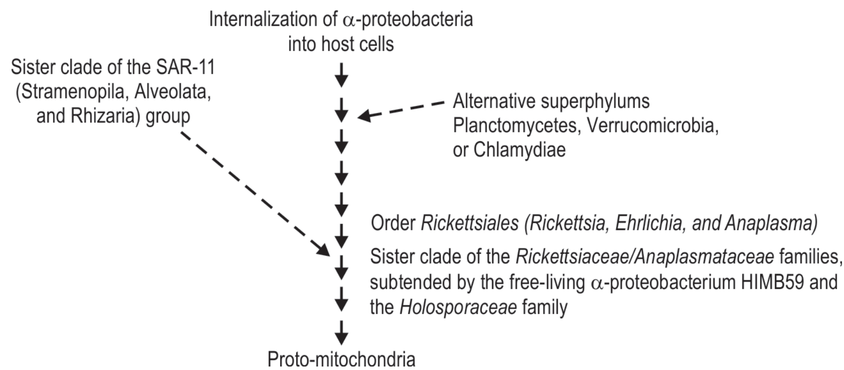


Fig. 6: Proteo-bacteria evolved into mitochondria following internalization into host cells

and mitosomes.[113] These MROs can generate H_2 via an ATP-producing, hydrogenase-mediated pathway.[114] The genome is relatively limited, and there are not many mtDNA-encoded genes similar to those seen in the respiratory complexes III, IV, and V.[115] These MROs also lack the ability to generate ATP via coupled electron transport and oxidative phosphorylation.[116] The MRO genome can contain organellar translation systems (rRNAs, tRNAs, ribosomal proteins) and a partial electron transport chain with subunits of electron transport complexes I and II.[111]

Evolutionary Precursors of Mitochondria

Mitochondria are generally believed to have evolved about 1.6–2 billion years ago from α-proteobacteria, a subgroup of the purple non-sulfur bacteria (Fig. 6).[81–83] These bacteria were internalization into host cells and then differentiated through several transitional forms of proto-mitochondria.[117–119] Increasing information places mitochondrial precursors in the order *Rickettsiales*, which is a subgroup of α-proteobacteria that include obligate intracellular bacterial parasites such as *Rickettsia, Ehrlichia,* and *Anaplasma*.[120] Phylogenomic analysis based on the 32 genes shared by mitochondria and these bacteria show similarities with the order *Rickettsiales*.[121] These similarities identify mitochondria to possibly be a sister clade of the *Rickettsiaceae/Anaplasmataceae* families, subtended by the free-living α-proteobacterium HIMB59 and the *Holosporaceae* family.[122]

Mitochondria also seem to show a sister-clade relationship with a group of free-living bacteria known as the Stramenopila, Alveolata, and Rhizaria (SAR)-11 group.[121] These are ubiquitous, free-living, small carbon-oxidizing bacteria with an estimated

global population of 2.4×10^{28} cells, present in nearly 25% of all oceanic plankton.[123,124] If confirmed, this relationship would suggest an alternative source of mitochondria in addition to those from *Rickettsiales*.[125] However, these findings need confirmation through accurate reconstruction of genome trees and evolutionary models, better statistical support without stochastic noise, identification of composition biases in the sequence data, or systematic errors such as long-branch attraction.[126–131]

Unfortunately, the identification of these relationships has been difficult.[130,132,133] There are many restrictions such as (a) weakness of the phylogenetic signal: Signals from small subunit rRNAs have weakened with time due to saturated mutations;[134] (b) long-branch attraction: Mitochondria and the obligate intracellular α-proteobacteria have more rapid rates of evolution than the free-living bacteria, and therefore, there are more artifacts;[135] and (c) sequence composition bias: AT-rich genome sequences in mitochondria can result in errors in phylogenetic reconstruction.[136]

Evolution of Prokaryotic Host Cells into Eukaryotic Ancestors

The evolutionary sequence in which the proto-mitochondrial bacteria entered an endosymbiotic relationship with prokaryotes is still uncertain. To place cellular evolution in perspective, prokaryotes are known to have acquired eukaryotic characteristics with differentiation.[117] The first eukaryotic common ancestor (FECA) matured through several stages to be identified as the last eukaryotic common ancestor (LECA) about 1–1.9 million years ago (Fig. 7).[113,137–139] These ancestor cells showed many features similar to modern eukaryotes.

Cells in the superphylum Asgard, which were the immediate descendants of the FECA, are considered to be the most likely hosts of the proto-mitochondria.[113,140] Some alternative host lineages have also been considered in the superphylum Planctomycetes, Verrucomicrobia, or Chlamydiae.[103,141] However, if the host cells were indeed proven to be Asgardian, these cells most likely evolved first to the domain archaea, and then to phyla such as Crenarchaeota, Thaumarchaeota, and Korarchaeota.[142] With some capability of metabolizing oxygen, these cells have been viewed as evolutionarily closer to eukaryotes and termed eocytes.[143–145]

The eukaryotic ancestors continued to differentiate during this process. The LECA possessed most of the eponymous components of eukaryotic cells, including the nucleus with nuclear pores, associated complexes, and nuclear lamina.[146] This nucleus is believed to have contained linear chromosomes with telomeres, encoding about 4,000 genes containing spliceosomal introns.[147] It likely possessed complex gene regulatory mechanisms, including RNA interference systems and small non-coding RNAs, and histone packaging that affected the accessibility to chromatin.[148] Transcription was uncoupled from translation and involved extensive RNA processing (including intron splicing, capping, and polyadenylation).[149] This ancestor also had an elaborate protein regulation and recycling system composed of a proteasome and a ubiquitin signaling system.[150]

The cellular environment of the LECA was compartmentalized with endomembrane systems such as the endoplasmic reticulum, the golgi apparatus, endosomes, lysosomes, and peroxisomes.[137] It displayed exocytic and various endocytic pathways such as phagocytosis.[151] There was an actin-tubulin cytoskeleton that enabled intracellular trafficking, cell motility, and a complex cell cycle.[152] The last eukaryotic common ancestor was likely able to synthesize phospholipids composed of glycerol 3-phosphate and fatty acids, as well as sterols and sphingolipids.[153] These cells also show many genes of bacterial origin that were likely acquired when the mitochondrial ancestor was engulfed.[154]

The host cells are covered in a bilayered lipid membrane, a simple cell wall (S-layer) rich in *N*-glycosylated proteins, and a relatively well-developed cytoskeleton with homologs of actin and tubulin.[155] During evolution into eukaryotes, the three most important changes were the acquisition of the nucleus, the endomembrane system, and the mitochondria.[154] However, the sequence of these events remains unclear. There are at two possibilities:

- Syntrophic consortium model: Simultaneous fusion of a symbiotic community that included the cytoplasm, nucleus, and mitochondria.[156]

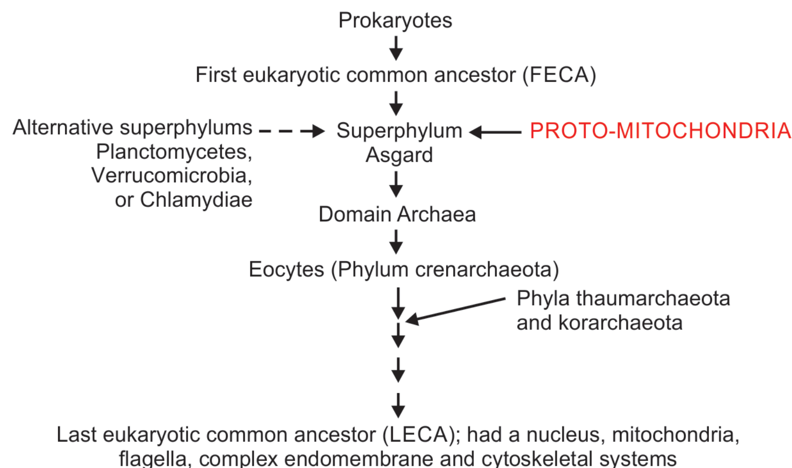


Fig. 7: Evolution of an endosymbiotic relationship between eukaryotic ancestors and proto-mitochondrial bacteria. The first eukaryotic common ancestors (FECAs) likely matured into the superphylum Asgard and internalized proto-mitochondrial bacterial ancestors. However, there is a possibility that the FECAs could have originated in other super-phyla. These cells matured over time, and the last eukaryotic common ancestors (LECAs) seen about 1–1.9 million years showed a nucleus, mitochondria, flagella, complex endomembrane, and cytoskeletal systems

- Endospore model: The nucleus evolved when a cell engulfed a sister following cell division. This model resembled endospore development in Gram-positive bacteria. Mitochondria were acquired later.[103] This model is so not well-supported by evidence.[62]

The nucleus was most likely not acquired from the internalization of another organism; phylogenomic analyses of the eukaryotic genome support the presence of an archaeal and a proteobacterial genome, but not the other genome donor(s) expected in nuclear endosymbiotic models (Fig. 8).[155] Such endosymbiotic models would also require supplemental theories to explain the origin of the endomembrane system, the physical continuity of inner and outer nuclear membranes, and the formation of nuclear pores.[62,104] Hence, the most compelling models suggest an autogenous origin of the nucleus. Infoldings/pinched-off sections of the plasma membrane formed the endoplasmic reticulum (ER) like internal compartments that later became organized around the chromatin to form the inner and outer nuclear envelope and enclosed a proto-nucleus.[155]

Acquisition of Mitochondrial Precursors into Host Cells

Mechanistic Perspective

Phylogenetic data suggest that the proto-mitochondria were likely acquired through an intimate mutualistic association between the archaeal host cells with bacterial ancestors of mitochondria that lived on the surface of these cells.[103] During evolution, these bacteria have exchanged genes to achieve lower GC contents.[157] The strongest evidence of this evolutionary process is the close homology between bacterial and mitochondrial respiratory chain complexes.[158,159] The mitochondrial endosymbiont gradually became less complex in its genome and proteome.[97,113] It also adapted gradually to anaerobiosis.[103] There are two possible mechanisms:

- Outside-in models: The symbionts living on the host surface might have been internalized in cell membrane vesicles (Fig. 9A). The inner and outer nuclear membrane was formed by the ER lamellae and the perinuclear space by the ER cisternae.[160] There is also a possibility that these bacteria could have been phagocytosed into food vacuoles, and then entered the cytoplasm by lysing the vacuolar membrane.[161] The cell membrane covering these symbionts contributed to the formation of the nuclear membrane;[155]
- Inside-out model: The host cells generated extracellular protrusions (blebs) to increase the total surface area[155] (Fig. 9B). The ancestral prokaryotic cell body remained intact as the eventual nuclear compartment during this evolution into eukaryotes.[155] The protrusions then fused and contained the cytoplasm and an endomembrane system, which evolved to make the outer nuclear and plasma membranes.[155] The mitochondrial precursors were initially trapped within the ER but then penetrated the ER membrane to move into the cytoplasm.[155] Finally, the formation of a continuous cell membrane closed off the ER from the exterior.[162]

The base of the cytoplasmic protrusions might have been stabilized by proteins homologous to the highly conserved coat protein II (COPII) in the outer ring of the nuclear pore.[163] Exchange of materials such as hydrogen, sulfur, hydrogen sulfide, organic acids, and ATP may have expanded these protrusions.[155] The blebs likely stabilized an outer ring of nucleoporins in the cell wall.[155] Proteins such as the linker of nucleoskeleton and cytoskeleton (LINC), which physically connect the cytoskeleton with the nucleoskeleton, might have stabilized the nuclear envelope and promoted nuclear bleb formation.[164]

Most structural lipids in eukaryotic cell membranes resemble bacterial, not archaeal lipids. Bacterial and eukaryotic membrane lipids carry glycerol-3-phosphate lipids with ester-linked, straight-chain fatty acids, whereas archaea contain a glycerol-1-phosphate backbone and ether-linked fatty acids (Fig. 10).[154,165] Additionally, eukaryotes and some bacteria, but not archaea, produce triterpenoids (for example, hopanoids and sterols) that modulate membrane fluidity.[166] Eukaryotes may

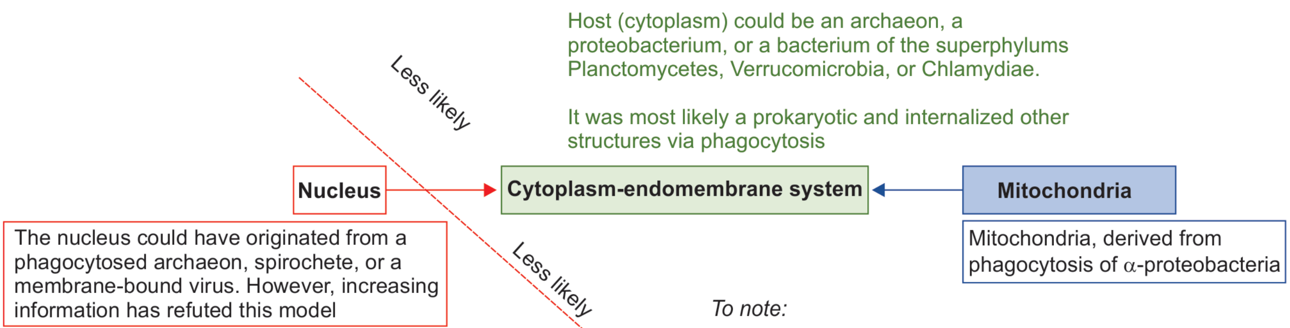
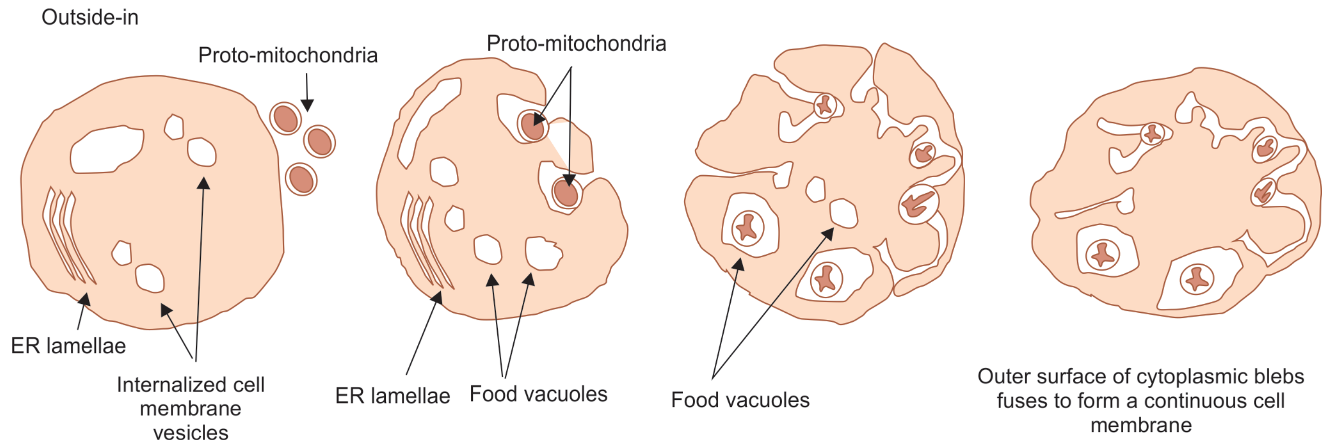
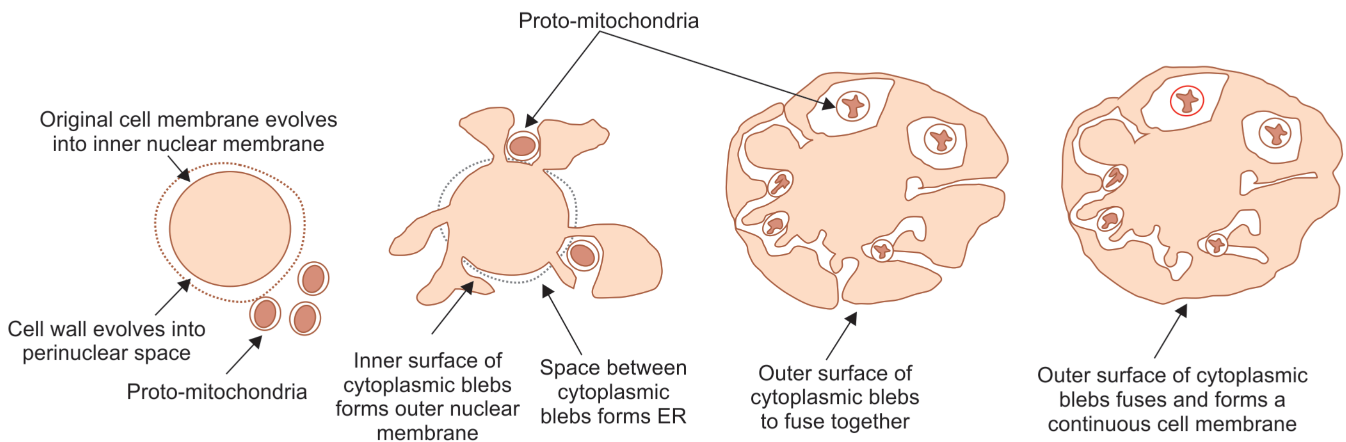


Fig. 8: Differentiating host cells internalized proto-mitochondrial bacterial ancestors. There have been considerations that the nucleus could also have originated in a phagocytosed archaeon, spirochete, or a membrane-bound virus. However, increasing information has refuted these possibilities and favor an endogenous origin of the nucleus



Autogenous outside-in model (visualized as entrapment at the eukaryotic cell membrane)	Endosymbiotic outside-in model (visualized as phagocytosis at the eukaryotic cell membrane)	Final structure in eukaryotic cells
Original cell membrane	Original cell membrane	Cell membrane
Internalized cell membrane vesicles	Internalized cell membrane vesicles	ER
Outer surface of ER lamellae	Food vacuole membranes	Outer nuclear membrane
ER cisternae	Food vacuoles	Perinuclear space
Inner surface of fused ER lamellae	Outer cell membrane of symbiont	Inner nuclear membrane

A



Inside-out model (visualized as starting at the eukaryotic nuclear membrane)	Final structure in eukaryotic cells
Original cell membrane	Inner nuclear membrane
Cell wall	Perinuclear space
Inner surface of cytoplasmic blebs	Outer nuclear membrane
Space between cytoplasmic blebs	ER
Outer surface of cytoplasmic blebs	Cell membrane

B

Figs 9A and B: Evolution of mitochondria. (A) Outside-in model: Symbionts could have been internalized in cellular vesicles. The inner and outer nuclear membrane may have originated in the endoplasmic reticulum lamellae, and the perinuclear space in the ER cisternae. There is also a possibility that these bacteria could have been phagocytosed into food vacuoles, and then entered the cytoplasm by lysing the vacuolar membrane; (B) Inside-out model: host cells generated extracellular protrusions (blebs). The ancestral prokaryotic cell body remained intact as the eventual nuclear compartment during this evolution into eukaryotes. The protrusions fused and formed the cytoplasm and an endomembrane system, which evolved to make the outer nuclear and the plasma membranes. The mitochondrial precursors moved from the ER into the cytoplasm. Finally, the formation of a continuous cell membrane closed off the ER from the exterior

have acquired bacterium-like lipids from mitochondria.[154] The genes for lipid biosynthesis from proto-mitochondria may have been transferred prior to the development of vesicle trafficking systems and phagocytosis.[155]

The analysis of archaeal lipids has provided some support to the possibility that phagocytosis evolved after the acquisition of mitochondria. Archaeal membranes typically retain their physical properties across a wide range of

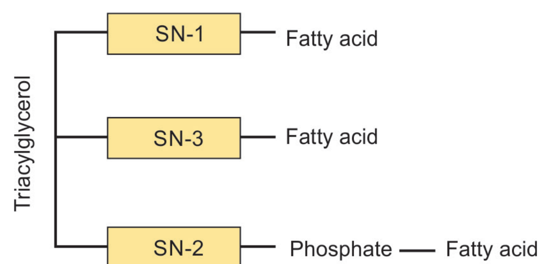


Fig. 10: Bacterial and eukaryotic membranes carry glycerol-3-phosphate lipids with ester-linked, straight-chain fatty acids, unlike the glycerol-1-phosphate backbone seen in archaea; SN, Stereospecific numbering

temperatures, whereas bacterial and eukaryotic membranes retain structures best at a narrow range of physiological temperatures.[167] These properties might be important for optimizing phagocytosis.

Phylogenetic Perspective

Mitochondria are seen in most eukaryotic host cells as the two may have evolved together.[97] Consequent improvements in efficiency in metabolic processes and the encoding of interacting gene products have created an obligate codependence. However, the number of mitochondria per cell has changed through evolution and differs across phyla.[168] Many unicellular eukaryotes contain only a few mitochondria, whereas others can contain up to 10^5.[169] The number of mitochondria can vary in multicellular eukaryotes from 80 to 2,000 per cell.[168]

In proto-mitochondria, the electron transport chain and pathways for β-oxidation of fatty acids were likely present, indicating that the mitochondrial endosymbiont had an aerobic metabolism.[170] The pathways for the synthesis of lipids, biotin, heme, and Fe-S clusters were also present.[113] This proto-mitochondria might have been capable of facultative aerobic respiration.[113]

Unlike the mitochondrial genome, the proteome shows only limited similarity with that in α-proteobacteria.[98] The mitochondrial ribosome also shows a high degree of evolutionary retailoring.[171,172] In many eukaryotes, the mitochondrial large- and small subunits have become smaller than their bacterial counterparts, and many new ribosomal proteins have been added.[97] The structure that was originally an RNA-rich complex has now become enriched in proteins.[171]

Mitochondrial Biogenesis

This process includes the growth and division of pre-existing mitochondria. Mitochondria are believed to have evolved from an α-protobacteria endosymbiont that became incorporated in a host cell.[173] Due to this bacterial origin, mitochondria contain a characteristic genome and also show auto replication.[174] Mitochondrial proteins are encoded by the mtDNA and specifically-encoded structures in the nuclear genome (described above).[94]

Major Molecular Components

A large number of mitochondrial proteins, nearly 1,000–1,500 are encoded in the nucleus.[175] The mRNAs are transcribed in the nucleus and translated into the cytosol. Most precursor proteins, whether folded or not, pass through the mitochondrial membranes assisted by protein translocases.[57] Many of these folded proteins are tagged with an *N*-terminal, positively-charged presequence.[176,177] Once this presequence is cleaved off by a matrix protease, these proteins may get folded with the aid of molecular chaperones.[176] Proteins moving to the other mitochondrial compartments may be transported by different protein-import pathways.[178] Many precursor proteins that do not contain the *N*-terminal signals may carry the targeting information within the actual protein sequence.[179] The mitochondrial membrane potential and the action of matrix Hsp70 (heat-shock protein 70) regulate this translocation.[180]

As mentioned above, the translocase of the outer membrane (TOM) protein is a universal entrygate for all proteins entering the mitochondria.[44] Many different pathways diverge at this point:

- translocase of the inner membrane (TIM), which sorts matrix-targeted precursors;[181]
- presequence translocase-associated motor (PAM) regulates matrix Hsp70 action to drive precursors into the matrix;[182]
- sorting and assembly machinery (SAM) on the outer membrane inserts β-barrel proteins into the outer membrane.[183]

These processes are an integral part of mitochondrial biogenesis, which may involve not only increased number but the size and mass. Mitochondrial biogenesis can also be altered by environmental stress as malnutrition, low temperature, oxidative stress, and cell division.[184]

An important regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor-gamma coactivator ([PGC]-1alpha) [PPAR (peroxisome proliferator-activated receptor)-γ coactivator-1α].[185,186] Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha stimulates the formation of new mitochondria by inducing UCP (uncoupling protein) 2, nuclear respiratory factor (NRF)-1, and NRF-2.[175] Nuclear respiratory factor (NRF)-1 and NRF-2 then induce key mitochondrial enzymes.[187] These also interact with Tfam, which drives transcription and replication of mtDNA and many nuclear-encoded mitochondrial components.[184] Nuclear respiratory factor (NRF)-1 activates the transcription of δ-ALAS (δ-aminolevulinate synthase), and NRF-2 that of cyclo-oxygenase (COX) IV.[188,189] Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha also interacts with other modulators of transcription such as PPARs, thyroid hormones, glucocorticoids, estrogen and ERRs (estrogen-related receptors)-α and -γ.[190,191] The ERRs are orphan nuclear receptors that target the gene networks involved in energy homoeostasis, and in mitochondrial biogenesis and function.[192–194] Mitochondrial subdivision also involves the dynamin-like and the fis-type proteins.[41]

Regulation of the numerical density of mitochondria in various cells: Metabolic needs may regulate the mitochondrial mass via-a-vis the cellular size across the cell cycle and the total body weight according to a power law.[168,195] In some tissues, the organelles appear as discrete units and the number and size may be related to the cell size. The cells resemble single-celled eukaryotes and may follow linear or sublinear scaling for mitochondria, not Kleiber's power law (an animal's metabolic rate scales to the $^{3}/_{4}$ power of the animal's mass).[196,197] Some cells show context-dependent mitochondrial morphology, and sometimes a large filamentous organelle reticulum.[34] The size of these organelles may not consistently scale strongly with cell size in single-celled eukaryotes, suggesting that the number of mitochondria per cell may be more important than the size of these organelles as a means of modulating cellular energetic requirements.[168] There might be a possibility of having an optimal per-cell mitochondrial mass given the cell size and the nature of mitochondrial biogenesis.

In humans, the mitochondrial count varies across different tissues and organs.[10,168] Mature erythrocytes do not contain any, whereas metabolically-active organs such as the liver, kidney, heart, and

brain tissues contain large numbers.[198] Mitochondria are important for metabolic functions and also for cellular maintenance.[199,200] Many mitochondrial genes have been transferred to the nuclear genome during evolution. These relocations might have improved the numerical efficiency, proximity to up- and/or downstream genetic systems, or improved utilization of cytoskeletal space by preventing redundancy in transcription sites.[201]

Even though there might be some variance in the expression of a subset of genes expressed in the mitochondria, most of these genes are transcribed at a specific, constitutive level.[91] One reason might be that the entire circular mitochondrial genome involves one strand at a time.[202] The number of mitochondria may also be important because of the variations in the lability of these organelles and that of energetic constraints across tissue types.[203]

Intercellular transfer of mitochondria: Mitochondria and mtDNA can be transferred between cells.[204] Transient focal cerebral ischemia can release mitochondria from astrocytes, which enter adjacent neurons via a calcium-dependent mechanism involving CD38 and cyclic ADP ribose signaling. This can amplify the survival signals.[205,206] Horizontal transfers of mtDNA have also been noted in cancer cells; extracellular vesicles containing mtDNA can pass through tunneling nanotubes and connexin 43 gap junctions between cells.[207,208]

Epigenetic Changes Involved in Mitochondrial Biogenesis

The epigenetic landscape is extensively reprogrammed during embryonic and fetal development.[209] Paternal genome also shows considerable DNA demethylation after fertilization.[210] Depletion of mtDNA leads to alteration in the metabolism of amino acids including methionine, leading to increased DNA methylation.[211] *S*-adenosylmethionine (SAM) acts as a co-factor in these methylation reactions; SAM is produced from methionine by methionine-adenosyl transferases (MATs).[212,213] Interestingly, the development of the embryo prior to implantation requires appropriate histone demethylation mediated by the JMJ (*jumonji*, or the Jarid2) deamylase, which removes the methyl group from lysine residues. The JMJ demethylases catalyze the histone demethylation in an α-ketoglutarate-dependent manner.[214,215] Thus, mitochondria regulate demethylation via α-ketoglutarate through the oxidation of glucose and glutamine in the mitochondrial citric acid cycle.[216]

Chromatin remodeling is also important in embryonic epigenetic programming.[216] Histone acetylation relaxes the condensed chromatin and promotes gene transcription.[217] Contrarily, deacetylation of histone condenses the chromatin and suppresses transcription.[217] Histone acetylation by specific histone acyltransferases requires acetyl-CoA, which is the product of oxidative decarboxylation of pyruvate produced by glycolysis, β-oxidation of fatty acids, and amino acid metabolism, and then shuttled out of mitochondria in the form of citrate, acetyl-CoA precursor.[218] In human embryonic stem cells, increasing acetylation suppresses differentiation, while inhibition of acetyl-CoA production from glucose results in the loss of pluripotency. The availability of nicotinamide adenine dinucleotide (NAD^+) controls the activity of the conserved NAD^+-dependent histone deacetylases, the sirtuins (SIRTs).[219] SIRTs are involved in blastocyst development as the inhibition of SIRT activity decelerates blastocyst development. NAD^+ can be synthesized de novo from the amino acid tryptophan or through the NAD^+ salvage pathway from nicotinamide.[220] However, cytoplasmic NAD^+ levels are normally very low, and blastocyst development and placental and fetal growth can be maintained only when NAD^+/NADH-reducing equivalents shuttle into mitochondria through either malate-aspartate or mitochondria glycerol 3-phosphate dehydrogenase.[221] Histone acetylation during development deserves further study.

Mitochondrial Function

The following section summarizes currently available information on various aspects of mitochondrial function(s) (Table 1):

Energy production: Mitochondria play an important role in energy production and its storage as ATP.[3] Glucose is broken down during glycolysis in the cytoplasm into two molecules of pyruvate, which are then translocated to the mitochondria by membrane-bound permeases.[222] There, pyruvate dehydrogenase processes these molecules via oxidative decarboxylation to produce acetyl coenzyme A (acetyl-CoA), which triggers the TCA as described above.[216] Metabolites enter the TCA cycle as acetyl-CoA, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. Nicotinamide adenine dinucleotide (NAD), a coenzyme central to metabolism, is reduced to form hydrogenated NAD (NADH).[223]

The mitochondrial respiratory chain has been conserved through evolution (as shown in Table 1 and details depicted in Fig. 11). Complex I and II oxidize NADH and Flavin adenine dinucleotide ($FADH_2$), respectively, and transfer the resulting electrons to ubiquinol, which carries electrons to Complex III (Fig. 12).[224] Complex III shunts the electrons across the intermembrane space to reduce cytochrome c (ubiquinone), which brings electrons to complex IV.[225] Complex IV or cytochrome *c* oxidase (COX) is the last electron acceptor of the respiratory chain, involved in the reduction of O_2 to H_2O. This multimeric complex includes multiple structural subunits encoded in two different genomes, several heme groups (heme *a* and heme a_3), and coordinated copper ions (Cu_A and Cu_B). About four electrons are removed from four molecules of cytochrome *c* and transferred to molecular oxygen (O_2) and four protons, producing two molecules of water. About eight protons are removed from the mitochondrial matrix (although only four are translocated across the membrane), contributing to the proton gradient.[226] Finally, mitochondrial ATP production is the main energy source for intracellular metabolic pathways. Complex V is a multi-subunit oxidative phosphorylation complex.[227] There are two functional domains, including (a) F_1, in the mitochondrial matrix; and (b) the F_0, located in the IMM.[228] This energy created in the proton electrochemical gradient is utilized to phosphorylate ADP to ATP.[227]

Reduced NAD^+ (NADH) is used by enzymes embedded in the mitochondrial cristae to produce ATP.[229] Beta-oxidation of fatty acids also produces acetyl-CoA, NADH, and reduced flavin adenine dinucleotide ($FADH_2$; FAD is a redox-active coenzyme involved in several metabolic pathways).[230] Oxidative degradation of certain amino acids can also contribute to this process; the mitochondria are also the key regulators of the biosynthesis of amino acids, lipids, and gluconeogenesis.[231] Under normal conditions, over 90% of ATP is made in mitochondria[232] but most of the genetic machinery needed to produce ATP has been translocated to the nucleus during evolution.[233] Only about 3% of the mitochondrial proteins are needed to synthesize ATP.[229]

Flavin adenine dinucleotide ($FADH_2$) is another energy carrier that is produced in the mitochondrial matrix and is processed by oxidative phosphorylation in the electron transport chain to regenerate FAD.[234] Protons are pulled into the intermembrane space by the energy of the electrons going through the electron transport chain. In the electron transport chain, 4 electrons are accepted by oxygen and the protons return to the mitochondrial

Table 1: Genetic components of mitochondria and associated inherited disorders seen in young infants

		Composition of the gene/gene complex	*Clinical features*
Mitochondrial respiratory chain complex	Complex I	Complex I contains 33 genes that have been mapped to various autosomes, 1 to the X chromosome, and 7 to mtDNA	Lethal infantile mitochondrial disease, lactic acidosis, Leber's Hereditary Optic Neuropathy, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome
	Complex II	Complex II (succinate: ubiquinone oxidoreductase) is the smallest complex in the respiratory chain and is composed of 4 subunits of the succinate dehydrogenase in the nucleus: SDHA, SDHB, SDHC, and SDHD	Leigh syndrome, mitochondrial leukoencephalopathy, Kearns-Sayre syndrome, cardiomyopathy, infantile leukodystrophy
	Complex III	Complex III contains 11 subunits, and only one, cytochrome b is of mitochondrial origin	Lactic acidosis, hypoglycemia, ketosis, hyperammonemia, cardiomyopathy, multisystemic dysfunction, encephalopathy; growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death (GRACILE) syndrome
	Complex IV	Complex IV is comprised of 4 cytochrome c oxidase genes, which form a large transmembrane protein complex. It is the last enzyme in the respiratory electron transport chain located in the mitochondrial membrane. It converts molecular oxygen to water and helps establish a transmembrane electrochemical potential that the ATP synthase then uses to synthesize ATP	Steatosis, encephalopathy, myopathy, hypertrophic cardiomyopathy, hepatomegaly, liver dysfunction and hypotonia, delayed motor development, mental retardation
	Complex V	Complex V contains 24 genes. Synthesizes ATP using energy provided by the proton electrochemical gradient across the IMM. It is an F-type ATPase and consists of two structural domains: F1, a soluble portion situated in the mitochondrial matrix; and F0, which spans the IMM. Protons pass from the inter membrane space to the matrix through F0, which transfers the energy created by the proton-motive force to F1; ADP is phosphorylated to ATP	Severe neonatal encephalopathy, neonatal respiratory distress, lactic acidosis, peripheral neuropathy, dysmorphism, cataract, pulmonary arterial hypertension, bilateral cataracts, Reye-like syndrome
Fatty acid metabolism	Carnitine palmitoyltransferase I		Hypoketotic hypoglycemia, hyperammonemia, elevated transaminases, and mild metabolic acidosis
	Carnitine-acylcarnitine translocase		Hypoglycemia, seizures, cardiomyopathy, cardiac arrhythmia, and apnea
	Carnitine palmitoyltransferase II		Nonketotic hypoglycemia, hepatomegaly, encephalopathy, seizures, respiratory distress, and metabolic acidosis. cardiomyopathy and arrhythmia
	Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency		Hypertrophic cardiomyopathy and fasting hypoketotic hypoglycemia
	Short-chain acyl-CoA dehydrogenase (SCAD) deficiency		Hypotonia, muscle weakness, and seizure
	Neonatal long-chain 3-ketoacyl CoA thiolase (LCKAT) deficiency		Lactic acidosis, pulmonary edema, and cardiomyopathy

(Contd...)

Table 1: *(Contd...)*

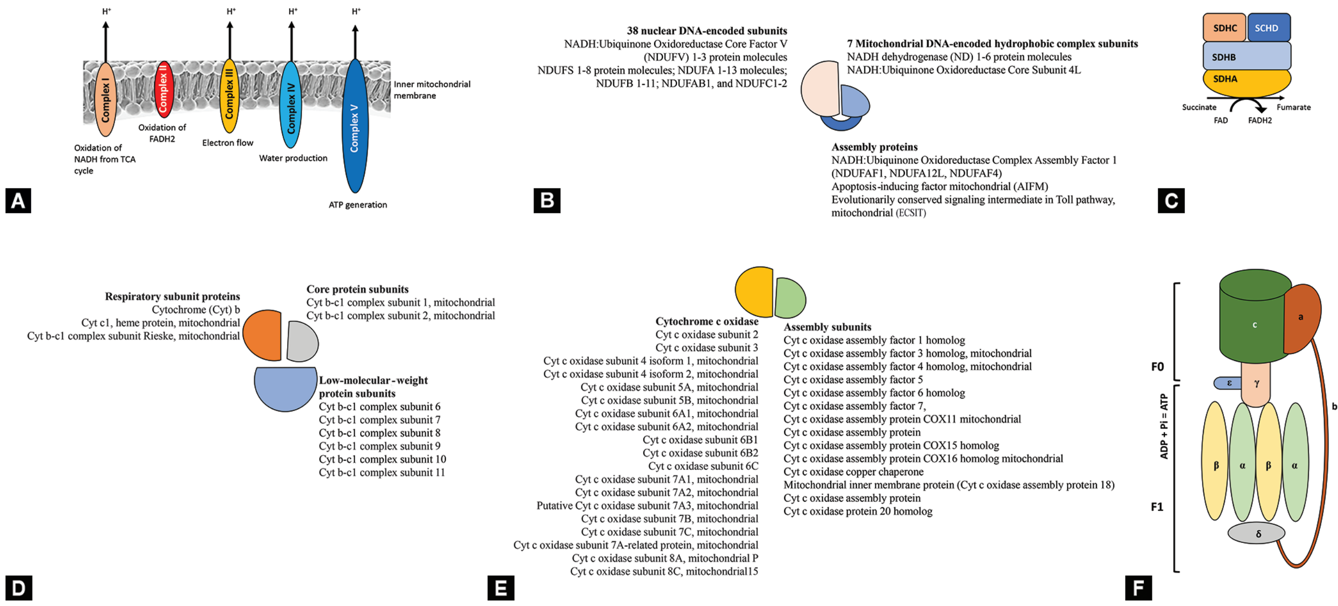
		Composition of the gene/gene complex	*Clinical features*
Amino acid metabolism	Phenylalanine hydroxylase Cystathionine synthase Branched chain ketoacid dehydrogenase SLC6A19 solute carrier family 6, member 19		Phenylketonuria Homocystinuria/Homocystinuria Maple syrup urine disease Hartnup disease
Pyruvate metabolism	PDHA1		Lactic acidosis, hypotonia, Seizure
	Pyruvate carboxylase		Hypercitrullinemia and hyperlysinemia
Krebs cycle metabolism	Dihydrolipoyl dehydrogenase		Severe persistent lactic acidosis, respiratory difficulties, seizures, dystonic movements, hypoglycemia, lethargy, hypotonia, vomiting, constipation, failure to thrive, and feeding difficulties
	α-ketoglutarate dehydrogenase		Choreoathetosis, opisthotonos, spasticity, hypertrophic cardiomyopathy, hepatomegaly, and sudden death
	Fumarase		Lethargy, microcephaly, hypotonia, axial dystonia or opisthotonos, areflexia, or developmental delay
Pathological disorders caused by complex genetic alterations	Deletions with combinations of Class I, II, III		Steatosis, fibrosis/cirrhosis
	Syndromes due to deletions and duplications in the mitochondrial genome	Usually sporadic, heteroplasmic, and unique. Frequently occur between directly repeated sequences, suggesting that many occur because of de novo arrangements during oogenesis or early development	Syndromes described in text

matrix through the protein ATP synthase.[224] The energy is used to activate ATP synthase, which then facilitates the passage of a proton to produce ATP.[234] The difference in pH between the matrix and intermembrane space creates an electrochemical gradient by which ATP synthase can facilitate the passage of protons into the matrix.[3] The oxidation of NADH and $FADH_2$ also produces GTP from succinyl-CoA synthetase-mediated signaling.[216]

In oogonia, mitochondria are the prime source of energy.[235] These mitochondria have a dense matrix and a few arch-like or transverse cristae, and are usually seen in the central cytoplasm.[236] Mitochondria in metaphase I and II of oocytes still resemble those in the germinal vesicle, with an even distribution in the cytoplasm and aggregation around the smooth ER.[236] At the pronuclear stage, mitochondria conglomerate around the pronuclei, and this persists up to syngamy.[237] In the 8-cell embryo, the morula, and the blastocyst, mitochondria are relatively less electron-dense and show clear areas in the matrices. In expanding blastocysts, trophoblast, embryoblast, and endodermal cells, mitochondria look elongated with the IMM arranged into transverse cristae.[3,236]

Growing oocytes preferentially utilize pyruvate to make ATP, and early embryos also use pyruvate, lactate, and amino acids to support development. [238–240] Mature oocytes contain the highest mitochondrial DNA copy number and mass of any cell.[241] Fertilized oocytes have even higher mtDNA copy numbers, Inhibition of mitochondrial metabolic activity blocks the maturation of oocytes and the subsequent embryonic development. [136,242–247] In humans, embryo development and implantation rates are closely correlated with ATP levels, and inhibiting mitochondrial activity suppresses embryonic stem cell differentiation. [247,248] After birth, there is a rapid increase in the density and activity of mitochondria in the heart and other metabolically active organs.[249] The expression of mitochondrial respiratory chain genes is also increased.[17]

The density of mitochondria in cells does seem to be important. Fibroblasts and other cells from obese mothers have been shown to carry fewer mitochondria, which relates to higher levels of triglycerides, free fatty acids, and more lipids.[250] The paucity of mitochondria alters the placental lipid metabolism and transfer of the lipids to the fetus, causing lipid-related diseases such as newborn adiposity.[251] Likewise, the reduced mitochondrial function has been noted in brain injury in newborns.[252] Decreased mitochondrial proteins may lower neonatal pyruvate dehydrogenase and oxidative phosphorylation activity, and increase the risk of morbidity.[253] Metabolic shifts are also important for the function of cardiomyocytes. Mutations in mtDNA and mitochondrial dysfunction were associated with dilated cardiomyopathy via transcription factor A, mitochondrial (TFAM).[254]



Figs 11A to F: Mitochondrial genes. (A) Overall pattern of organization in the 5 mitochondrial gene complexes; (B) Complex I is comprised of 38 subunits that are encoded in the nuclear DNA, 7 in mitochondrial DNA, and several assembly units. This complex oxidizes NADH by transferring electrons to ubiquinol. NADH stands for "nicotinamide adenine dinucleotide (NAD) + hydrogen (H); (C) Complex II converts succinate to fumarate and reduces FAD to $FADH_2$ during this process. The released electrons are transferred to ubiquinol. SDH = succinate dehydrogenase. The figure shows four components of the SDH complex, A-D; (D) Complex III (coenzyme Q: cytochrome *c* – oxidoreductase, or the cytochrome bc_1 complex), contains 11 subunits: 3 respiratory subunits, 2 core proteins and 6 low-molecular-weight proteins; (E) Complex IV (cytochrome *c* oxidase or cytochrome AA3), contains two hemes, a cytochrome a and cytochrome a_3, and two copper centers, the Cu_A and Cu_B centers; and (F) Complex V (mitochondrial ATP synthase) is a multisubunit oxidative phosphorylation complex. Complex V is composed of two functional domains: F_1, which is situated in the mitochondrial matrix, and F_o, located in the inner mitochondrial membrane. Complex V uses the energy created by the proton electrochemical gradient to phosphorylate ADP to ATP. ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; Pi, inorganic phosphate

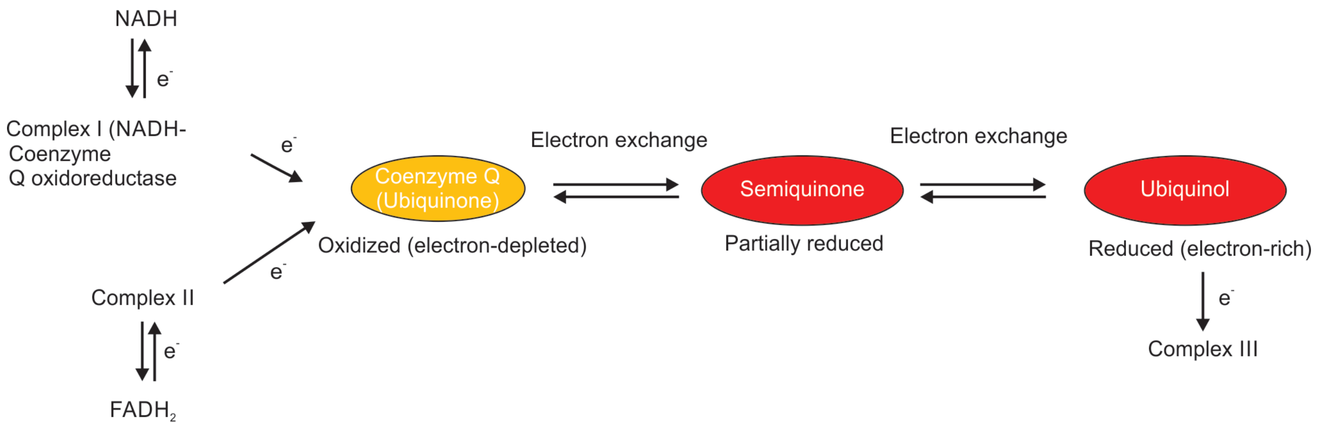


Fig. 12: Ubiquinol is an electron-rich (reduced) form of coenzyme Q (ubiquinone). The term most often refers to ubiquinol-10 that has a 10-unit tail; it exists in three redox states, the fully reduced (ubiquinol), partially reduced (semiquinone or ubisemiquinone), and fully oxidized (ubiquinone) forms. Ubiquinol can serve a redox function in cellular energy production and in antioxidant protection based on the ability to exchange two electrons in redox cycles. Complex I (NADH-Coenzyme Q oxidoreductase, or NADH dehydrogenase) can accept high energy electrons from NADH, and complex II interacts with $FADH_2$.

Changes in mitochondrial function based on maternal age: Mitochondria have important roles in oocyte maturation, fertilization, and early embryo development.[255] In women of advanced reproductive age, aging oocytes often show less ATP and mtDNA copy number, mutations in mtDNA, and ultrastructural abnormalities.[256]

Urea Cycle

The initial steps of the urea cycle take place in the mitochondrial matrix, particularly in hepatocyte and renal epithelium (Fig. 13).[257] In the first step, the carbamoyl phosphate synthetase I enzyme utilizes two ATP molecules to convert ammonia into carbamoyl phosphate.[258] In the second, ornithine transcarbamylase converts carbamoyl

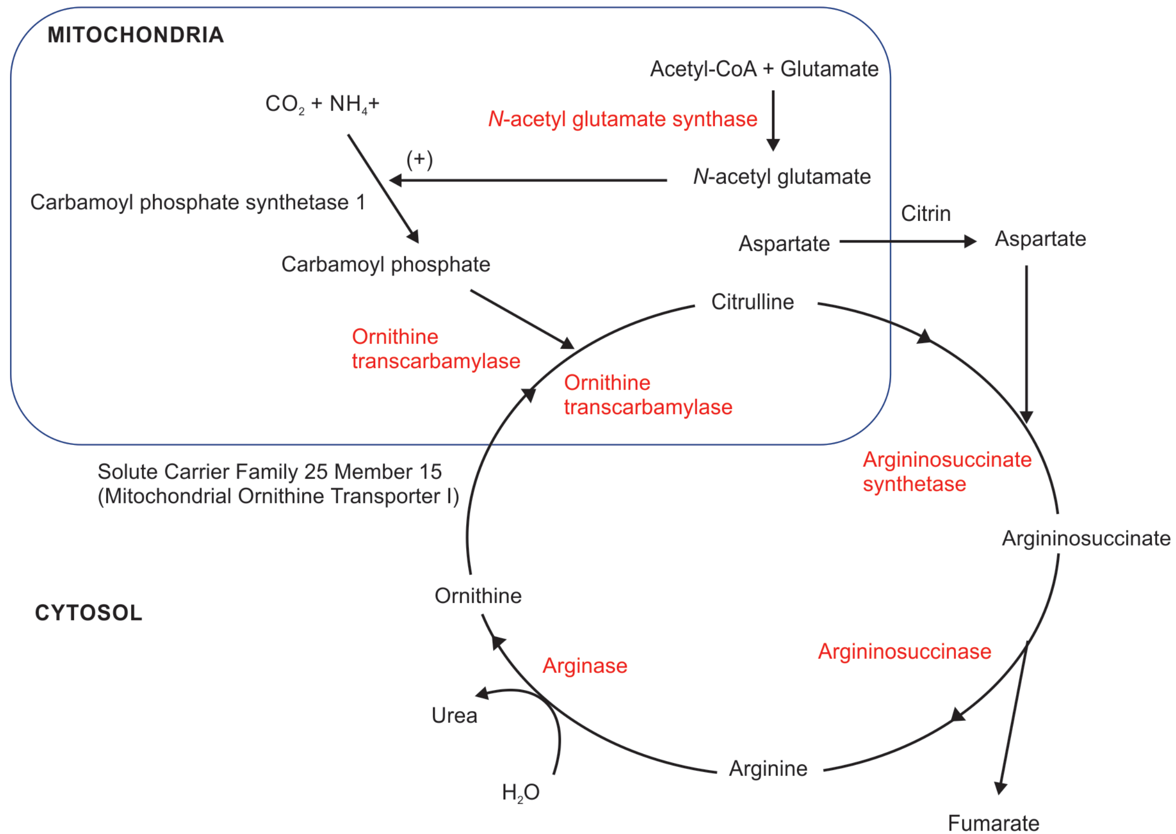


Fig. 13: The initial steps of the urea cycle take place in the mitochondrial matrix. Carbamoyl phosphate synthetase I (CPS I) combines ammonia with carbon dioxide to from carbamoyl phosphate and ornithine transcarbamylase promotes citrulline synthesis. *N*-acetyl glutamate (NAG) synthase increases the formation of NAG, which activates CPS I.

phosphate and ornithine into citrulline.[259] Subsequent steps continue in the cytoplasm until ornithine is re-transported into the matrix.[257]

Transamination

Oxaloacetate can be transaminated to produce aspartate and asparagine in the matrix.[260] Similarly, transamination of α-ketoglutarate produces glutamate, proline, and arginine.[261]

Metabolic Regulation

The concentrations of ions, various metabolites, and energy charge in mitochondria are closely regulated. Ca^{2+} ions regulate the TCA cycle (as shown in Fig. 4) by activating pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase.[262] The concentration of intermediates and coenzymes in the matrix also influences the rate of ATP production.[263] NADH can inhibit TCA enzymes α-ketoglutarate dehydrogenase, isocitrate dehydrogenase, citrate synthase, and pyruvate dehydrogenase.[264] Adenosine triphosphate inhibits isocitrate dehydrogenase, pyruvate dehydrogenase, the electron transport chain, and ATP synthase.[265] In contrast, ADP acts as an activator.[266]

Apoptosis

Mitochondrial apoptosis is the most common form of programmed cell death.[267] It is mediated through proteins of the B-cell lymphoma-2 (bcl-2) family.[268] There are two sub-classes of bcl-2 proteins: (a) the pro-apoptotic bcl-2 associated X-protein (bax) and bcl-2 antagonist/killer 1 (bak) proteins, which oligomerize to create "pores" on the OMM in response to apoptotic stimuli.[269] Through these pores, proteins from the mitochondrial intermembrane space (IMS) reach the cytoplasm and activate the caspase cascade;[51,270,271] (b) anti-apoptotic bcl-2 proteins such as bcl-2, bcl-x_L or myeloid cell leukemia (MCL)-1, which inhibit bax and bak signaling.[272]

Apoptosis is induced when various conformations of activated bax accumulate on the mitochondrial surface.[267] The ratio of mitochondrial/cytosolic levels of bak and bax determine the cellular response to apoptosis stimulation.[273] Increasing information suggests that bax, either alone or complexed with other proteins such as cytochrome *c*, Smac/direct IAP binding protein with low pI (Diablo), HtrA Serine Peptidase 2 (HtrA2)/Omi, and apoptosis-inducing factor (AIF), forms pores in the OMM to release IMS proteins.[274] Bcl-2 associated X-protein (Bax) can also modulate the function of permeability transition pore complexes formed with other mediators such as the voltage-dependent anion channels (VDAC–1, –2, –3; mitochondrial porin), the adenine nucleotide transporter cyclophilin D, and the F_1F_o ATP synthase.[275] These complexes promote the transport of nucleotides, phosphocreatine, Ca^{2+}, and other small ions across the OMM.[276] Some investigators have noted the resemblance between active bax/bak and the holin proteins that are involved in host cell membrane lysis by bacteriophages.[277,278] The bax/bak oligomers form membrane lesions, which release endolysin, a muralytic enzyme, through these lesions to attack the cell wall.[278,279]

Ionic Content of Various Tissues

Mitochondria can absorb calcium ions and play an important role in the regulation of calcium content in various cells.[11,280] Increased intracellular calcium can regulate many cellular processes. In neurons, these levels can alter neurotransmitter release.[281] It can also alter other processes such as endocrine changes. muscle function, and coagulation.[282] In infants, mitochondria can alter non-shivering thermogenesis in brown fat through proton leaks.[283]

Mitochondrial Genetic Defects in Neonates

Epidemiology and Clinical Features

Mitochondrial dysfunction affects one in 6,000–8,000 newborns.[284–286] The diseases may occur in patterns consistent with autosomal recessive, autosomal dominant, mitochondrial, and random mutations.[287,288] Young infants require mitochondrial energetic metabolism to support rapid growth. Key organs such as the muscle, heart, and brain require mitochondrial function/aerobic metabolism for adaption to extra-uterine life.[289] Disorders of mitochondrial metabolism caused by defects in fatty acid oxidation; pyruvate metabolism; and those in the respiratory chain, including mitochondrial complex I, II, III, IV, and ATP synthase, can become symptomatic in the neonatal period.[230] Mutations of both nuclear genes and mtDNA can cause mitochondrial dysfunction with adverse, outcomes in neonates.[224–228,290]

Neonatal-onset mitochondrial disease is associated with considerable, early mortality.[291] In a recent study, Ebihara et al.[285] reviewed the records of 281 patients with mitochondrial disease. The multisystem disease was noted in 194, Leigh syndrome in 26, cardiomyopathy in 38, and hepatopathy in 23 patients. Of the 321 with initial symptoms, 236 were recognized to have illness within two days of birth. The disorders were recognized by altered mitochondrial respiratory chain enzyme activity rate in 182, and abnormal oxygen consumption rate in 89. The remaining 10 patients were diagnosed using a genetic approach. Genetic analysis showed 69 to have nuclear DNA variants in 36 genes; 11 of 15 had mtDNA variants in 5 genes, and 4 had a single large deletion. Cyclo-oxygenase (Cox) proportional hazards regression showed significant differences in survival in those with Leigh syndrome [hazard ratio (HR) = 0.15, 95% confidence interval (CI) 0.04 to 0.63, $p = 0.010$] and in others with a molecular diagnosis (HR = 1.87, 95% CI 1.18 to 2.96, $p = 0.008$).

In the outpatient setting, mutations of mitochondrial complex I mutations can be seen with Leigh Syndrome, lethal infantile mitochondrial disease, lactic acidosis, MELAS syndrome, and Leber's Hereditary Optic Neuropathy.[292,293] Mutations in succinate dehydrogenase (SDH)-A, -B, and -AF1 genes in Complex II can cause mitochondrial leukoencephalopathy, cardiomyopathy, infantile leukodystrophy, and Kearns-Sayre syndrome.[294] Mutations in Complex III can cause severe lactic acidosis with hypotonia, irritability, and muscle wasting.[295] Complex III deficiency is mainly caused by mutations in maternally-transmitted mitochondrial chaperone BCS1 (BCS1L), Ubiquinol-Cytochrome C Reductase-Binding Protein (UQCRB), Ubiquinol-Cytochrome C Reductase Complex III Subunit VII (UQCRQ) and mitochondrially-encoded Cytochrome B (MTCYB) genes.[296,297] Mutations in Complex IV are associated with neonatal hypertrophic cardiomyopathy, liver dysfunction, myopathy, hypotonia, developmental delay, and encephalopathy.[294] The biogenesis and assembly of cyclooxygenase (COX) in Complex IV depends on numerous ancillary factors, including copper chaperones, all nuclear-encoded.[298,299] Specifically, disease-causing mutations were found in the gene encoding the Surfeit locus protein 1 (SURF1), which is essential for the formation of early assembly intermediates.[300,301] Mutations in Complex IV have been associated with neonatal encephalopathy, respiratory distress, pulmonary hypertension, lactic acidosis, peripheral neuropathy, dysmorphism, and cataracts.[302] Defects in ATP synthase can also cause fatal encephalopathy in neonates.[303,304]

Pyruvate dehydrogenase complex (PDHc) catalyzes the oxidative decarboxylation of pyruvate to produce acetyl-CoA and initiates the TCA cycle.[305] Pyruvate dehydrogenase complex (PDHc) deficiency is most often due to mutations in the first component of the enzyme complex, pyruvate dehydrogenase E1α (responsible for 70% of PDH deficiencies).[306] There is a spectrum of clinical presentations in E1α mutations;[307] the most severe mutations can manifest with lactic acidosis within a few hours of birth.[308] Other infants may show hypotonia, lethargy, feeding, respiratory difficulties, and encephalopathy.[309,310]

Mitochondrial Disorders

- *Infections*: The sepsis syndrome is a systemic host inflammatory response accompanied by organ dysfunction in response to invading microbial pathogens.[311] The host recognizes both danger and pathogens through its pattern recognition receptors on immune cells.[312] These receptors bind to pathogen associated molecular patterns (PAMP) and danger (DAMP) associated molecular patterns derived from microbes and host tissues, respectively.[313] These DAMPs and PAMPs activate the formation of inflammasomes, which bind to the apoptosis-associated speck-like protein (ASC) containing a caspase recruitment domain (CARD).[314–316] This forms a platform for the activation of caspase-1 and induction of interleukin (IL)-1β and IL-18.[317,318] Caspase-1 triggers mitochondrial damage.[319] It also inhibits mitophagy, a process that clears damaged mitochondria, leading to accumulation of defective mitochondria and damaged cells.[319] Mitochondrial DNA (mtDNA) has also been detected in the extracellular traps formed by innate immune leukocytes in these infected lesions.[320,321]
- *Oxidative phosphorylation*: Mitochondria are the site of oxidative phosphorylation in eukaryotes; the energy is produced by means of electron flow between four enzymes, of which three are proton pumps, in the inner mitochondrial membrane.[322] NADH generated in the TCA cycle is oxidized and activates the electron transport chain, which is comprised of complexes I–IV, and ATP synthase.[158,216] Acute inflammation may curtail these pathways.[323] Tumor necrosis factor (TNF) induces microRNAs that damage the mitochondrial complex-I, inhibit oxidative phosphorylation, and reduce ATP levels.[324,325]
- *Inflammation:* Inflammatory stimuli can promote mitochondrial fragmentation by increased protein unfolding, ER stress, phosphorylation of pro-fission proteins, and decreased respiratory capacity.[326–330] There is also increased oxidative stress; mitochondrial complex III generates superoxide during the ubiquinone (Q)-cycle.[331] Some lesions may show mitochondrial fission, mitophagy, and decreased fusion.[330,332,333] The intrinsic dynamicity of mitochondria also plays a role in proinflammatory signaling, identifying these organelles as a central platform for the control of innate immunity and the inflammatory response.[334]

During inflammation, cytokines such as TNF, interleukin (IL)-1, and IL-18 can promote necroptosis, a form of programmed necrosis mediated by various cytokines and pattern recognition receptors (PRRs).[335–338] Cells dying by necroptosis show necrotic phenotypes, including swelling and membrane rupture, and release damage-associated molecular patterns (DAMPs), inflammatory cytokines, and chemokines, thereby mediating extreme inflammatory responses.[339] Mitochondrial proteins such as the phosphoglycerate mutase (PGAM)-5 and dynamin-related protein (Drp)-1 have been identified as important activators of the receptor-interacting serine-threonine kinase (RIPK)-3 and consequent mitochondrial fission and necroptosis.[340–342] Mitochondrial reactive oxygen species (ROS) may regulate TNF-mediated cell death in other diseases.[343–345]

Mitochondrial mediators such as the DNA polymerase γ (POLG) and the protein growth factor erv1-like can alter physiological mediators for cellular self-renewal and suppress signaling mediators such as the octamer-binding protein 4 (OCT4), nanog homeobox (NANOG), and the putative thiosulfate sulfurtransferase (SSEA).[346–350]

- *Mitophagy in birth asphyxia and neurological disorders*: In asphyxiated infants with hypoxic-ischemic encephalopathy, neural energy failure is being increasingly documented.[351] Currently, the options for timely diagnosis and treatment are limited. Mitochondrial dysfunction with increased permeability, altered dynamics with changes in fission and fusion, mitophagy, and biogenesis have been observed in many studies.[352–355] Mitoprotective therapies may help prevent/treat brain injury and reduce the incidence of lifelong disabilities.[355,356]

  In other neurological disorders, mitochondrial transmembrane potential loss with the involvement of PTEN (phosphatase and tensin homolog)-induced putative kinase 1 (PINK1), which then recruits Parkin, the E3 ubiquitin ligase, to the damaged mitochondria.[357–360] The BCL2 (B-cell lymphoma 2 genes)-interacting protein 3-like (BNIP3L), a mitophagy receptor that recruits LC3 family proteins to the damaged mitochondria, can be altered.[361,362] The FUN14 domain-containing 1 (FUNDC1) is another mitophagy receptor located on the outer mitochondrial membrane.[363,364]
- *Mutations in mitochondrial genes*: Many deletions and duplications in the mitochondrial genome can be seen sporadically. These may develop *de novo* or during early development (Table 1):[365–367]

  Leber's hereditary optic neuropathy (LHON) is the most common, maternally-inherited mitochondrial disorder in the respiratory chain, which causes degeneration of retinal ganglion cells, associated axons, and optic atrophy within a year of disease onset.[368,369,319] An intriguing feature of LHON is that only 50% of males and 10% of the females with the mtDNA mutations actually become symptomatic.[370] This incomplete penetrance and gender bias imply that additional mitochondrial and/or nuclear genetic factors must be modulating the phenotypic expression of LHON.[371] It typically begins as a unilateral progressive optic neuropathy with the central visual loss with sequential involvement of the fellow eye months to years later.[220] In about 90% of clinical cases, the disease is associated with three mutations in mtDNA complex I subunit genes, namely the G3460A, G11778A, and T14484C. These mutations are absent or very rare among normal controls.[371–374]

  Pearson syndrome is a rare fatal mitochondrial disorder caused by single large-scale mitochondrial DNA deletions. Most patients present with sideroblastic anemia during infancy, followed by multi-organ dysfunction including lactic acidosis, pancreatic insufficiency, renal tubulopathy, failure to thrive, muscle hypotonia, and endocrine disorders.[375,376] Bone marrow cytology shows vacuolization in erythroid and myeloid precursors and ring-sideroblasts; the diagnosis is established by the detection of mitochondrial DNA deletions.[375,377,378] Most cases have a lethal outcome. Some survivors go on to develop Kearns-Sayre syndrome, a progressive cardio-encephalo-myopathy caused by a large deletion or rearrangement of mtDNA.[379–382]

  Leigh syndrome, also termed subacute necrotizing encephalomyelopathy, is a rare, inherited progressive neurodegenerative disorder that usually manifests in infancy or early childhood.[383,384] Many cases can be diagnosed in early infancy and present with developmental delay, pyramidal and extrapyramidal symptoms, leukodystrophy, and brainstem dysfunction.[385,386] Neuroimaging shows focal, symmetrical, necrotic lesions in the thalamus, the brainstem, and the posterior columns of the spinal cord. Histopathology shows symmetric spongiform lesions with degeneration of basal ganglia, particularly in the corpus striatum; and demyelination, vascular proliferation, and astrocytosis in the brainstem.[387–389] The lesions typically appear hyperintense on T2-weighted MRI.[390] Pathogenic mutations are often seen in flavoprotein of complex II.[391]

  Alpers-Huttenlocher syndrome manifests with seizures, developmental delay, hypotonia, and liver disease.[326,327] It is a maternally-inherited disease with variable penetrance. It is usually caused by mutations in polymerase gamma (POLG).[392,393] Mutations in mitochondrial tRNA synthetase genes, including the phenylalanyl-tRNA synthetase 2, mitochondrial (FARS2), asparaginyl-tRNA synthetase 2, mitochondrial (NARS2), and the prolyl-tRNA synthetase 2, mitochondrial (PARS2) have also been linked.[394,395]

  A syndrome associated with MELAS syndrome is caused by mutations in mitochondrial transfer genes such as the mitochondrially-encoded tRNA leucine 1 (MT-TL1).[396,397] It is characterized by mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.[398,399]

Our Current Understanding

Mitochondria provide energy and regulate key cellular events not only during the embryonic, fetal and neonatal period, but and throughout life.[4] After birth, the newborn infant's organs may show major changes in the number and function of mitochondria.[400] There is some evidence that epigenetic changes in mitochondrial DNA during the perinatal period may be protective.[401,402] These changes are most noticeable in the muscles, heart and brain.[403] Disorders of mitochondrial metabolism related to nuclear/mtDNA defects in mitochondrial complexes I, II, III, IV, and ATP synthase may present in the neonatal period.[404,405]

There is a need for focused studies of mitochondrial function and its modulators in the fetal/neonatal period to ascertain the need for interventions.[406] We still have major gaps in our understanding of the long-term effects of mitochondrial dysfunction in neonatal period and infancy.[353] A framework is needed to focus future research on altered mitochondrial function as a mechanism of perinatal adaptation.

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REVIEW ARTICLE

Congenital Chikungunya Virus Infections

Srijan Singh[1], Astha Amrit[2] , Sushant Mane[3], Gangajal Kasniya[4], Mohd Mozibur Rahman[5], Atnafu Mekonnen Tekleab[6], Akhil Maheshwari[7]

Received on: 16 January 2023; *Accepted on:* 08 February 2023; *Published on:* 06 April 2023

Abstract

Structure: Chikungunya virus (CHIKV) is an arthropod-borne ribonucleic acid (RNA) virus, classified in the genus alphavirus in the family Togaviridae.

Clinical presentation: Perinatal/neonatal infections are rare, but some infants can develop fever, thrombocytopenia, lymphopenia, pigmentary changes, and a maculopapular rash. The neurocognitive outcome of some infants with vertically transmitted mother-to-child perinatal infections and CHIKV neonatal encephalopathy can be poor.

Diagnosis: The diagnosis of CHIKV infections can be confirmed by the detection of chikungunya viral RNA via real-time reverse-transcription polymerase chain reaction (RT-PCR) and/or specific immunoglobulin (Ig)M and IgG serology.

Treatment: Currently, no specific antiviral treatment(s) are available for CHIKV, and management is limited to supportive care by maintaining adequate intravascular volume by intravenous fluids and oral rehydration. Infants exposed *in utero* or during the perinatal period need to be monitored for adverse neurocognitive outcomes.

Keywords: *Aedes aegypti*, *Aedes albopictus*, Brownie nose, Chikungunya sign, Chikungunya virus encephalitis, Infant, Neonate, Newborn, Thrombocytopenia, Vertical transmission.

Newborn (2023): 10.5005/jp-journals-11002-0054

Key Points

- Chikungunya virus is widely transmitted in tropical and subtropical areas by Aedes (Ae.) mosquito vectors: *Aedes aegypti* and *Aedes Albopictus*.
- Pregnant mothers with recent CHIKV infections can transmit the virus to the fetus *in utero* or to the newborn infant during the perinatal period. These infants are diagnosed as infected if they test positive for viral RNA or specific IgM antibodies before postnatal day 10 in blood or day 15 in the cerebrospinal fluid (CSF). The virus is carried throughout the body in infected monocytes.
- Chikungunya virus encephalitis can show as white matter (WM) hyperintensities on T1-weighted magnetic resonance imaging (MRI) in the choroid plexus, leptomeninges, and ependyma. The long-term neurocognitive outcome of these children is poor.
- There is no specific treatment. Supportive management includes close monitoring of vital signs and maintenance of adequate intravascular volume.
- A live-attenuated, measles-vectored vaccine expressing CHIKV structural proteins (MV-CHIK), a chikungunya (CHIK) virus-like particle (VLP) vaccine, and a messenger RNA (mRNA)-based vaccine (VLA-181388) are under trials.

[1,3]Department of Pediatrics, Grant Government Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India

[2]Department of Neonatology, Bai Jerbai Wadia Hospital for Children, Mumbai, Maharashtra, India

[4]Department of Neonatology, Ochsner Hospital for Children, New Orleans, Louisiana, United States of America

[5]Department of Neonatology, Institute of Child and Mother Health, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh

[6]Department of Pediatrics and Child Health, Saint Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia

[7]Global Newborn Society, Clarksville, United States of America

Corresponding Author: Srijan Singh, Department of Pediatrics, Grant Government Medical College and Sir JJ Group of Hospitals, Mumbai, Maharashtra, India, Phone: +91 7011033174, e-mail: srijanstar89@gmail.com

How to cite this article: Singh S, Amrit A, Mane S, *et al*. Congenital Chikungunya Virus Infections. Newborn 2023;2(1):45–59.

Source of support: Nil

Conflict of interest: Dr. Mohd Mozibur Rahman and Dr. Akhil Maheshwari are associated as the Editorial Board Members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial Board Members and their research group.

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne (arbovirus) classified in the genus alphavirus, the arthritogenic Semliki forest virus serocomplex, and the family Togaviridae.[1–3] In adults, it has been associated with acute febrile polyarthralgia, inflammatory arthritis, and dermatologic and systemic presentations.[4,5] It was first isolated by Ross in 1952 in the Newala district of Tanzania[6] and then described in more detail in 1955 by Robinson and Lumsden after an earlier outbreak on the Makonde Plateau, along the border between Tanganyika and Mozambique.[7,8] The name Chikungunya is derived from the Kimakonde language spoken in southeast Tanzania and northern Mozambique, meaning "that which bends up" referring to the debilitating arthralgia caused by this disease.[9,10] It has now increasingly been recognized as a global health concern.[11]

Viral Structure

The chikungunya virus originated in Tanzania and is closely related to the O'nyong'nyong virus, which originated in Uganda.[12–14] It

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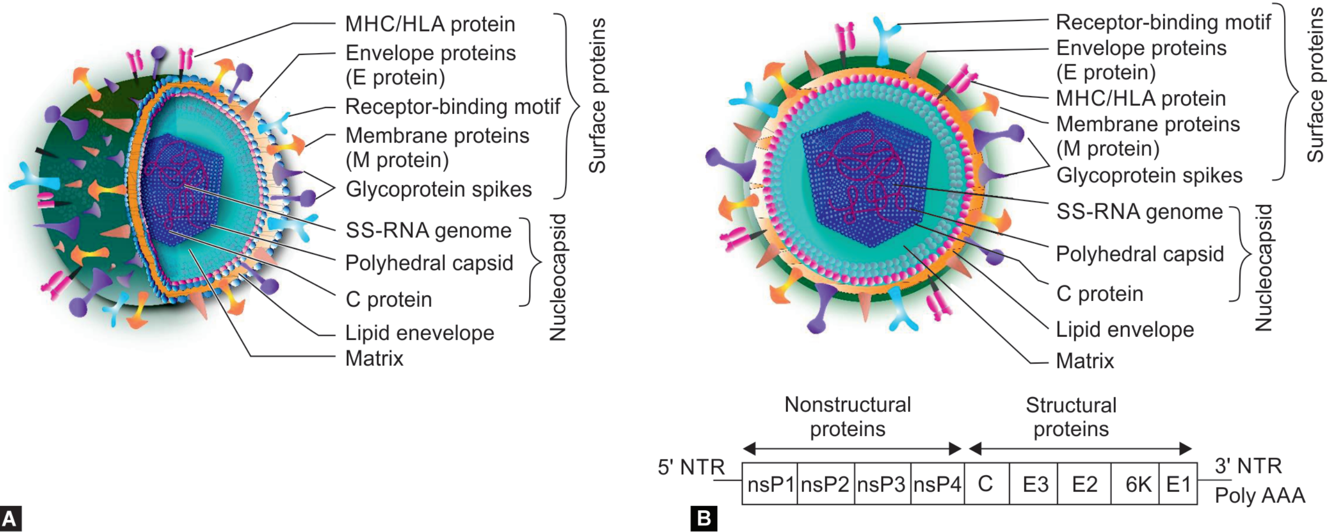


Figs 1A and B: Schematic diagrams showing (A) surface and side dissection and (B) cross-section of the chikungunya virus

is an enveloped, positive-sense, single-chain linear ribonucleic acid (RNA) virus with a diameter of 60–70 nm (Fig. 1).[15] The RNA genome of around 11.8 kb is divided into two open reading frames (ORFs) surrounded by 5′ and 3′ nontranslated regions.[15,16] The 5′ ORF encodes four nonstructural proteins (nsPs): nsP1, nsP2, nsP3, and nsP4. The 3′ ORF also encodes four structural proteins: capsid (C), envelope 3 (E3), envelope 2 (E2), and envelope 1 (E1).[17,18] The nsPs are important mediators in viral pathogenesis and neuroinvasiveness due to their role in viral replication inside the host cytoplasm. The structural proteins facilitate the recognition of host cells, binding, and entry.[19,20] The E2 subunit of the E protein binds the Mxra8 receptor on fibroblasts and skeletal muscle cells and promotes viral entry into host cells by clathrin-mediated endocytosis.[20–24]

In RNA viruses, the replacement of only a few amino acids can bring about major changes in biological properties.[25,26] The RNA-dependent RNA polymerase (RdRp) of CHIKV is a low-fidelity enzyme, and hence can promote the formation of new viral variants during successive cycles of RNA replication.[27–29] These features promote adaptation to varying hosts and determine their pathogenicity.[30–32] The CHIKV has been traditionally classified into three lineages based on the sequences of the envelope E1 gene.[33,34] Since 2006, a new classification with four geographic lineages has been recognized: (1) the West African, (2) the Asian genotypes with varying E protein expression, (3) the East/Central/South African (ECSA) with mutations in the E1 protein, and (4) the Indian ocean lineage that has diverged from the ECSA.[35–38] The ECSA variants have also been isolated in Rio de Janeiro, Brazil, and in Reunion Island.[39] These variants can cause severe cutaneous lesions and multisystem disease in neonates.[19,33] Some mutants may show enhanced transmission via *Aedes albopictus* mosquitoes[26] and are associated with neuroinvasive disease by upregulating Toll-like receptor-3 in neuronal cells.[40,41] The ECSA strains with an arginine-to-valine mutation on position 226 seem to be the most pathogenic, CHIKV-Western Hemisphere (CHIKV-WH) as moderately so, and the unmodified ECSA and West African strains the least pathogenic lineages.[42,43] Table 1 summarizes the information on the major viral components.

Epidemiology

Geographical Areas at Higher Risk of CHIKV Infections

Chikungunya virus infections can be both endemic and epidemic.[10,44] The virus is transmitted via the *Aedes* mosquito vectors with a typical incubation period of 3–7 days, although the infectious period may range from 1 to 12 days.[45,46] It is endemic in West Africa, but outbreaks have also been recorded in other parts of Africa, Asia, Europe, islands in the Indian and Pacific Oceans, and in the Americas.[47,48] Over one-third of the inhabitants of La Reunion Island, a French territory in the Indian Ocean, were affected in the 2005–2006 outbreak.[49] Most outbreaks in the tropics occur during rains.[11] The CHIKV may rarely be transmitted by blood products.[50,51] The chikungunya (CHIK) viremia can precede the onset of symptoms and disappears after 6–7 days of illness.[52,53]

Mother-to-Fetus Transmission

Pregnant women infected with CHIKV before 16 weeks' gestation can develop deep trophoblastic invasion and consequently, fetal sequelae and deaths. In these women, the viral genome is detectable in high titers in the amniotic fluid, placenta, and/or brain of the fetuses.[54] The mothers are usually asymptomatic other than the occasional miscarriage.[55,56] During the second trimester, the placenta is a strong barrier to CHIKV and maternal-to-fetal transmission of the virus is generally infrequent.[47] In the third trimester, transmission is infrequent; even most of the stillborn fetuses born to mothers with CHIKV fever do not test positive for the virus.[57]

We do not have consistent and detailed epidemiological data implying a strong association between first-trimester infections and increased risk of miscarriage or congenital malformations. Chikungunya virus infections may occur with higher frequency in mothers from the lower socioeconomic strata of society.[58]

Maternal viremia is seen frequently in the peripartum period, particularly in the preceding 2 to the subsequent 2 days after delivery.[56] Vertical transmission rates during this period may range between 27.7% and 49%;[24,55,59] the risk is higher when peripartum

Table 1: Major structural components of CHIKV

Structure	*Available information*
Lipid envelope	The lipoprotein envelope is derived from the nuclear membrane of an infected host cell and covers the nucleocapsid.[191]
Glycoproteins	Glycoproteins, E1 and E2, form membrane spikes in an icosahedral shell on the virion surface. Glycoprotein E1 is a class II fusion protein that mediates low pH-triggered membrane fusion during infection. E2 is a type I transmembrane glycoprotein and binds cell surface receptors.[192,193] It is derived from furin cleavage of p62 precursors.[66,194]
Receptor-binding motifs	Receptor-binding motifs are involved in virion attachment to host cell surface receptors during the process of infection and endocytosis. Receptor binding is facilitated by the E2 glycoprotein of CHIKV,[195,196] which contains recognized receptor binding sites.[194,197] E2 domain B contains a class III PDZ-binding motif,[198,199] which mediates protein–protein interactions.[200,201] A phosphatidylserine residue in the viral envelope also binds cell–surface receptors on the cell surface.[202]
Envelope protein	Envelope proteins, E1 and E2, form membrane spikes on the viral surface.[203] These spikes facilitate attachment to cell surfaces and viral entry into the cells.
Membrane protein	E1 protein contains three β-barrel domains. Domain I is between domains II and III, and the fusion loop is at the distal end of domain II.[194] Heterodimers of the E1 and E2 proteins assemble into spikes on the virion surface and facilitate the infection of target cells.[194] The E1 protein contains a hydrophobic fusion peptide and is necessary for viral and cellular membrane fusion.[21]
Major histocompatibility complex (MHC) or human leukocyte antigens (HLA) proteins	Conserved B- and T-cell epitopes of CHIKV structural proteins may play an important role in evoking immune responses against CHIKV. B-cell epitopes "PPFGAGRPGQFGDI" is highly immunogenic, while among T-cell epitopes, MHC class I peptides "TAECKDKNL" and MHC class II peptides "VRYKCNCGG" are important. All T-cell epitopes are conserved between CHIKV genomic sequences belonging to 17 different countries.[204]
Spike protein	Glycoproteins, E1 and p62, bind to form heterodimers that subsequently trimerize into a viral spike in the endoplasmic reticulum. The CHIKV spikes show intraspike contacts between three constituent E2 molecules. The glycoprotein E1 wraps around E2 and contributes to interspike interactions with E3 being located at the periphery of the E2 molecules. The spikes undergo a structural rearrangement during maturation, with the cleavage of p62 into E2 and E3, thereby exposing the fusion loop on E1 and arranging the glycoprotein spikes into a mature conformation. The association of mature spikes with the nucleocapsid makes these less compact and nucleocapsid disassembly upon release into the host cell cytoplasm corresponding to the release of the genome into a host cell after virus entry.[199]
Surface tubules	Either not expressed or relevance unclear fetal/infantile disease.
Palisade layer	Either not expressed or relevance unclear fetal/infantile disease.
Viral tegument	Either not expressed or relevance unclear fetal/infantile disease.
Lateral bodies	Either not expressed or relevance unclear fetal/infantile disease.
Capsid	The capsid is composed of 240 copies of specific proteins and encloses the viral genomic RNA in nucleocapsid cores. These cores interact with the E1–E2 glycoproteins produced in the endoplasmic reticulum and the Golgi. The mature virions bud from the plasma membrane.[205]
Capsomeres	Structural subunits of the capsid and can be seen in electron micrographs.[206]
Core membrane	Either not expressed or relevance unclear fetal/infantile disease.
Protein core	The polyprotein is expressed from the ORF1 of CHIKV. It is processed into four nsPs (nsP1, 2, 3, and 4), which undergo proteolysis and assemble into the viral replication complex.[201] Mature nsPs function collaboratively to replicate the viral genomic RNA and to transcribe the subgenomic RNA, which encodes the structural genes for virus particle assembly.[207]
Core fibrils	Either not expressed or relevance unclear fetal/infantile disease.
Matrix	Either not expressed or relevance unclear fetal/infantile disease.
Enzymes	The cerebral palsy (CP) sindbis virus (SINV) is divided into three regions: region I (residues range: 1–80), region II (residues range: 81–113), and region III (residues range: 114–264). The regions I and II are part of the N-terminal domain of CP and are involved in the encapsidation of the genomic RNA.[205] The region III is part of the C-terminal domain, which is responsible for the serine protease activity of CP. The CP has a cis-proteolytic activity that cleaves itself from the nascent structural polyprotein precursor.[206] The nsP1 displays the unique N7-guanine-methyltransferase and guanylyltransferase activities required for viral RNA 5′ cap-0 synthesis. The nsP2 is the largest nsP that has the N-terminal RNA helicase/nucleoside triphosphatase/RNA triphosphatase domain and the C-terminal cysteine protease domain. The nsP4 is the RNA-dependent RdRp.[201]
RNA elements	A transient double-stranded replicative RNA intermediate composed of viral plus- and minus-strand RNAs is synthesized by a replicase complex formed by the non-structural proteins nsP1–4.[208] The newly synthesized minus strand serves in turn as a template, allowing the RNA-dependent RdRp to synthesize additional plus-strand genomic RNA.[17] Following the complete processing of the ns-polyprotein, the replicase then promotes the synthesis of the viral genomes and production of the subgenomic RNAs that encode the viral capsid and envelope proteins.[209]

(Contd...)

Table 1: *(Contd...)*

Structure	*Available information*
Nucleus	Either not expressed or relevance unclear fetal/infantile disease.
Nucleosome	Either not expressed or relevance unclear fetal/infantile disease.
DNA	No DNA genome exists.
RNA	The CHIKV virion contains a positive-sense RNA genome ~11.8 kb in length, which is translated into a large polyprotein during the infectious life cycle. The genome contains two ORFs flanked by 5′- and 3′-untranslated regions (UTRs) and separated by a noncoding intergenic region. The 5′-UTR is 76 nt in length and contains a 5′ type-0 N 7-methylguanosine cap for initiation of cap-dependent translation. The 3′-UTR varies in length between ~500 and ~900 nt and includes a 3′ polyadenylate tail.[17]
Genome-associated polyprotein	RNA genome is translated into a replicase complex consisting of four nsPs that are expressed as a polyprotein precursor. These nsPs are initially produced as a nonstructural polyprotein precursor that is processed by the viral protease.[210]
DNA polymerase	Either not expressed or relevance unclear fetal/infantile disease.
RdRp	The C-terminal domain of nsP4 acts as an RNA-dependent RdRp and catalyzes the formation of negative-sense, genomic, and subgenomic viral RNAs. Viral replication begins with the synthesis of minus-strand RNA from the positive-strand RNA genome, which then acts as a template for the formation of plus-strand RNA genomes. Production of new viral particles is catalyzed by the RNA-dependent RdRp.[211]
Reverse transcriptase	Either not expressed or relevance unclear fetal/infantile disease.
Head	Either not expressed or relevance unclear fetal/infantile disease.
Base plate	Either not expressed or relevance unclear fetal/infantile disease.
Integrase	Either not expressed or relevance unclear fetal/infantile disease.
Tail	Either not expressed or relevance unclear fetal/infantile disease.
Tail fiber	Either not expressed or relevance unclear fetal/infantile disease.
Neck	Either not expressed or relevance unclear fetal/infantile disease.

PDZ, post-synaptic density-95, disks-large and zonula occludens-1

maternal viremia coincides with breaches in the placental barrier and, consequently, results in high placental viral loads.[47,52,55] Cesarean sections are not protective and are, therefore, not recommended.[54–56,59] However, even though the epidemiological data are scant, there are many reports of infants who got infected during the peripartum period as developing neurocognitive delays and arrested head growth after birth.[47,54,60]

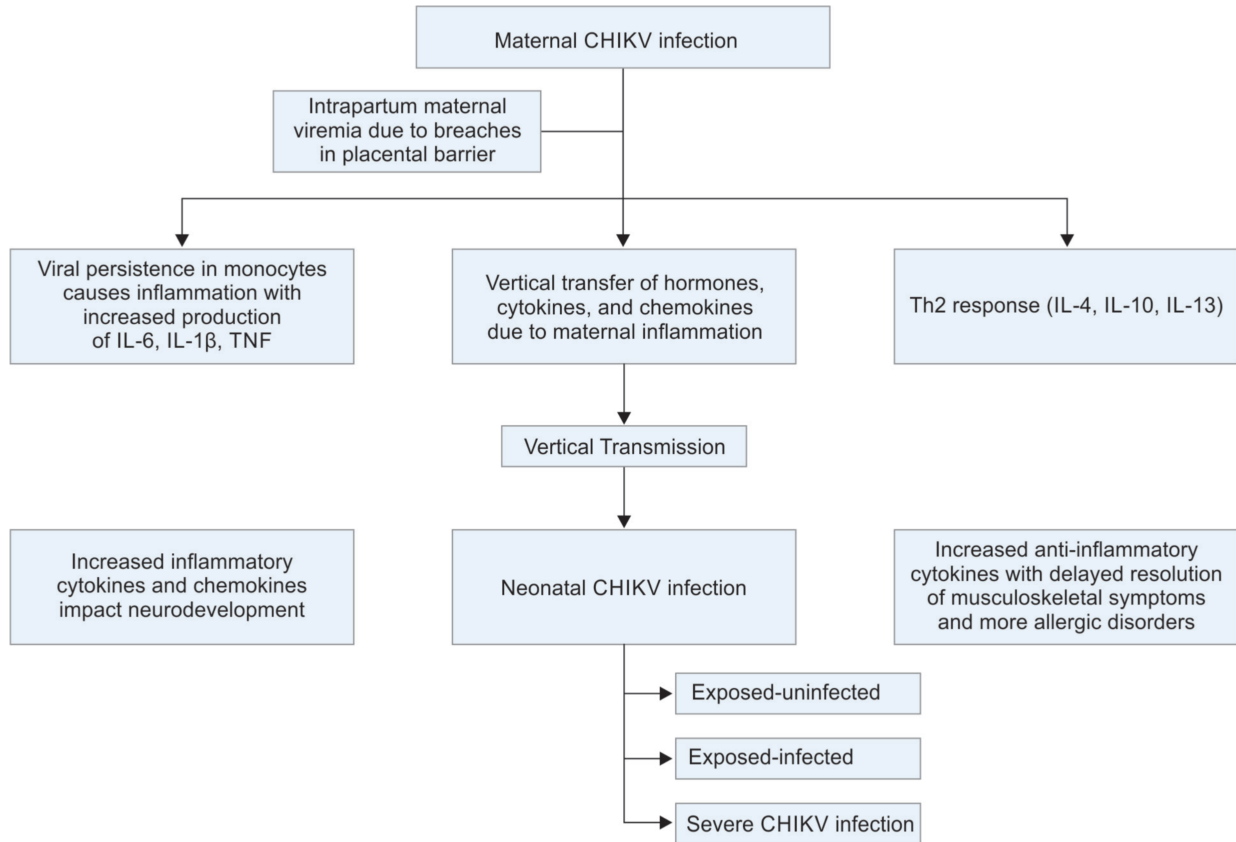
Pathogenesis

The CHIKV infection is followed by viremia within a few days of infection. Animal models of CHIKV infections suggest that the virus first infects the synovium, tenosynovium, and muscle, and then may persist in joints for several days to weeks.[61] This promotes the recruitment of leukocytes, particularly monocytes, and increased expression of inflammatory cytokines, chemokines, and other inflammatory mediators.[62–64] Disease severity correlates with the persistence of CHIKV in monocytes and the systemic inflammatory response with increased production of interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF), and CC ligand 2 (CCL2, monocyte chemoattractant protein-1) (Flowchart 1).[24,47,65,66]

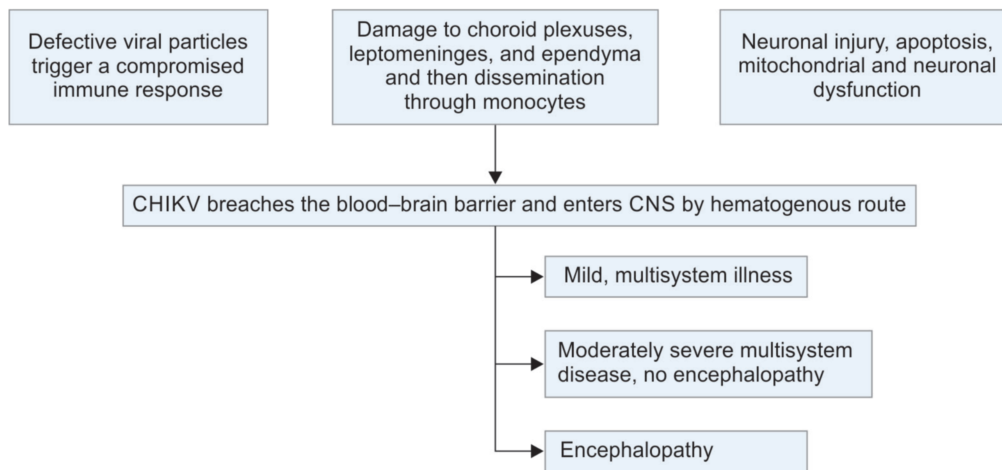
The CHIKV infections during pregnancy can manifest with maternal sepsis, preterm delivery, premature rupture of membranes, decreased fetal movements, intrauterine death, oligohydramnios, and preterm labor pains.[67,68] Cluster of differentiation 163 (CD163), an activation marker,[69] is detected in the CHIKV-infected placenta as an indication of the presence of Hofbauer cells.[70] The placenta is hyperplastic with enlarged CD163$^+$ cells due to immunological activation. Mitochondrial swelling, a characteristic of apoptosis,[71] and dilated endoplasmic reticulum cisterns are seen in the cytotrophoblasts of CHIKV-infected placenta, thereby affecting cell homeostasis and signaling.[72] There is a thickening of the endothelial basement membrane, which can alter the absorption of gases and nutrients in the placenta.[73] Maternal viral infections during gestation can cause epigenetic changes and alter the inflammatory microenvironment leading to developmental changes; there is a need for long-term follow-up.[74,75]

The CHIKV-induced maternal inflammation can influence fetal development through the vertical transfer of cytokines, chemokines, and hormones.[76] Neonates, with still evolving specific adaptive immunity, depend on innate immune responses with exaggerated inflammation and considerable morbidity.[77] The CHIKV-exposed infants have high levels of inflammatory cytokines such as TNF, which can impact neurodevelopment, and inhibit the proliferation and differentiation of neuronal cells (Flowchart 2).[78,79] However, the effects of various cytokines are not consistent. The CHIKV-exposed infants may have increased circulating chemokines such as chemokine (C-X-C motif) ligand 8 (CXCL-8), chemokine (C-C motif) ligand 3 (CCL3) macrophage inflammatory protein, (MIP)-1α and CCL4 (MIP-1β), which recruit neutrophils and monocytes.[80–82] Elevated plasma/cerebrospinal fluid (CSF) levels of CXCL9, CXCL10, and the eotaxins (CCL11, CCL24, and CCL26) can promote neuronal damage and possibly be associated with Zikavirus (ZIKV) microcephaly.[47,83–86] CXCL10 and eotaxins can also promote neurological damage in these patients.[47,84] Low levels of T-helper cell 2 (Th2)-related cytokines such as IL-4, IL-10, and IL-13 can delay the resolution of musculoskeletal symptoms.[87,88] However, CCL3/MIP-1α may be neuroprotective.[82] Interferon (IFN)-γ and IL-12p70 may also be

Flowchart 1: Pathogenesis of perinatal chikungunya infection



Flowchart 2: Pathogenesis of neurological manifestations



neuroprotective in some situations.[79,89,90] However, neonates have limited expression of Toll-like receptors that induce IFN production.[91] Type I IFN (IFNα, −β, and −ω) are an important component of anti-CHIKV immunity and can suppress viral replication in the early stages of the disease.[92]

Asymptomatic CHIKV-infected infants may present with serum ferritin > 600 ng/dL, which is a by-product of IL-8 activation in viral infections.[93] It has been noted earlier as a predictor of the severity of dengue fever.[94] Compared to plasma, CSF samples frequently contain different or defective viral mutants with insertions/deletions and stop codons in nonstructural genes. Defective viral particles can trigger an atypical immune response that may cause placental or blood–brain barrier damage, followed by vertical or brain transmission.[95]

Murine models of CHIKV infection show increased expression of genes presumably involved in neuronal injury, mitochondrial and neuronal dysfunction, and apoptosis.[96] Even though only a minority of infected patients develop neuroinvasive disease, immune imbalance may play an important role in neurodegeneration. Because in addition to the CHIKV infection

itself, some immuno mediators such as TNF and IL-6 are known to cause neuronal death.[96]

The CHIKV encephalopathy is associated with neurotropism as evidenced by white matter (WM) and corpus callosum atrophy, microglial activation and demyelination, neuronal loss due to WM damage and leading to microcephaly, cerebral palsy, or neurocognitive dysfunction, which is similar to neonatal encephalitis caused by enterovirus or parechovirus infections.[97,98] These encephalopathic changes involve damage to the highly vascular choroid plexuses, leptomeninges, and the ependyma,[92,99] and are followed by the dissemination of the virus in monocytes.[100] This is seen as WM hyperintensities on T1-weighted magnetic resonance imaging (MRI), consistent with microglial activation leading to demyelination.[47] On MRI, the most distinctive lesion of CHIKV neonatal encephalopathy is reversible diffusion restriction of WM associated with transient ischemia with cytotoxic edema.[101] In an animal model, viral infection is mainly detected at the meningeal and ependymal levels rather than in the brain parenchyma.[92] The CHIKV affects stem cell production by ependymal cells, neuron migration,[102] and myelin sheath production.[13,103,104] Demyelination is the hallmark of CHIKV neonatal encephalopathy, which is caused by autoreactive $CD8^+$ T lymphocytes to clear infected cells.[105,106] $CD8^+$ T cells are frequently seen in the CSF of cynomolgus macaques, the only nonhuman primate model challenged by CHIKV.[107]

Clinical Presentations

For infants presenting during the neonatal period, the median age of presentation is 9.5 (range: 3–15) days, whereas for babies presenting after the neonatal period, the median age is between 1 and 3 months.[47,108] In the Réunion outbreak, neonates presented earlier, within 3–7 days after delivery with fever, poor feeding, rash, and peripheral edema with 89% having thrombocytopenia.[55] Some infants present with meningoencephalitis, cerebral edema, and intracranial hemorrhage or myocardial disease.[54]

Vertically transmitted CHIKV infections in neonates typically present during the first week of life but not at the time of birth and present with fever, polyarthralgias, limb edema, irritability, poor feeding, and rash. Twenty-five percent show skin manifestations such as maculopapular rashes (Fig. 2), freckle-like pigmentation in the centrofacial area, and vesiculobullous lesions. Acute inflammatory lesions typically last 5–7 days and are often followed by hyperpigmentation due to a postinflammatory response or CHIKV-induced intraepidermal melanin retention.[109] The pigmentary changes are seen most frequently in the axilla, perioral, and genital areas. Some infants show tenderness and edema of the hands and feet. Arthritis is seen very rarely.[108] There may be mucosal changes such as nasal blotchy erythema and multiple aphthous-like ulcerations. Some infants may show purpuric or hemorrhagic vasculitic lesions, toxic epidermal necrolysis-like rash, or nail changes like black lunulae, longitudinal melanonychia, and transverse pigmented bands.[109–111]

Severely afflicted infants may have sepsis-like syndrome with multiorgan dysfunction, meningoencephalitis, recurrent apnea, shock, and/or disseminated intravascular coagulation. Unlike dengue, hemorrhagic manifestations and shock are infrequent in CHIKV infections.[112]

One systemic review[47] showed that the pooled combined disease impact on the fetus and newborn was 17%. The overall risk of symptomatic disease in neonates born to mothers with active infection was 15.5%. The risk was higher, nearing 50%, among intrapartum maternal infections. The pooled risk of long-term neurodevelopmental delays in infants with symptomatic neonatal infections neared 50%. The mean interval between the onset of maternal illness and the onset of neonatal illness was 5 days (range: 3–9). The most frequent clinical signs in neonates were fever (79%), pain (100%), rash (82%), and peripheral edema (58%). Thrombocytopenia (76%), lymphopenia (47%), altered coagulation (65%), and elevation of aspartate aminotransferase (77%) were detected with some neonates developing complications such as seizures, hemorrhage, and hemodynamic disorders. Reverse transcription-polymerase chain reaction (RT-PCR) in CSF was positive in 22 of 24 cases. The brain MRI showed WM lesions, intraparenchymal hemorrhages, or both. Echocardiography showed myocardial hypertrophy, ventricular dysfunction, pericarditis, and coronary artery dilatation. One neonate died of necrotizing enterocolitis.[47]

In neonates, the incidence of symptomatic infections varies by region, although most have no or relatively mild symptoms. In contrast to adults with CHIKV infections, infants have a fever lasting only for 24–48 hours, and this is followed by the appearance of the maculopapular rash. Some may develop vesicles and bullae by the fourth day along with acrocyanosis without any hemodynamic alteration.

Figs 2A and B: Clinical manifestations in two neonates with congenital chikungunya. (A) Images from one infant show prominent pigmentary changes comprising the "CHIK sign" of congenital chikungunya infection on the central part of the face. (B) The pigmentary changes in a second infant extended to the chest

One report described 12% of those infected vertically as symptomatic.[113] Joint involvement can be seen in a few cases.[114] One case of neonatal CHIKV infection has been reported from India with a fixed flexion deformity of the right thumb on follow-up at 6 months, suggestive of tenosynovitis manifesting as a sequela of arthritis.[115] In another report, the authors have described painful arthralgia in 78–100% neonates, associated with distal joint edema and persistent prostration.[55] In highly endemic zones, neonates can acquire CHIKV after birth, coincidental with other family members.[116] There is a reported case of congenital CHIKV infection who had hyperpigmented macules and extensive dystrophic calcifications at birth, suggestive of *in utero* skin affliction.[117] These skin lesions resolved without any sequelae with supportive therapy (Table 2).[118]

The severe neonatal disease is frequently associated with thrombocytopenia, where low platelet counts were seen in the more severe neonatal diseases.[119] Another report has described a newborn who was infected postnatally, confirmed by positive immunoglobulin (Ig)M in the neonate and a negative IgM serology on the mother.[120] A few infants with high viral concentrations developed severe manifestations such as meningoencephalitis and disseminated intravascular coagulation.[56,121,122]

In the CHIMERE cohort study of CHIldren Exposed to Perinatal MothEr-to-Child Chikungunya Virus Infections on the REunion Island,[54] 33 children with maternal–fetal transmission of CHIKV at birth and 135 uninfected controls during the Reunion outbreak were evaluated. Neurodevelopmental follow-up at 2 years showed that 51% of infected children had a global neurodevelopmental delay compared to 15% of controls. These findings suggested that there might be a causal relationship between perinatal CHIKV infection and neurocognitive outcomes. Both the encephalopathic and nonencephalopathic forms of CHIKV infections have been associated with early cytotoxic and late vasogenic cerebral edema along with the presence of viral genome in CSF.[55,123] Pregnant women who acquired CHIKV long before delivery delivered healthy neonates.[55,58,124,125] In 12 cases of CHIKV neonatal encephalopathy, 5 have been identified as having microcephaly and 4 matched the definition of cerebral palsy. The MRI scans showed severe restrictions of WM areas, predominant in the frontal lobes in these children.[54]

Eighteen months after the Reunion outbreak of CHIKV infections, a retrospective cohort TELECHIK survey was performed on a random representative sample of the SEROCHIK population-based sero survey.[126] The TELECHIK cohort study revealed that 10% of CHIKV patients had light cerebral disorders (headache, sleep, memory, and depression) on 18-month follow-up.[126] Preterms were at risk of severe neurologic damage,[92] as exemplified by brain swelling and WM injury on MRI.[55,123] Coordination and language skills were frequently affected followed by movement/posture and sociability. The CHIKV neonatal encephalopathy shows low *N*-acetyl aspartate peaks on magnetic resonance spectroscopy, indicating WM hypometabolism, especially in the frontal lobes, thereby affecting coordination and language centers.[127]

Case definitions used in perinatal chikungunya are summarized in Table 3.

Table 2: Pigmentary changes noted in chikungunya infection[117]

Generalized hyperpigmentation.
Striking pigmentation on the nose is called "brownie nose" or the "Chik" sign of CHIKV disease.
Macular type.
Freckle-like pigmented macules that tend to coalesce with each other Pinpoint confetti-like macules.
Irregular flagellate or whiplash pattern of brownish pigmentation seen over trunk and extremities.
Periorbital hypermelanosis.
Addisonian-type palmar pigmentation

Laboratory Diagnosis

The diagnosis of CHIKV is done by detection of chikungunya viral RNA via real-time RT-PCR or IgM- and IgG-specific serology.[128]

Reverse-transcription Polymerase Chain Reaction

The RT-PCR is usually positive during the viremic phase, which continues till 1 week after the onset of symptoms.[129] For individuals presenting 1–7 days following the onset of symptoms, a positive CHIKV RT-PCR is diagnostic of infection.[129] The RT-PCR has 100% sensitivity and 98% specificity.[40,50]

Serology

Serologic testing is done by enzyme-linked immunosorbent assay (ELISA) or indirect fluorescent antibody for those presenting ≥8 days following the onset of symptoms. Immunoglobulin M anti-CHIKV antibodies (detected by direct ELISA) are detected on the fifth day (range: 1–12 days) after disease onset and persist for several weeks to 3 months, whereas specific IgG antibodies begin to appear on the 15th day and persist for years.[4,130] A plaque-reduction neutralization test can help to quantitate virus-specific neutralizing antibodies and to discriminate between cross-reacting antibodies such as those reactive with the Mayaro and o'nyong'nyong viruses.[131] The absence of positive CHIKV serology at birth does not exclude neonatal CHIKV infection because the development of CHIKV IgG and IgM antibodies in infected infants can be delayed in the first 3–4 weeks of life.[132] Hence, serial serologic monitoring may be helpful in the follow-up of these infants.[133] Transplacentally transferred CHIKV-IgG antibodies disappear by around 8 months of age in uninfected neonates.[132,134,135] The time to neonatal seroconversion is inversely related to the time of maternal infection as evidenced by IgG positivity of approximately 75%, 30%, and <1% for maternal infection in the first, second, and third trimesters, respectively.[132] Uninfected neonates may achieve full seroconversion (IgG-negative status) by 24 months.[132] Infants who are vertically infected with CHIKV may be seronegative at birth but specific IgM and IgG antibodies may appear by 3–4 weeks later.[132]

Viral Culture

The CHIKV isolation has a high specificity and high sensitivity in early infection but reduces after day 5 of onset of illness. It is expensive and labor intensive, hence usually done for research purposes.[50,129,136] Virus isolation takes around 7–10 days.[137] However, it can help identify the viral strain which is of value in the assessment of risk and for collecting epidemiological data.[10] Immunohistochemical staining can detect specific viral antigens in fixed tissue.[138]

Laboratory Evaluation

The CHIKV-exposed neonates are not symptomatic at birth but become ill before day 7, thereby making observational care in the postnatal ward mandatory at least for a week with serial

Table 3: Definitions used in perinatal CHIKV

Neonatal CHIKV infection	Defined as RT-PCR detection of the viral genome in the neonate's serum and/or CSF during the first week of life and/or detection of serum anti-CHIKV IgM.[123]
Maternal CHIKV infection	Defined by RT-PCR detection of the viral genome in maternal serum and/or the presence of serum anti-CHIKV IgM.[123]
Prepartum maternal CHIKV infection	Maternal symptoms lasting between day 7 and day 3 before delivery and diagnosed by RT-PCR (or IgM seroconversion when RT-PCR not available).[55]
Intrapartum maternal CHIKV infection	Maternal symptoms between preceding 2 to subsequent 2 days after delivery and with positive RT-PCR (or IgM seroconversion when RT-PCR not available).[55]
CHIKV perinatal mother-to-infant infection (p-CHIKV infection) or exposed-infected (EI)	Diagnosed if infants of mothers infected during pregnancy have a positive RT-PCR result and/or presence of CHIKV-specific MAC-ELISA IgM antibodies before day 10 (or day 15 in the CSF).[56]
Exposed-uninfected (EU)	Neonates exposed to maternal CHIKV infection and testing negative for RT-PCR and CHIKV-specific IgM antibodies at birth, for whom CHIKV-specific IgG seroreversed during follow-up.[54]
Severe CHIKV infection	Presence of convulsions, coma requiring mechanical ventilation, or abnormal MRI scans indicative of cytotoxic or vasogenic cerebral edema during the acute phase of the disease.[55,56]
Mild, multisystem illness	CHIKV-infected neonates who have difficulty in feeding, tachypnea, and vomiting/diarrhea.[56]
Nonencephalopathic, moderate-to-severe multisystem illness	Moderate severity of illness. May need ventilatory support. There may be some alterations in the laboratory evaluation of liver and renal function. No encephalopathy.[56]
Encephalopathy	Newborns show encephalopathy-related neurologic signs during the acute phase of the disease, such as convulsions, altered sensorium, and abnormal MRI scans indicative of cytotoxic/vasogenic cerebral edema.[212]

measurements of white blood cell and platelet counts, with urgent transfer to the neonatal intensive care units upon the appearance of symptoms, lymphopenia, or thrombocytopenia.[55] Laboratory investigations to be done are complete blood count, metabolic parameters (blood sugar, calcium, sodium, and potassium), liver function tests, sepsis screen, cultures, and CSF analysis.[139] Thrombocytopenia, leukopenia or leukocytosis, hypoalbuminemia, and transaminitis with direct hyperbilirubinemia and altered coagulation are seen in symptomatic infants.[47] Lymphocytopenia has been noted in nearly 70% of neonates with CHIKV infection.[55,70] Thrombocytopenia has been seen in 89% of infected neonates and is a marker of disease severity. Steroids and intravenous Igs have been tried to reduce the risk of bleeding complications but the benefits remain unproven.[140,141] In Salvador-Brazil, sera and urine samples have tested positive on RT-PCR for CHIKV during the first postnatal week in neonates and their mothers.[142]

Neurological Evaluation

The infant may require the ultrasound of the skull or MRI of the brain, CSF analysis, RT-PCR of CSF, and basic metabolic workup (blood sugar, calcium, magnesium, and sodium) to rule out other causes of encephalopathy.[143,144] In cases with signs of meningeal involvement, lymphocytic pleocytosis with normal CSF glucose and proteins is seen.[95]

Histopathology of Placenta

The CHIKV RNA and antigens can be detected in the placental tissue seen as histopathological (deciduitis, fibrin deposition, edema, fetal vessel thickening, and chorioamnionitis) and ultrastructural alterations (cytotrophoblast with mitochondrial swelling and dilated cisterns in the endoplasmic reticulum, vesicles in syncytiotrophoblasts, and thickening of the basement membrane of the endothelium).[145,146]

Table 3 presents the case definitions of neonatal chikungunya.

Differential Diagnosis of CHIKV Infection

Dengue and CHIKV have similar clinical manifestations and geographic distribution.[147] The CHIKV is more likely to cause high fever, severe arthralgia, arthritis, rash, and lymphopenia, while neutropenia, thrombocytopenia, hemorrhage, shock, and death are commoner in dengue.[148] Chikungunya virus manifests with higher fevers and more intense joint pain than Zika.

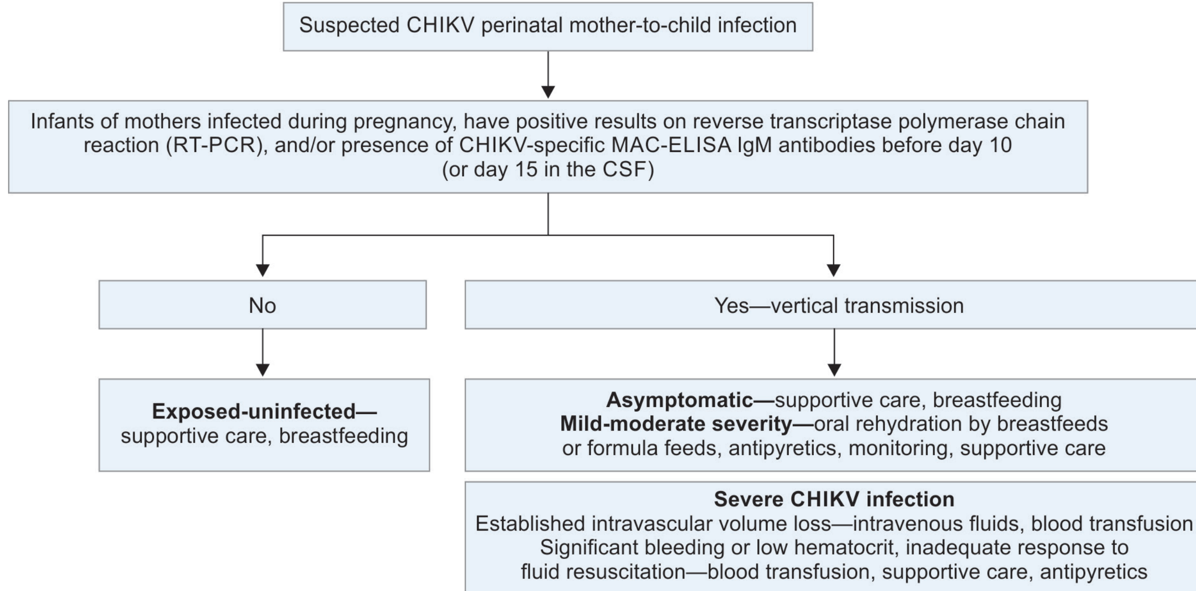
The CHIKV outbreaks have occurred concurrently with outbreaks of dengue, Zika virus,[149,150] and yellow fever.[150] Coinfection with CHIKV and other pathogens has been reported, namely, CHIKV, dengue, and Zika;[49] CHIKV and dengue;[151] and CHIKV and Zika,[152] and CHIKV and yellow fever.[153] Neonatal CHIKV infections can mimic meningoencephalitis, bacterial sepsis, or metabolic encephalopathy.[154]

Treatment

There is no antiviral treatment available for CHKV infections[155–157] and, therefore, primary treatment is supportive care by maintaining adequate intravascular volume (Flowchart 3). Oral rehydration by breastfeeds or formula feeding should be done. Acetaminophen (maximum 60 mg/kg/day) can be used for the management of fever. In a patient who could have a dengue virus infection, aspirin, and other non-steroidal anti-inflammatory drugs (NSAIDs) should not be used until dengue has been excluded in view of the bleeding complications associated with dengue and the potential risk of Reye's syndrome in children. Coinfection with dengue and CHIKV can occur. So, dengue infection needs to be excluded even if the diagnosis of CHIKV infection is confirmed.

Infants with CHKV infections should be monitored closely for vital signs, input–output, oxygen saturation, and sensorium. Administration of intravenous fluid is required in babies with established intravascular volume loss. Blood transfusion is

Flowchart 3: Management of perinatal chikungunya infection



warranted in patients with significant bleeding or low hematocrit and inadequate response to fluid resuscitation. There is no role for corticosteroids, intravenous Igs, or antivirals in the treatment of CHIKV.[24]

Pregnancy and Breastfeeding

Pregnant women should avoid travel to *Aedes* spp. endemic regions.[158] Post-travel laboratory testing should be reserved for symptomatic patients.[158] Whether CHIKV is secreted in human milk is uncertain, although CHIKV RNA has been detected in human milk.[45] Transmission by breastfeeding has not been reported and, therefore, breastfeeding should be continued even in areas with the circulation of the CHIKV.[159] Breast milk also contains antiviral antibodies that may provide protection.[160] Asymptomatic neonates may be discharged after a week; while in symptomatic cases, discharge can be considered when afebrile for 24–48 hours, hemodynamically stable, with good urine output and accepting feeds well.[120]

Outcomes

The case fatality rate in congenital CHIKV infection may vary between 0.8 and 37.5%.[58,59,123,161] Maternal–fetal transmission of CHIKV may result in severe neonatal complications. Among three symptomatic neonates with serologically confirmed, vertically transmitted CHIKV infection in Curaçao, two developed neurological complications, including convulsions and intracranial bleeding, while one newborn, in whom maternal infection occurred 7 weeks before delivery, had a fatal outcome after birth.[89] Exposed/infected children can have poor neurocognitive outcomes and must be monitored throughout childhood and early intervention therapy should be provided wherever feasible for "CHIKV-driven disability".[126,157,161,162] Long-term sequelae have been described including neurologic sequelae at 6 months of follow-up[56] or 2 years after CHIKV encephalitis.[159] CHIKV-infected pregnant women and neonates should be followed-up for sequelae such as chronic inflammatory rheumatism, which may persist for up to 6 years.[157,161] Children infected with CHIKV later during the first 2 years of life as determined by ELISA for antigens and/or specific antibody tests have 2-year neurodevelopmental outcomes similar to children who were not infected.[57,163–168]

Prevention

Approaches for the prevention of dengue virus (DENV) infection in endemic areas may include mosquito control, personal protective measures, and vaccination. Prevention of chikungunya virus infection consists of minimizing mosquito exposure through personal protection and environmental control measures.[169]

Vaccine Development

There are no licensed vaccines for CHIKV, but 15 candidate vaccines are currently under preclinical and clinical development.[113,170–175] In an randomised controlled trial (RCT), a live-attenuated, measles-vectored vaccine expressing CHIKV structural proteins (MV-CHIK) induced neutralizing antibodies against CHIKV after one to two immunizations.[175] Seroconversion rates varied between 50 and 93% after one and 86 and 100% after 2 doses. Immune responses lasted till 6 months of these doses, and the vaccine was safe and well-tolerated. Further studies are required for vaccine efficacy and cross-protection against multiple CHIKV strains.

Phase 2 RCTs of a CHIK virus-like particle (VLP) vaccine have revealed a 4-fold rise from baseline neutralization titers in 88% of recipients after an intramuscular dose.[176] The immune response lasts 72 weeks after vaccination, and the vaccine is safe and well-tolerated. Phase 3 trials are required. A messenger RNA (mRNA)-based vaccine (VLA-181388) is still in phase 1 clinical trials.[177,178]

Future Directions

Further efforts are needed to develop specific antiviral agents and vaccines for the management of chikungunya infections.[179] There is also a need for planned urbanization with efforts for mosquito control.[180] Public health agencies and clinicians should be aware of the existence of maternal–fetal transmission of chikungunya and be prepared to diagnose and treat these neonatal infections.[47,169]

Recent efforts to control mosquito populations through genetic strategies appear promising.[181,182] Several genetics-based approaches focused on male sterilization are being tried.[183–186] Recombinant DNA methods provide a step change in our ability to design and build specific genetic systems.[187,188] Several *Aedes*

species have now been transformed, either by recombinant DNA methods using transposon vectors or by artificial infections with various *Wolbachia*, a diverse group of intracellular bacteria.[189,190] These techniques may help control chikungunya and other vector-borne diseases.

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REVIEW ARTICLE

Innate Immune Memory in Macrophages

Akhil Maheshwari

Received on: 28 February 2023; *Accepted on*: 22 March 2023; *Published on*: 06 April 2023

Abstract

Macrophages have been recognized as the primary mediators of innate immunity starting from embryonic/fetal development. Macrophage-mediated defenses may not be as antigen-specific as adaptive immunity, but increasing information suggests that these responses do strengthen with repeated immunological triggers. The concept of innate memory in macrophages has been described as "trained immunity" or "innate immune memory (IIM)." As currently understood, this cellular memory is rooted in epigenetic and metabolic reprogramming. The recognition of IIM may be particularly important in the fetus and the young neonate who are yet to develop protective levels of adaptive immunity, and could even be of preventive/therapeutic importance in many disorders. There may also be a possibility of therapeutic enhancement with targeted vaccination. This article presents a review of the properties, mechanisms, and possible clinical significance of macrophage-mediated IIM.

Keywords: Chromatin, Development, Fetus, Fumarate, Lipoprotein(a), MMP-2, MMP-9, Neonate, Newborn, Succinic acid, α-ketoglutaric acid.

Newborn (2023): 10.5005/jp-journals-11002-0058

Key Points

- Macrophages have so far been recognized as the primary mediators of innate immunity. However, emerging information suggests that macrophage responses may be altered, either enhanced or suppressed, based on earlier infectious or other immunological stimulation.
- The memory of prior stimulation in macrophages is less accurate in terms of antigen specificity, but is analogous to that seen in adaptive immune responses. It has been described as "trained immunity" or the "innate immune memory (IIM)".
- The likely mechanism(s) of IIM in macrophages are rooted in epigenetic reprogramming and metabolic alterations.
- Understanding macrophage IIM may be particularly important in the context of the maturing fetus/neonates who are yet to develop protective levels of adaptive immunity.

Introduction

Macrophages are viewed as key sentinels in the innate immune system throughout the body that contribute to both homeostasis and disease.[1–4] These cells identify, phagocytose, and eliminate invading pathogens; ensure the timeliness of defense reactions by secreting antimicrobial peptides, cytokines to recruit and activate leukocyte present in the vicinity, chemokines to recruit leukocytes from the circulation and other tissues; and promote the resolution of inflammation prior to the onset of illness and by eliminating the pathogens and severely-damaged cells.[1,5–17] These cells also coordinate immune activation by presenting antigens to adaptive immune cells.[18–20]

Macrophages play a crucial role in immune responses in neonates and young infants, who are yet to acquire protective levels of neutrophil function and adaptive immunity. These cells begin to resemble adult macrophages in many host defense functions by the late 2nd trimester, and are therefore likely to be important even in premature infants. However, macrophages have been studied mostly in the context of innate immunity, not as carriers of immune memory that could enhance the efficiency of elimination of pathogens.[21–23] But now, this perception is changing.[23–26] Preclinical and clinical data indicate that macrophages do retain some memory of previous encounters through epigenetic reprograming and show quicker and more robust responses in secondary infections.[21,23,27–34] This progressive enhancement in macrophage-mediated defenses has been described as "trained immunity" or "innate immune memory (IIM)".[23,32,35,36] Innate immune memory can activate circulating macrophages and those located in the lungs, and suppress many in the intestine.[23,37]

This immunological memory of macrophages may constitute one of five patterns where immune cells learn to mount quicker and enhanced responses to "known" antigens[38,39] (Fig. 1): (1) systemic acquired resistance seen in plants;[40,41] (2) transgenerational immune priming,[42,43] which may include vertical transmission of immune experience from parents to the offspring; horizontal transfer between individuals, and between individuals and other parents' offspring; (3) natural killer (NK)-cell immune memory;[44,45] (4) classical adaptive memory in vertebrates;[46,47] and finally, the increasingly appreciated (5) IIM in myeloid cells (monocytes, macrophages, and dendritic cells).[23,30] In this article, we have focused on the IIM macrophages with a particular focus on the relevance of these cells in the fetus and newborn infants. The dendritic and adaptive immune cells are still evolving in the fetus and neonates,[48] and so we did not include these details in the

Founding Chairman, Global Newborn Society, Clarksville, Maryland, United States of America

Corresponding Author: Akhil Maheshwari, Founding Chairman, Global Newborn Society, Clarksville, Maryland, United States of America, Phone: +708-910-8729, e-mail: akhil@globalnewbornsociety.org

How to cite this article: Maheshwari A, Innate Immune Memory in Macrophages. Newborn 2023;2(1):60–79.

Source of support: Supported by work done as part of the NIH projects HD059142 and HL124078 (to AM).

Conflict of interest: Dr. Akhil Maheshwari is associated as Editor-in-Chief of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of the Editor-in-Chief and his research group.

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Systemic acquired resistance. Seen in plants. Transmitted via chemical signals such as salicylic acid or its derivatives.

Trans-generational immune priming. Seen in invertebrates and early vertebrates. (a) maternal peptides such as vitellogenin or microRNAs transferred from the maternal intestine to the progeny; seen in early invertebrates; (b) mRNAs expressing antimicrobial immune effectors in the developing embryo and/or surrounding serosa; in insects and some fish; (c) immune effector proteins such as lectins, lipopolysaccharide (LPS)-binding protein/bactericidal permeability-increasing proteins, and antimicrobial peptides present in the mother's hemolymph or actively transferred through provision of specialized cells. Transferred via the yolk in birds, fishes, and reptiles, or through the placenta or milk in mammals; (d) anti-microbial peptides such as gloverin and defensin-like tenecin-1 transferred in invertebrate mothers into eggs. Can promote colonization of the embryo by symbiotic bacteria. Responses not consistent between all hosts and all pathogen challenges; and (e) parents-to-progeny transfer of epigenetic reprogramming such as histone acetylation or DNA methylation following exposure to pathogens. Has been seen in crustaceans, although not all studies have been consistent.

NK-cell immune memory in advanced vertebrates. No rearrangement of genes encoding activating receptors; a selective education process with expansion of long-lived clones against previously-encountered pathogens. Can last up to 6 months.

Classical adaptive memory in advanced vertebrates. Specific, long-lived; mediated via rearrangement of genes encoding their activating receptors.

Innate Immune Memory in myeloid cells such as monocytes and macrophages; mediated through epigenetic mechanisms that last for weeks to months

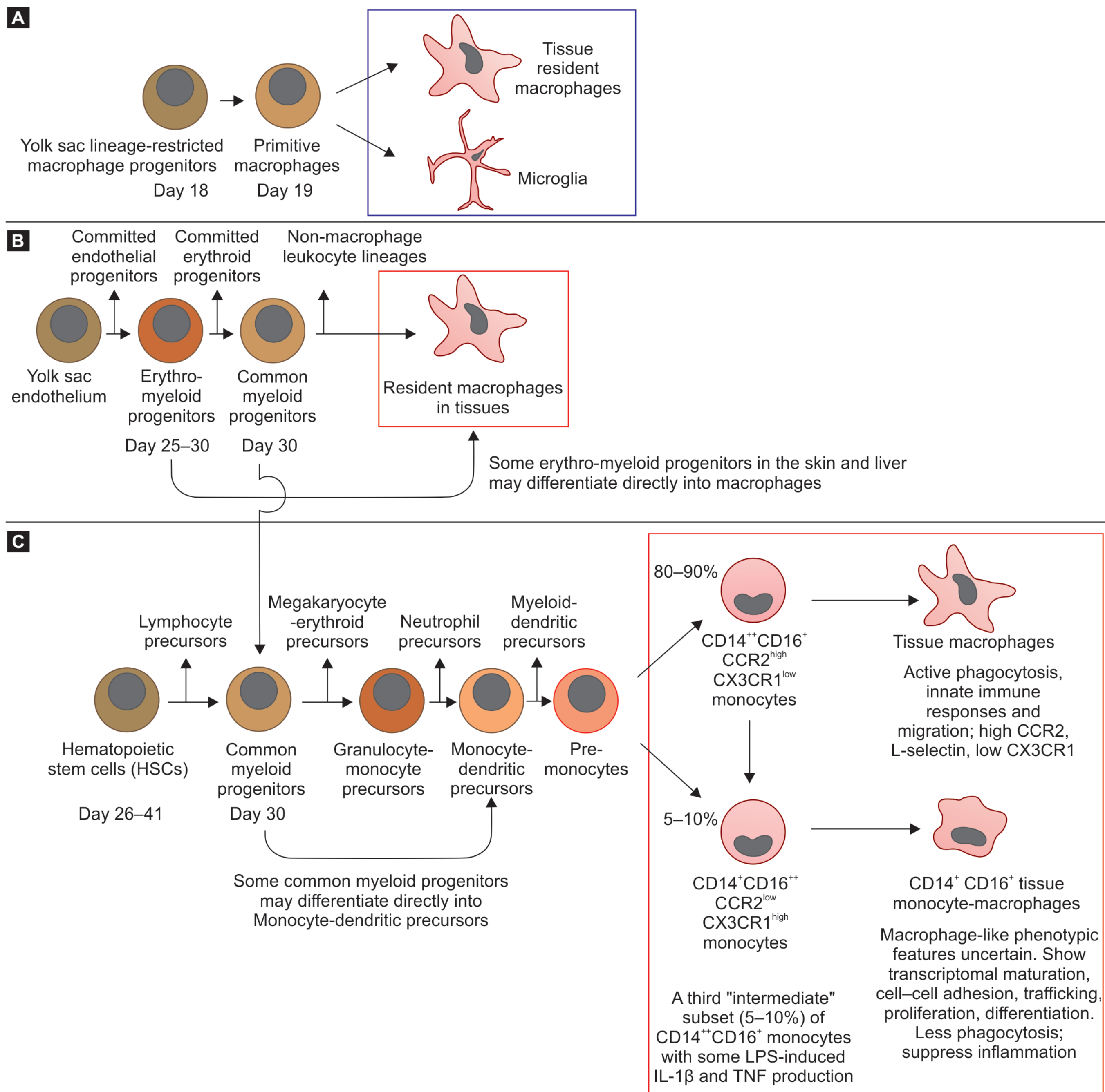
Fig. 1: Phylogenetic evolution of immune memory. Five categories of immune memory have been recognized: (1) Systemic acquired resistance, as seen in plants; (2) Transgenerational immune priming, which may include vertical transmission of immune experience from parents to the offspring; horizontal transfer between individuals, and between individuals and other parents' offspring; (3) NK-cell immune memory; (4) Classical adaptive memory, as seen in vertebrates; and (5) IIM in myeloid cells. The broken line separates NK-cell immune memory, classic adaptive memory, and the IIM myeloid cells as these are seen in evolutionarily advanced vertebrates. The IIM myeloid cells are the focus of the current article and have been highlighted in a red-outlined box

present article. We included information from some of our own preliminary studies with an extensive literature search in EMBASE, PubMed, and Scopus.[49] To avoid bias in identification of studies, keywords were short-listed *a priori* from PubMed's Medical Subject Heading (MeSH) thesaurus.[50]

Development of Macrophages in the Fetus and Neonate

All tissues contain a complement of yolk sac (YS), hepatic, and bone marrow-derived macrophages.[2,51] The numbers are considerable in many tissues and may reach 5,000–10,000 per cubic mL.[23,52] During evolution, macrophages appeared earlier than the lymphocytes known for classical immune memory (details in Mezu-Ndubuisi and Maheshwari).[1] The following graphic (Fig. 2) shows the three major pathways of macrophage differentiation; the terminal stages of development with noted findings of IIM have been highlighted in each pathway:

- **Macrophage differentiation from lineage-restricted YS progenitors:** Hemocytoblasts resembling myeloblasts are first seen in the secondary YS (Fig. 2A) on day 18.[53] On day 19, some hemocytoblasts differentiate directly into embryonic macrophages.[54] During the days 25–30, many erythro-myeloid progenitors (EMPs) also differentiate into macrophages.[55] Around this time, some hematopoietic stem cell (HSC) clusters of differentiation (CD) 45 and 34 ($CD45^+ CD34^+$) migrate from the peri-aortic region to the central nervous system (CNS) and differentiate into microglia.[56]
- **Macrophage differentiation in the aorta-gonad-mesonephros (AGM) zone:** The vascular endothelium here (Fig. 2B) produces $CD45^+ CD34^+$ HSCs,[57] which can differentiate first into common myeloid progenitors (CMPs) and then into tissue macrophages. These macrophages migrate to all the embryonic organs except the CNS. These cells express characteristic markers such as the angiotensin-converting enzyme, T-cell acute lymphocytic leukemia 1/stem cell leukemia (Tal/SCL) gene, and the myeloblastosis oncogene (c-Myb).[58]
- **Macrophage differentiation in the liver and the bone marrow:** On day 32, the $CD45^+ CD34^+$ HSC precursors of macrophages migrate from the AGM zone to the liver and the bone marrow



Figs 2A to C: Macrophage differentiation. Schematic shows macrophage development from lineage-restricted embryonic progenitors. The terminally differentiated embryonic and hepatic macrophages, and bone marrow-derived monocytes and macrophages are highlighted in rectangular borders as these are the stages of differentiation where some cells get committed for innate immune memory. (A) lineage-restricted embryonic progenitors; (B) YS endothelium, which differentiates into EMP and then into CMPs. Some CMPs differentiate into macrophages and other primitive leukocytes, whereas others differentiate into GMPs and then in sequential steps into macrophages as shown in panel C; (C) HSC in sequential stages of CMPs GMPs, monocyte-dendritic precursors, pre-monocytes, M1 or M2 (and possibly an intermediate subtype) monocytes and then into corresponding macrophages. The stages at which IIM appears have been highlighted by enclosing those in rectangular borders

(Fig. 2C).[59] Some of these cells may arise from EMPs. Hepatic HSCs are known to differentiate into monocytes and macrophage precursors between 8 and 20 weeks' gestation and then involute during the 20–23 weeks period. After birth, the hepatic HSCs migrate to the bone marrow for further definitive hematopoiesis.

Increasing information suggests that most tissue macrophages, even in adults, likely originate from the EMP and AGM progenitors acquired during embryonic development, not from circulating monocytes.[2,59,60] However, the ontogeny of monocyte-derived macrophages (MDMs) is best lineated in marrow-derived monocytes. $CD45^{+}$ $CD34^{+}$ HSCs in the bone marrow clearly differentiate into CMPs, granulocyte-monocyte precursors (GMPs), common monocyte and DC precursors (MDPs), pre-monocytes (committed monocyte progenitors), monocytes, and then into

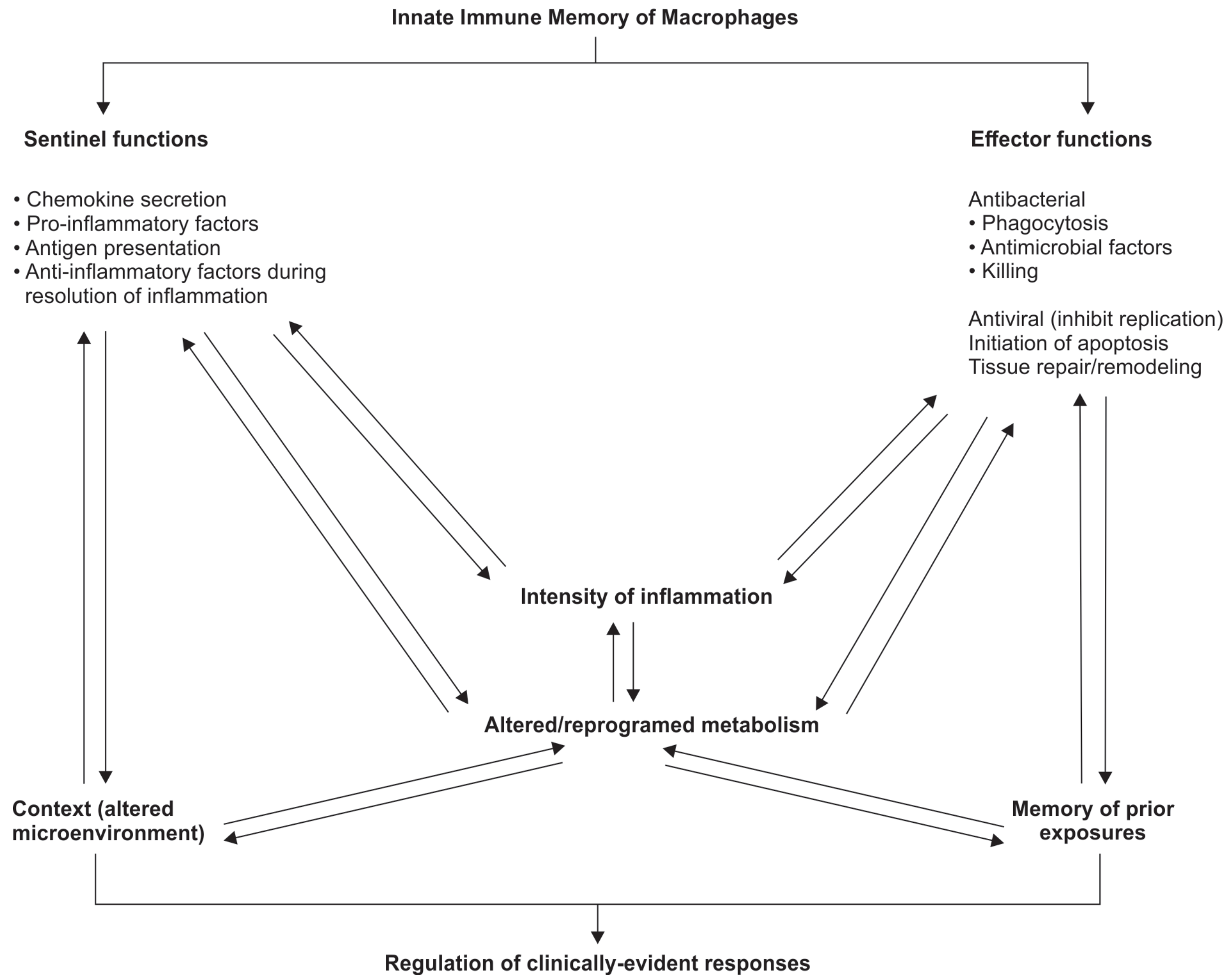


Fig. 3: Innate immune memory of macrophages affects both the sentinel and effector functions of these leukocytes. The context (altered microenvironment) and memory of prior exposures are important variables in the regulation of clinically evident responses

macrophage precursors by the 7th week of gestation.[61] These hematopoietic lineages can be detected in other tissues such as the brain, heart, liver, and skeletal muscle.

In the bone marrow, more than 90% of HSCs differentiate into classical monocytes with strong CD14 expression ($CD14^{++}$). These cells mature into M1 macrophages that strongly react to toll-like receptor (TLR) ligands, and express inflammatory cytokines and reactive oxygen species (ROS). About 10% develop into a nonclassical, $CD16^{++}$ subset. These cells produce some inflammatory cytokines, but not much ROS. These cells patrol and assess endothelial integrity and infiltrate normal tissues.[62] A third, intermediate $CD14^{+}$ $CD14^{+}$ population may show both inflammatory and tissue healing properties; these cells may express MHC-II, show strong phagocytic activity, present antigens, and contribute to T-lymphocyte activation.[62]

In premature and young infants, macrophages show developmental changes in antigenic profiles. These cells express high levels of CD11b, chemokine receptors CCR1, CCR2, CCR5, CXCR1, CXCR2, and other molecules such as CD115, glycan structures containing 6-sulfo *N*-acetyl lactosamine, and triggering receptors expressed on myeloid cells (TREM) are high. There might be some immaturity in movement, phagocytosis, and regulation of inflammation. These cells can be stimulated by many endogenous triggers such as cytokines; oxidized lipids; ROS and reactive nitrogen species (RNS); metabolic products, and débris released from dying cells such as heat-shock proteins (HSPs) and damage-associated molecular patterns (DAMPs).[63] There are also multiple well-known exogenous activators such as microbial products, microparticles, and chemicals.[63]

Innate Immune Memory in Neonatal Macrophages

Increasing information indicates that macrophages do retain some memory of previous encounters and show quicker, more robust responses in secondary infections (Fig. 3). This immunological memory very likely enhanced the survival of early multicellular eukaryotes by enhancing the defense responses.[31] Innate immune memory macrophages may not fit in the current dualistic model of classic (M1) or alternative (M2) macrophage polarization, and may need to be classified in a distinct category (Fig. 4, Table 1). There is increased expression of CD43 and CD206, but other surface markers can differ in specific model(s). In mice treated with Bacillus Calmette-Guérin (BCG), peritoneal macrophages showed enhanced expression of CD43, CD206, CCR2, CXCR4, CD80, and TLR2.[64] Low doses of lipopolysaccharide (LPS) induced an overlapping profile with increased CD206 and CD43, but less CCR2, CXCR4, and CD80. Innate immune memory macrophages also show a shift toward increased glycolysis and altered energy metabolism.[32,65,66]

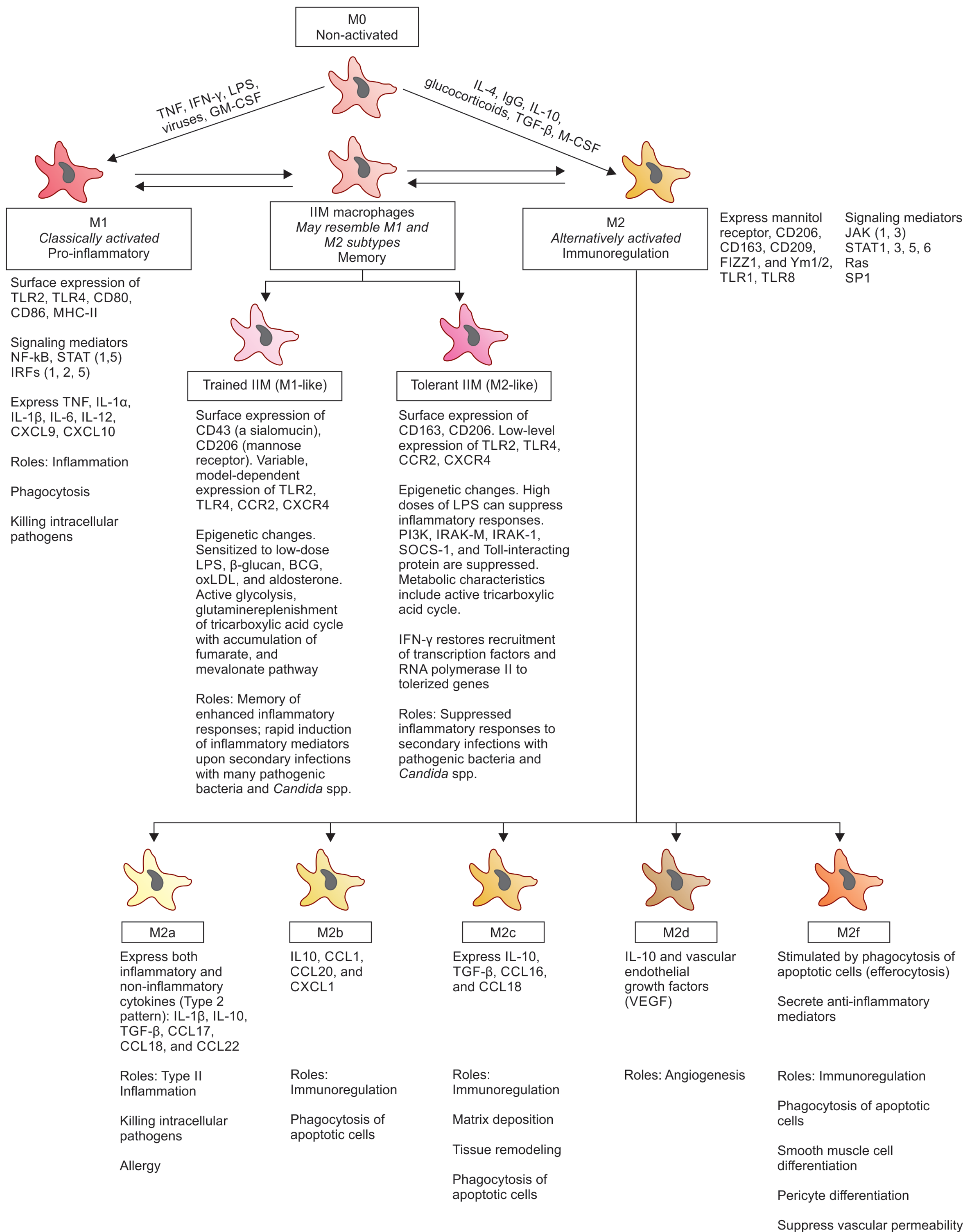


Fig. 4: Differentiation of MDMs. Schematic shows differentiation of naïve macrophages into classically activated M1, the IIM macrophages, and the alternatively activated M2 subclasses. The surface markers and key signaling mediators are depicted with each group. The IIM macrophages, including the trained (M1-like) and the tolerant (M2-like) subgroups, do not match the other categories and may need to be classified separately. The M2 macrophages may be comprised of 5 subgroups with distinct inflammatory functions and physiological roles

Table 1: Macrophage subpopulations

Macrophage subpopulation	*Activation*	*Function*	*Biological processes*
M0	Naïve, unstimulated macrophages		
M1	Inflammatory macrophages		
	– LPS and interferon-γ. – macrophage-produced inducible nitric oxide synthase.[299] – macrophage-produced IL-12, IL-18, and IL-23.[300]	– pro-inflammatory, antimicrobial. – regulate angiogenesis.[299,301,302] – matrix composition; express MMP-1, MMP-3, and MMP-10.[303]	– activate Tie-signaling.[304] – promote endothelial cell chemotaxis, and migration of other cells involved in angiogenesis.[305]
IIM	Innate immune memory macrophages		
Trained (M1-like)	– low-dose LPS, β-glucan, BCG, oxLDL, and aldosterone-trained macrophages.[155]	– memory of previous infections, which can rapidly recruit and activate innate immune cells.[30] – rapid induction of inflammatory mediators upon secondary infections with pathogenic bacteria and *Candida* spp.[306]	– host defense. Particularly important in neonates and young infants before adaptive immunity becomes functionally adequate.[307]
Tolerized (M2-like)	– epigenetic changes involved in development. High doses of LPS can suppress inflammatory responses.[155]	– memory of previous infections; can suppress unduly severe inflammatory responses.[23]	– host protection. May protect young infants, who are still developing adaptive responses, from severe tissue damage.[23]
M2	Anti-inflammatory, pro-healing macrophages		
M2a	Cytokines, IL-4, IL-13.[308]	– regulate the expression of platelet-derived growth factor-BB and transforming growth factor-β.[299]	– support pericyte and smooth muscle cell differentiation.[304]
M2b	– immune complexes, IL-1β and molecules with PAMs.[309] – immune complexes and TLR ligands.[310]	– express inflammatory cytokines (IL-1, IL-6, and TNF), and anti-inflammatory IL-10.[10]	– altered regulation of the PI3K/Akt/FoxO3a pathway.[311]
M2c	– IL-10, TGF-β, and glucocorticoids.[312]	– express MMPs.[312] – express IL-10, TGF-β, and pentraxin-3.[313]	– vascular remodeling.[299]
M2d	– TLR agonists.[224] – adenosine A2A receptor agonists.[314]	– suppress inflammatory responses.[115]	– regulate the expression of IL-10 and VEGF.[315]
M2f	– phagocytosis of apoptotic cells.[316] – upregulate TGF-β_1.[304]	– express anti-inflammatory mediators.[304]	– regulate vascular permeability.[304]

Macrophages recognize most antigens through the pattern recognition receptors (PRRs) expressed on the cell surface. These receptors can recognize pathogen-associated molecular patterns (PAMPs) in structural débris or secreted products. Some PRRs can identify DAMPs, the endogenous danger signals expressed on or released from dying cells.[67] Pathogen-associated molecular patterns are important for microbial survival and have been evolutionarily conserved with minimal diversification.[68] The best-known examples are LPS and porins of Gram-negative bacteria; peptidoglycans of Gram-positive bacteria; flagellins; β-glucans and mannans from fungi; and bacterial and viral nucleic acids.[69–76] The specificity for classes, not individual microbes, has helped in evaluation of molecular dynamics in pathogens. Damage-associated molecular patterns can be seen in intracellular proteins such as the HSPs and the high-mobility group box 1 (HMGB1); extracellular matrix components such as hyaluronan fragments; and non-protein components such as adenosine triphosphate (ATP), uric acid, heparin sulfate, and deoxyribonucleic acid (DNA).[77]

The traditional view of macrophage function as limited to the first line of defense may indeed be too restrictive.[6] However, macrophage IIM is still less robust than the classical adaptive memory of T- and B-lymphocytes.[31] Despite all possible differences in ontogeny and genetic expression (as noted in epigenomic or transcriptome profiles), there are notable similarities in functional responses to immunological challenges. The consistency of these responses, the context, the microenvironmental cytokine *milieu*, and the evidence supporting stimulus memory suggest a possibility of convergent evolution.[20,78,79] These host-defense responses may not be as perfectly antigen specific as in lymphocytes, but these do seem to gain in efficiency with repeated exposures.[23,35,36,79] Innate immune memory seems to alter inflammatory responses more than its effects on phagocytosis and other motor activities.[28,80]

Increasing evidence suggests that immune memory may include a full spectrum of responses ranging from the IMM seen in macrophages to the classical adaptive immune memory of lymphocytes. When re-exposed to defined stimuli, other leukocytes

Table 2: Innate and adaptive immune memory

	IIM in macrophages	*Cells with intermediate properties*	*Adaptive immune memory*
Cells	IIM in monocytes/ macrophages	Seen in B1 and marginal zone B-cells; invariant natural killer (iNKT)-cells; innate lymphoid cells, and γδ T-cells	Seen in circulating αβ T- and B-lymphocytes; CD8αα-expressing intestinal intraepithelial lymphocytes
Phylogeny	Plants, invertebrates, early vertebrates	Vertebrates	Higher vertebrates
Mechanism	Epigenetic reprogramming, cell metabolic change	Genetic programming and restrictions; produce IgM. Invariant NKT cells interact with a few lipid antigens; γδ T-cells recognize antigens without the major histocompatibility complex	Genetic programming; antigen-specific immunity through gene rearrangement. Produce immunoglobulins, particularly IgG and IgD
Human age groups	All	B1 cells in fetal-neonatal period. Other cells seen in all ages	All
Duration	Weeks to months	Weeks to months	Weeks to months
Specificity	No	Limited; initiate and amplify both innate and adaptive immune responses	Yes

such as the B-1 and marginal zone B-cells, invariant NK, innate lymphoid cells, and γδ T-cells also show some enhancement of secondary responses. However, these responses are not as consistent as in myeloid cells (Table 2).[31,81,82] The differences between IIM and classical immune memory of lymphocytes are more clearly noticeable. Upon antigen exposure, naïve lymphocytes undergo genetic rearrangements and evolve into specific, mature clones with increased sensitivity to the original antigens.[83,84] These mature lymphocytes, in turn, can recruit more naïve lymphocytes to differentiate into the needed clones and thereby establish feed-forward loops.[85] Most lymphocytes become effector cells that provide host defense, but some evolve into longer-living memory cells.[86] If exposed to the same antigen at a later time-point, the memory cells proliferate to form large pools of effector and memory cells. Some memory T-cells can also transgress into effector cells.[87]

Macrophage IIM is largely mediated via epigenetic changes, and its kinetics differs from that of lymphocyte-mediated adaptive immunity.[88] Sensitized macrophages display a rapid, potentiated activation following secondary exposures to the same or similar antigens.[89,90] These responses are typically last only for a few weeks to months, and may either be systemic or limited to just the tissue of origin.[35] In contrast, the adaptive immune memory seen in lymphocytes may last for the lifetime of the cells or even that of the organism as it is rooted in genetic mutations, antigen-specific gene rearrangements, and recombinations.[23,84,91] Some of these changes show developmental changes, and further work is needed to understand the functional and clinical importance of macrophage-mediated vs. adaptive immune memory at various stages of fetal/neonatal development.[1]

Macrophage PRRs may be important in immune memory.[89] Administration of BCG might be detected by intracellular PRRs such as the nucleotide-binding oligomerization domain 2 (NOD2), which may protect these cells against secondary infections.[87,92] Nucleotide-binding oligomerization domains are germline-encoded receptors that respond to microbial danger signals.[93,94] These belong in the broader category of conserved cytosolic PRRs, the so-called *NOD*-like receptors (NLRs). Nucleotide-binding oligomerization domains-like receptors sense microbe-associated molecular patterns (MAMPs) during viral and bacterial infections.[95–97] These receptors can sense that MAMPs in the cytoplasm and occasionally in the extracellular space, especially if virulence factors such as muropeptides are transported into the cytoplasm.[98,99] Upon ligand binding, NLRs oligomerize and recruit adaptor proteins to form the so-called inflammasomes, which can activate the production of inflammatory cytokines, antimicrobial peptides, and in some cases, precipitate cell death.[100,101]

Macrophages previously exposed to PRRs ligands, such as dectin-1 ligand, β-glucan, NOD2 ligand muramyl dipeptide, and flagellin show memory and express more tumor necrosis factor (TNF) and interleukin (IL)-6 on secondary stimulation.[102–108] In some conditions, LPS and flagellin can also induce long-term tolerance with less intense inflammatory responses,[109–111] although such tolerance may not always be detectable in premature and critically ill neonates.[1,14,15,112–114] The expression of IIM mediators does not change with cell differentiation, except perhaps for decreased production of TLR2 in specific subsets.[23,115]

Types of IIM in Macrophages

Innate immune memory macrophages show rapid appearance at the sites of infection, phenotypic plasticity, and the ability to sample the inflammatory environment.[28] Changes in surface markers such as the PAMPs and DAMPs may alter function/phenotype of these macrophages in complex and context-specific ways.[68]

Macrophage IIM seems to be comprised of multiple steps. After an initial stimulus primes the inflammatory response, a second one can result either in training and potentiation, or in tolerance (Fig. 5). The details of these training and tolerance responses are provided below:

(a) Training: Low doses of bacterial LPS from Gram-negative bacteria, β-glucans from the *Candida albicans* cell wall, and certain parasites and viruses can sensitize macrophages to show enhanced inflammatory responses to secondary infections with many pathogenic bacteria and *Candida* spp.[116–118] Such "training" increased expression of inflammatory cytokines such as TNF and IL-1, IL-6, ROS, and various other cytokines and chemokines. Macrophage training may enhance tissue damage in acute infections, but improves host defense and survival.

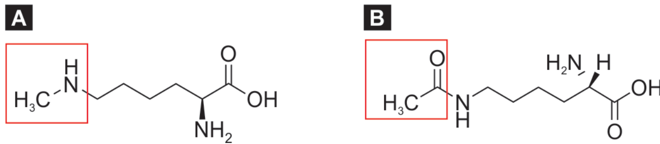


Fig. 5: Schematic figure showing (A) methylated (CH_3) lysine (K). On histone 3, lysine (K) residues on positions 4 (H3K4) and 27 (H3K27) can be mono- [(6-*N)*-methyl lysine], di- [(6-*N*,6-*N*) dimethyl lysine], or tri-methylated [(6-*N*,6-*N*,6-*N*) trimethyl lysine]. These H3K4 sites are usually located close to the transcription start sites or enhancers of various genes; (B) acetyl [C(O)CH_3] lysine (or acetylated lysine) is an acetyl-derivative of the amino acid lysine. These residues are important in epigenetics as regulators of binding of histones to DNA in nucleosomes and thereby controlling the expression of genes on that segment of DNA

In mice lacking T- and B-cells, *Candida* infections can prevent repeated infections with pathogenic bacteria.[119] In other studies, administration of the BCG to simulate vaccination can expanded the pool of IIM macrophages with H3K4me3.[119–121]

(b) Tolerance: Repeated exposure to high doses of LPS can dampen the inflammatory responses to later encounters with these bacteria, particularly on mucosal surfaces in the gastrointestinal tract.[122–124] Prior infections with the influenza or respiratory syncytial viruses can promote immune tolerance lasting weeks to months to subsequent bacterial infections of the lungs. These viruses desensitize TLRs, particularly TLR5, and the lectin and mannose receptors. It also inhibits NF-κB signaling in alveolar macrophages (AMs), resulting in lower levels of inflammatory factors TNF and IL-17 following exposure to bacterial pathogens. Interferon (IFN)-α/β, IFN-γ, and IL-10 produced during viral infection can further suppress antibacterial resistance by inhibiting the production of free oxygen radicals.[125–128] This tolerance memory in macrophages may be related to a few epigenetically-active histone tags on the promoters and enhancers of antibacterial resistance genes. Interestingly, β-glucan can reinstate cytokine production and partially reverse macrophage immune tolerance by reinstatement of the histone tags.[129]

Epigenetic Changes that Promote Priming in Macrophages

The origin of macrophage IIM is still being investigated, but it is generally visualized as a pattern of consistent, progressively quicker phenotypic shifts in these cells following repeated exposures to specific environmental stimuli.[130–132] Transgenerational memories might require genomic changes, whereas moderate-term memories could be generated by changing the number of cells available to produce a response or by epigenetic modification of the programing of existing cells.[3,43,133] Short-term memories could be generated by the ephemeral changes that are transient, but show diverse concentrations or molecular modifications of signaling components.[109] Taken together, the medium-term duration of IIM of macrophages has brought the focus on epigenetics (Table 3).

Many epigenetic changes in macrophages have been identified as altering the heritable “memory” with specific changes in the three-dimensional structure and compaction of the daughter macrophages. There are at least three categories of such changes: (1) DNA methylation; (2) histone modifications; and (3) regulation of gene expression by non-coding RNAs.[27] The timing of these epigenetic changes in macrophages during development is still unclear. Even though fusing gametes are presumed to be epigenetically reprogramed during fertilization with erasure of all epigenetic tags, about 1% of these tags are imprinted and retained across generations.[134,135] Maternal epigenetic information in the oocyte could also directly influence the primordial germ cells.[136,137]

In a fetus or young infant, some HSCs in the bone marrow differentiate into monocytes and macrophages.[1,138] These monocytes are released into the peripheral blood, where these cells circulate for up to 5 days[3,139] and then enter various tissues other than the CNS, to differentiate into macrophages.[140] The PRRs in these HSCs get epigenetically programed and display altered responses to infections. The innate inflammatory pathways seem generally suppressed in the HSCs, but a large repertoire of metabolic enzymes is active.[21,140,141] Most of this genetic imprinting occurs within the first 24 hours.[142] In infants with bacterial infections, the MDMs) may display IIM traits for a few weeks.[23,27,125,131] In comparison, adult macrophages get primed sooner and show specific memory traits for longer periods.[27,143] However, these changes may be altered by infections or vaccination in all age groups.[3]

Macrophages have traditionally been perceived as relatively plastic cells.[144] However, recent data combining fate-mapping, single-cell transcriptomics, and epigenetics show that prolonged residence in tissue-specific niches can rewire or override their transcriptional program in the local microenvironment.[145] These cells likely also get imprinted from the conditions at the time of recruitment.[35,146] The accessibility of the promoters/enhancers in the cellular DNA to transcription factors and RNA polymerases can result in chromatin remodeling.[147,148] The remodeling may include DNA compaction, DNA methylation, histone modifications (methylation, acetylation, phosphorylation, and citrullination), and gene priming by regulators such as the upstream master long non-coding ribonucleic acid (lncRNA) of the inflammatory chemokine locus (UMILILO).[35,149–152]

Histone Modifications

Epigenetic modifications of histones plays an important role in IIM in macrophages.[123,153,154] Histone modifications can affect histone–histone and histone–DNA interactions, binding to chaperones, and chromatin structure (Fig. 6).[155,156] The most dynamic histone epigenomic mark is histone acetylation in the nucleosomes.[157] This mark is frequently located close to gene promoters and enhancers, and therefore correlates well with changes in gene expression. Histone methylation in actively expressed gene promoters can affect both the levels and the plasticity of transcription.[149,157]

The effects of histone acetylation on the promoters and enhancers of inflammatory genes have evoked considerable interest; H3K27ac seems to be a key determinant of the expression of immune response factors;[123] it is often seen in the enhancers and promoters of many genes that are typically inactive.[158–160] H3K9ac and H3K56ac are involved in nucleosome–DNA interactions and are rapidly and reversibly reduced in response to DNA damage.[161,162] H4K91ac leads to nucleosome instability.[163] Many histone modifications can be identified even after the primary stimulus is no longer active, and can facilitate the transcription of inflammatory genes upon restimulation.[164] Some of the so-called “latent” enhancers are not pre-marked in naïve cells but acquire histone modifications upon primary stimulation.[123,165] After the removal of the stimulus, some of these latent enhancers still retain the histone modifications and show rapid, stronger activation upon restimulation.

The effects of histone methylation are also important. These vary with the particular types of histones that are methylated, the

Table 3: Signaling programs in macrophage "training"

Stimulant	*Receptor*	*Training immunity signaling*	*Metabolic remodeling*	*Epigenetic remodeling*
β-glucan	Dectin-1	Akt-mTOR-HIF-1α IL-1, GM-CSF/CD131	Glycolysis	H3K4me1[129]
			Glutaminolysis	H3K4me3[317]
			Mevalonate synthesis	H3K27ac[318]
BCG	NOD2	Akt-mTOR, IFN-γ, IL-32	Glycolysis	H3K9me3[319]
			Glutaminolysis	H3K4me3[319]
			Mevalonate synthesis	H3K27ac[320]
OxLDL	TLRs, oxLDL receptor	mTOR-dependent ROS	Glycolysis, mevalonate synthesis	H3K4me3[275]
LPS	TLR4	IRAK-M, Tollip, JNK-miR24, ATF7	Glucose and cholesterol metabolism	H3K4me1, H3K4me3, H3K9me2, H2K27me[24]
Aldosterone	Mineralocorticoid	Fatty acid synthesis pathway	Fatty acid synthesis	H3K4me3[321]
HMGB1	TLR RAGE	IRAK-M		Inhibits methylation of H3K9 and other histones.[322] C-terminal tail of HMGB1 interacts with the core histones, including H3 and H2A-H2B dimers to stimulate transcription[323]
Fungal chitin	Several possible receptors, including TLR2, TLR3, TLR8, TLR9, FIBCD1, LYSMD3, NOD2, mannose receptor	Binds TLR2 Endosomal ligands of TLR3 (ligand Poly I:C), TLR8 (risiquimod), TLR9 (CpG)		Histone methylation.[154] Limited details so far
Uric acid	Clec12a (negative receptor)	IL-1β, Akt		Histone methylation.[324] Limited details so far

Akt, Ak strain transforming serine/threonine-protein kinase ("Ak" in Akt refers to the AKR mouse strain that develops spontaneous thymic lymphomas, "t" stands for "thymoma"); GM-CSF, granulocyte macrophage-colony stimulating factor; H3K14ac, histone 3 lysine 14 acetylation; H3K27ac, histone 3 lysine 27 acetylation; H3K4m3, histone 3 lysine 4 trimethylation; H3K9m2, histone 3 lysine 9 dimethylation; H3K9me2, histone 3 lysine 9 dimethylation; HIF-1α, hypoxia-inducible factor 1α; HMGB1, high mobility group box 1; IFN-γ, interferon γ; IRAK-M, IL-1 receptor-associated kinase M; LPS, lipopolysaccharides; mTOR, mammalian target of rapamycin; NOD2, nucleotide-binding oligomerization domain-containing protein 2; Tollip, toll-interacting protein; oxLDL, oxidized low-density lipoprotein; PLZF, promyelocytic leukemia zinc finger; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; TLR, toll-like receptor; FIBCD1, fibrinogen C containing domain 1 (FIBCD1); LYSMD3, LysM domain containing 3; Clec12a, C-type lectin domain family 12 member A; CpG, cytosine and guanine nucleotides with the "p" representing the linking phosphate

number of methyl groups added, and the presence of acetylation in nearby regions.[149] For instance, trimethylation of lysine 4 in histone 3 (H3K4me3) and H3K4me1 can activate promoters and enhancers, respectively.[166,167] In unstimulated macrophages, chromatin regions containing inflammatory genes are compacted and largely not accessible for transcription. Primary stimulation with the antigens/pathogens recruits various transcription factors, such as activator protein 1 AP-1; the signal transducers and activators of transcription STATs; and nuclear factor-kappa B (NF-kB) to the promoters and enhancers, which are already pre-marked in the naïve cells by the lineage-specific PU.1 transcription factor.[168–171] When challenged again with the same or a different antigen/pathogen, the chromatin shows increased decondensation, demethylation of DNA, and modifications of histone 3 (H3) such as tri-methylation of lysine 4 (K4; H3K4me3), mono-methylation (H3K4me1), and acetylation of lysine 27 (H3K27ac).[172,173] These epigenetic changes lead to enhanced transcription and translation of immune response factors (Fig. 7).[174]

H3K27 methylation has been associated with both gene activation and repression.[175–177] Many models show concomitant methylation and acetylation, and the effects have not been easy to predict.[123,155] The silencing effects of histone methylation might not always be independent and could involve additional regulators such as the polycomb group proteins.[27,177–181] Trained macrophages show H3K4me1 and H3K27ac in the enhancers and promoters of many genes that are typically inactive.[158–160]

Bacillus Calmette-Guérin inoculation increases resistance to *Staphylococcus aureus* by upregulating H3K4me3 levels associated with inflammatory genes IL-1β and TNF.[120,182] In contrast, β-glucan training increased H3K4me3 and H3K27ac in at least 500 gene promoters.[154,183] Upon secondary stimulation, these leukocytes showed increased expression of transcription factors, cytokines, and phenotypic/functional changes seen in acute inflammation.[23,183] The temporal stability of various changes is also variable. H3K4me1 persisted for long periods but H3K27ac was eliminated sooner after the stimulus was removed.[184,185]

Age, both of the cells and of the host, is an important determinant of the effects of LPS on IIM macrophages.[186] The intensity of immune responses is higher in the developing fetus and neonate.[1,9,14,15,112–114,187–189] Ageing in macrophages impacts many processes including TLR signaling, polarization, phagocytosis, and wound repair.[190–192] Even though the innate immune system is in a "quiescent" mode at birth,[193,194] the mucosal surfaces in the lung and the gastrointestinal tract contain a large number of macrophages. Most of these cells show low baseline expression of MHC-II, F4/80,

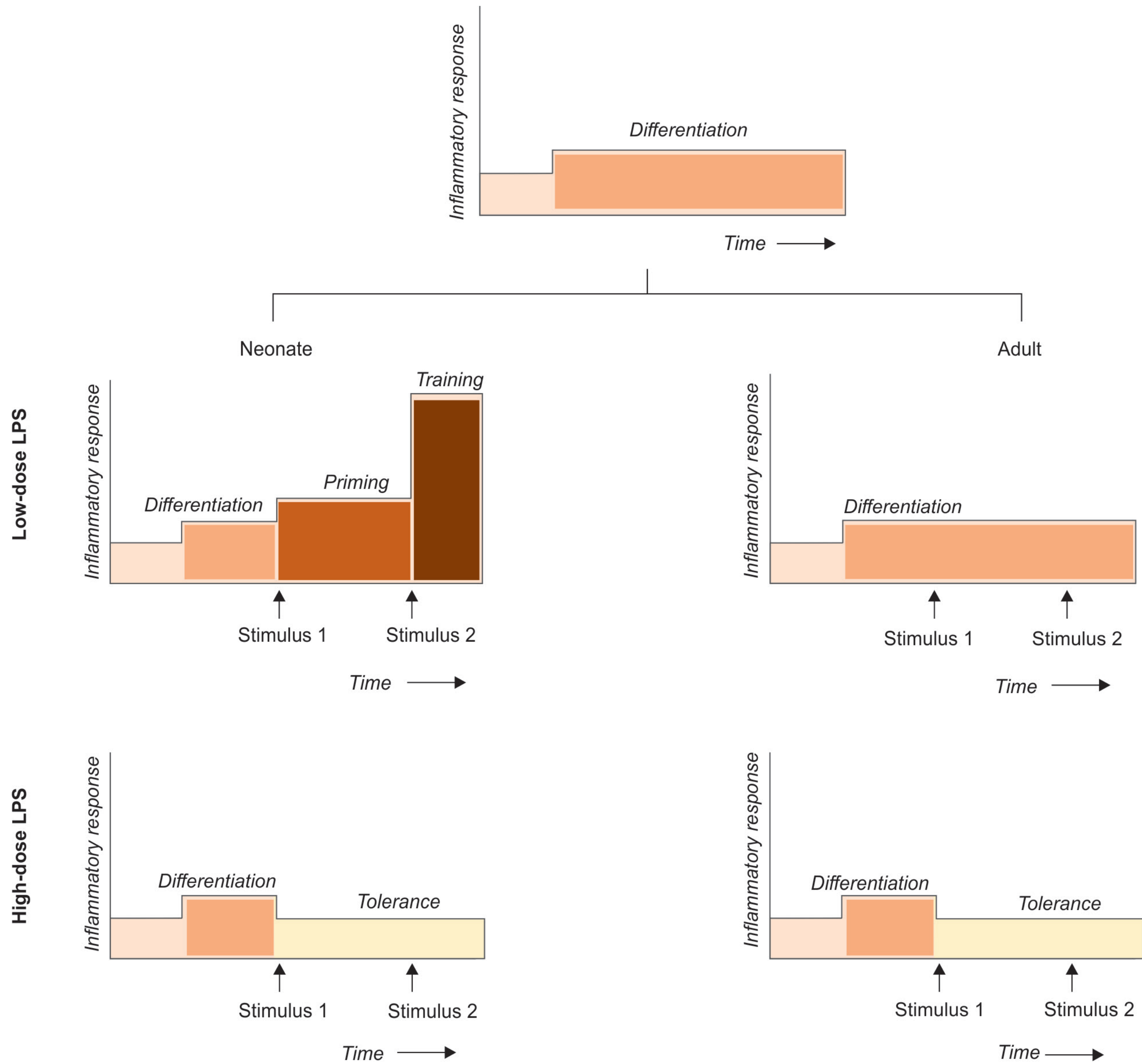
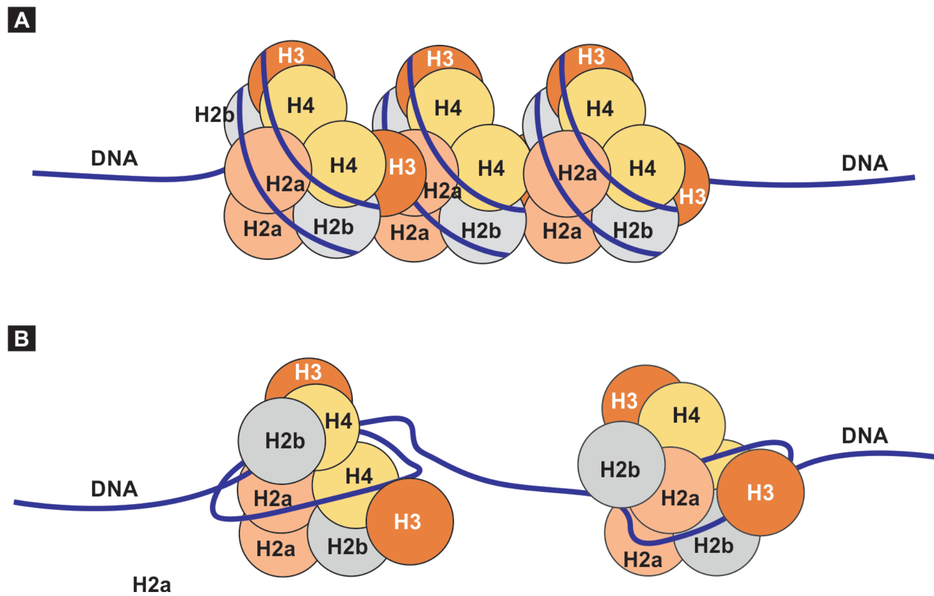


Fig. 6: Effect of age on the effects of LPS on macrophage IIM. Differentiation of naïve macrophages leads to a baseline increase in the expression of inflammatory cytokines such as TNF and/or IL-6. Subsequently, an initial application of LPS in low-doses primes neonatal macrophages for expression of inflammatory mediators. Re-application of LPS in these same doses trains the macrophages and can induce a hyper-inflammatory response. Such induction of these mediators is not seen in mature macrophages in adults. Application of LPS in higher doses suppresses the inflammatory responses in both neonatal and adult macrophages

CD68, CD80, and CD86[193,194]; these low levels of expression may be teleologically important to minimize inflammation when exposed to various environmental and physical challenges soon after birth.[193,195] However, these cells express an M1-like phenotype which can get quickly primed and display highly enhanced immune responses with proinflammatory cytokines, iNOS, and CD86 following LPS stimulation at much higher levels than in adults.[186,193] Arginase-1, which plays anti-inflammatory roles, is also decreased.[193] These characteristics are consistent with the high protein levels of the inducible nuclear factor NF-κB and the pro-inflammatory characteristics seen in neonatal macrophages.[171,193]

The number of macrophages in various mucosal organs in neonates also differs from that in adults in various organs.[115,193] Even though LPS is recognized as the primary pathogen-associated molecule that triggers host innate immune responses to bacterial invasion, the phenotypical modulation of macrophages in response to the various components of the microbiome may vary.[115] M1 is the predominant mucosal macrophage subtype in most such responses.[115,193]

Compared to naïve macrophages, differentiation of these cells leads to a baseline increase in the expression of inflammatory cytokines such as TNF and IL-6. An initial exposure to low doses of LPS primes neonatal macrophages, and a later secondary application further stimulates the expression of inflammatory mediators. Such induction of these mediators is not seen in adult macrophages. In contrast, the application of LPS in high doses suppresses the inflammatory responses in both neonatal and adult macrophages (Fig. 5).[122,155,196] These changes have



Figs 7A and B: Chromatin condensation state affects gene expression. (A) Chromatin housing the immune response genes in naïve (unstimulated) macrophages is highly condensed (heterochromatin state) due to high methylation of DNA, making these genes inaccessible to the transcription factors. These genes are completely silenced or transcribed at very low levels. (B) Stimulation with a pathogen/danger signals demethylates DNA, decondenses chromatin (euchromatin state), and makes these genes accessible for transcription

been associated with increased H3K9me2 and H3K27me2, which downregulated TNF and other inflammatory cytokines.[155,197–199] Lipopolysaccharide-induced tolerance was marked by increased phosphorylation of the transcription factor cyclic associated molecular pattern (AMP)-dependent transcription factor 7 (ATF7).[200,201] H3K9me2 levels were decreased.[200–202]

In newly recruited monocytes in various tissues, there may be up to 8,000 epigenetically dynamic regions where histone acetylation is the most prominent change.[3,154] Histone methylation H3K4me1 is increased in distal regulatory regions, which are relatively stable and might represent decommissioned regulatory elements.[141] β-glucan priming can induce up to 3,000 distal regulatory elements, whereas LPS-tolerization may induce H3K27ac at 500 distal regulatory regions.[3,141] Gene modules that mediate LPS tolerance are more active in monocytes than in naïve macrophages.[3,155] About 12% of known human transcription factors displayed variation in expression during macrophage differentiation, training, and tolerance.[3]

Several other mechanisms are also being studied. Cytokines such as IL-12 may play an important role.[35] A reverse adaptive-to-innate directionality of memory formation is another possibility, as noted in a respiratory adenoviral infection model.[125] In lungs, memory AMs can develop and sustain independently of blood monocytes. The CD8-T cells, which are known adaptive effectors, can help prime, but not maintain, memory AMs by producing IFN-γ. Memory macrophages can also help maintain antibacterial immunity by stimulating the neutrophil populations.[203]

Effects of MicroRNAs

MicroRNAs (miRNAs) can promote prolonged epigenetic changes and LPS tolerance in IIM macrophages.[204,205] High miR-155 levels were associated with inflammatory activation.[206,207] Prolonged exposure to LPS increased miR-221 and miR-222 levels.[208,209] These miRNAs silenced the inflammatory genes through switch/sucrose non-fermentable (SWI/SNF) and signal transducer and activator of transcription (STAT)-mediated chromatin remodeling.[210–212]

As currently understood, miRNAs silence gene expression by repressing cap-dependent translation.[213] These also destabilize the target mRNAs through deadenylation, decapping, and then degradation from the 5´ to the 3´ ends.[214] The miRNA-induced silencing complexes (miRISCs) involve interactions of the conserved GW182 proteins (named after the glycine and tryptophan repeats and the molecular weight) with the argonaute proteins (discovered in *Arabidopsis thaliana*) and downstream deadenylases.[215] These protein–protein interactions, in turn, increase (a) biogenesis of small RNAs[216]; (b) insertion of tryptophan residues into hydrophobic pockets on the surface of argonaute proteins[217]; (c) displacement of the translation initiation factors 4A[218]; and/or (d) recruitment of the translational repressor and decapping of the activator DEAD box protein 6.[219]

Effects of Metabolic Changes

Classically activated M1 macrophages produce energy largely through glycolysis, whereas M2 macrophages utilize oxidative phosphorylation and the tricarboxylic acid cycle (TCA; citric acid cycle).[220,221] Treatment with β-glucan or BCG augment aerobic glycolysis via the Akt/mechanistic target of rapamycin (mTOR)/hypoxia-inducible factor-1α (HIF-1α) pathway.[222,223] In M1 macrophages, oxidative phosphorylation begins after the acute phase response ends.[224,225]

Cellular metabolism in macrophages is closely related to epigenetic changes.[150,226] The epigenetic profile of histones is closely related to the activity of two sets of enzymes, the histone acetyltransferases (HATs) and the histone deacetylases (HDACs).[227,228] These induce posttranslational modifications on histones, which in turn, can alter chromatin structure and function.[229,230] HATs acetylate the *N*-terminal histone tail to induce a "relaxed" chromatin structure that allows transcriptional activation.[227,231] In contrast, HDACs repress transcription by tightening the chromatin structure and rendering the associated DNA less accessible for transcription.[232,233]

Histone deacetylases 1 and 6 promote the development of the immune phenotype of macrophages.[234–236] Trained monocytes

typically show high levels of histone acetylation, which correlates with the acetyl-coenzyme A (acetyl-CoA) levels.[65,154] Tricarboxylic acid cycle intermediates such as fumarate, succinic acid, and α-ketoglutaric acid (α-KG) can also promote IIM.[66,237] These cells typically show low demethylase activity but high levels of cholesterol synthesis, which promote epigenetic reprogramming by activating the mTOR pathway.[25,154,238] Glutamine metabolism is also associated with increased succinic acid and α-KG, which activate epigenetic enzymes to enhance M2-related H3K27me3, which in turn, suppresses these genes and turns memory macrophages into an anti-inflammatory phenotype.[66,123,224] In cells with LPS-induced endotoxin tolerance, α-KG promotes M1 activation of macrophages.[224,239] These results suggest that cellular metabolism can alter immune memory.

Role of IIM Macrophage in Diseases in Adult Patients/Animal Models

Innate immune memory in macrophages can alter the responses to many pathogenic stimuli.[23,240] Most work has been done in diseases of adulthood, but these data could provide useful insights into the susceptibility and pathogenesis of many neonatal conditions.[241–243]

Acute Inflammation

Inflammatory macrophages can both express and promote the expression of TNF, IL-1β, and IL-8 in neighboring cells.[10] Interestingly, mice treated with IL-1β prior to a second bacterial infection showed increased IIM macrophages and improved survival.[244] In this model, IIM macrophages express higher H3K4me3 levels (unpublished data from our laboratory). β-glucan is another inducer of IIM macrophages; it can reprogram macrophages by curtailing the activation of inflammasomes containing the NOD-like receptor family pyrin domain-containing-3 (NLRP3).[245,246] NLRP3 can detect markers of cellular damage such as extracellular ATP and crystalline uric acid.[4,247]

Infectious Diseases

Macrophages provide innate immunity against bacterial and viral infections, and IIM macrophages can enhance the defenses against *S. aureus* skin infections.[4,28,248] In murine models, these macrophages showed increased monocyte recruitment, bacterial killing, healing, and resistance to secondary infections.[248,249] In the lungs, AMs can be activated by a primary respiratory syncytial virus infection with improved host defense against pneumococcal superinfections.[250] Memory AMs express major histocompatibility complex (MHC)-II and chemokines at higher levels, and show more glycolysis and bacterial killing.[4,203,249–251]

Infection-induced IIM has been associated with molecules such as NOD2; possibly viral RNA; and proteins containing a leucine-rich repeats (*LRR*)-containing domain are evolutionarily conserved in many *proteins* associated with innate immunity.[252] Similarly, *NLRP3* (NOD-, LRR- and pyrin domain-containing protein 3), which is an intracellular sensor that detects many microbial molecules may also be associated.[253,254] The BCG vaccine can activate NOD2-dependent pathways to protect against secondary infections through epigenetic reprogramming of monocytes/macrophages.[121,255] In the resulting memory macrophages, the promoters of IL-6 and TNF genes can increase H3 trimethylation (H3K4me3) and induce the expression of these cytokines.[121,256]

Allergic Disorders

Infectious agents can induce IIM in macrophages, but similar changes are frequently seen in allergic and other type 2 inflammatory conditions.[257] M2-polarized macrophages may play a role in asthma[258]; AMs in these patients express chemoattractants such as CCL17,[259–261] and eicosanoids, particularly leukotrienes, which can stimulate T helper-2 cells.[262,263] Pathogen molecules, sterile inflammatory stimuli, and respiratory viruses can induce epigenetic and metabolic reprogramming in macrophages, and thereby alter responsiveness and effector functions similar to those seen in allergic disorders.[257] These IIM changes can be seen both in tissue macrophages and myeloid progenitors.[4,257,264,265] Evaluation of epigenetic/histone-profiles such as *H3K27me3* and H3K9me3 may help develop focused therapies.[4,266]

Transplant Rejection

Innate immune memory macrophages may increase the risk of transplant rejection by activating innate and adaptive immunological responses and consequent inflammation.[267,268] Macrophages may recognize MHC-I molecules and generate memory.[269] In murine kidney and heart transplantation, deletion of recipient [type A paired immunoglobulin-like receptors (PIR-A)] or blocking the binding of PIR-A to donor MHC-I molecules can block the memory response and alleviate the rejection reaction.[270,271] Such IIM has also been seen in human transplant cases.[27] Macrophages can acquire IIM for recognizing alloantigens, and blocking this memory may improve the outcomes of transplantation.[272,273]

Atherosclerosis

Innate immune memory macrophages can protect against atherosclerosis.[274] In addition to the classical inducers of innate immunity such as β-glucan, BCG, and LPS, endogenous non-microbial atherogenic stimuli such as high cholesterol levels, oxidized low-density lipoprotein (oxLDL), and lipoprotein(a) can also promote IIM in macrophages.[275]

Oxidized low-density lipoprotein is a recognized DAMP; it can increase macrophage recruitment, inflammation, and interstitial fibrosis.[276,277] It recruits macrophages binds the CD36 receptor to, increases glycolysis, increases the production of pro-inflammatory factors, and induces IIM.[278] Upon stimulation by TLR2 and TLR4 ligands, oxLDL-stimulated macrophages produce inflammatory factors such as TNF, IL-6, and collagenases such as matrix metalloproteinase (MMP)-2 and -9. These mediators can destabilize atherosclerosis plaques.[279] Tumor necrosis factor promoters are enriched in H3K4me3 markers.[280]

Neoplasms

Innate immune memory macrophages have been detected in several tumors.[281,282] These findings might not be clinically relevant in neonates but may still provide important mechanistic insights. Inflammatory M1 macrophages can provide anti-tumor immunity; β-glucan can induce type I IFN signaling, and BCG can be useful for directly stimulating macrophages.[4,65,120,283,284] Innate immune memory macrophages with M1-like properties can promote tumor progression with angiogenesis, fibrosis, and consequent tissue remodeling.[65,140] These macrophages show histone modifications such as H3K4me3 and H3K9me3, and upregulated expression of inflammatory and other genes associated with tumor progression.[285]

Conclusions

With adaptive immune responses still maturing, macrophages are a much-needed component of immune responses in the fetus and the newborn infant.[1,9,112–114] Innate immune memory macrophages

may be crucial for trained/acquired host immunity in the fetus/young infant, but we still have major gaps in our understanding of the functional maturation of these cells.[1] These details will be of translational importance for developing therapeutic interventions in various inflammatory diseases.

Single-cell transcriptomics and epigenomics have helped identify IIM macrophage precursors.[286] Studies of tumor-associated macrophages may also be useful; understanding the developmental regression with persistent activation of these macrophages can provide useful clues into the ontogeny of macrophage subpopulations, macrophage memory, and the involved molecular mechanisms.[287,288] These findings can then be evaluated in appropriate fetal and genetically altered animal models.[123,289–298]

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REVIEW ARTICLE

Lung Ultrasound in Neonates: An Emerging Tool for Monitoring Critically Ill Infants

Arjun Verma[1], Abhishek Paul[2], Atnafu Mekonnen Tekleab[3], Abhay Lodha[4], Kei Lui[5], Akhil Maheshwari[6], Jan Klimek[7], Pradeep Suryawanshi[8]

Received on: 19 January 2023; *Accepted on*: 04 March 2023; *Published on*: 06 April 2023

Abstract

Context: Neonatal lung ultrasound is emerging as a useful clinical tool for the assessment of lung anatomy and management of various lung pathologies. In this review, we summarize normal lung ultrasound (LUS) findings and specific features of various lung morbidities.

Evidence acquisition: A comprehensive literature search was conducted across multiple sources with relevant keywords with an additional filter of the age-group between 0 and 28 days.

Findings: Apart from the description of normal newborn lungs, clinical and radiological features of a variety of lung pathologies were evaluated and incorporated in the review. Bedside LUS has evolved to be an important point-of-care imaging modality that can help in day-to-day clinical decision-making. It can be used in differentiating respiratory distress syndrome from transient tachypnea on the newborns, in the detection of pneumothorax, and in diagnosing pneumonia, pulmonary hemorrhage, and pleural effusion. Evidence supports the use of LUS scores to decide on the need for early rescue surfactant therapy with high sensitivity and specificity. Lung ultrasound scores obtained during the first 2 weeks after birth can help predict the likelihood of chronic lung disease/bronchopulmonary dysplasia. Once validated, it could be valuable for guiding early intervention and evaluation of new treatments.

Conclusion: Neonatal lung ultrasound is emerging as a vital monitoring tool in critically ill infants with lung disease. It will be valuable in the early diagnosis, management, and prognosis of these patients.

Keywords: Bronchopulmonary dysplasia, Conventional lung ultrasound score, Modified lung ultrasound score, Pneumothorax, Point-of-care lung ultrasound, Respiratory distress syndrome, Transient tachypnea of newborn.

Newborn (2023): 10.5005/jp-journals-11002-0057

Key Points

- Point-of-care lung ultrasound, a new modality for assessing the severity of lung disease and the need for surfactant treatment in a timely fashion, has the potential to revolutionize the management of respiratory distress syndrome (RDS).
- In the early neonatal period, LUS can help in differentiating transient tachypnea of newborn (TTN) from RDS. The characteristic findings in TTN include a double lung point, confluent or compact B-lines, and the alveolar interstitial pattern.
- In later weeks, sonography can help differentiate pneumonia from atelectasis. Pneumonia is marked by the presence of dynamic air bronchograms and large-size consolidations with irregular margins.
- Point-of-care LUS can easily diagnose pneumothorax with very high sensitivity and specificity. These bedside assessments can help in the timely evaluation and clinical management of air leaks.
- Sonography is emerging as a consistent, reliable tool for the assessment and monitoring of chronic lung disease/bronchopulmonary dysplasia. Several scoring systems are under evaluation. The ease of this non-invasive point-of-care imaging promises to be an exciting tool in guiding early management decisions and evaluation of new treatment strategies.

[1]Department of Neonatology, Mahatma Gandhi Medical College, Mahatma Gandhi University of Medical Sciences and Technology, Jaipur, Rajasthan, India

[2]Department of Neonatology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India

[3]Department of Pediatrics and Child Health, Saint Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia

[4]Department of Pediatrics and Community Health Sciences, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada

[5]Discipline of Pediatrics and Child Health, School of Clinical Medicine, University of New South Wales, Sydney, Australia

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

[7]Department of Neonatology, Westmead Hospital, Westmead, Australia

[8]Department of Neonatology, Bharati Vidyapeeth University Medical College, Hospital and Research Center, Pune, Maharashtra, India

Corresponding Author: Pradeep Suryawanshi, Department of Neonatology, Bharati Vidyapeeth University Medical College, Hospital, and Research Center, Pune, Maharashtra, India, Phone: +91 9923540500, e-mail: drpradeepsuryawanshi@gmail.com

How to cite this article: Verma A, Paul A, Tekleab AM, *et al.* Lung Ultrasound in Neonates: An Emerging Tool for Monitoring Critically Ill Infants. Newborn 2023;2(1):80–90.

Source of support: Nil

Conflict of interest: Dr. Abhay Lodha, Dr. Kei Lui and Dr. Akhil Maheshwari are associated as the Editorial Board Members of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of these Editorial Board Members and their research group.

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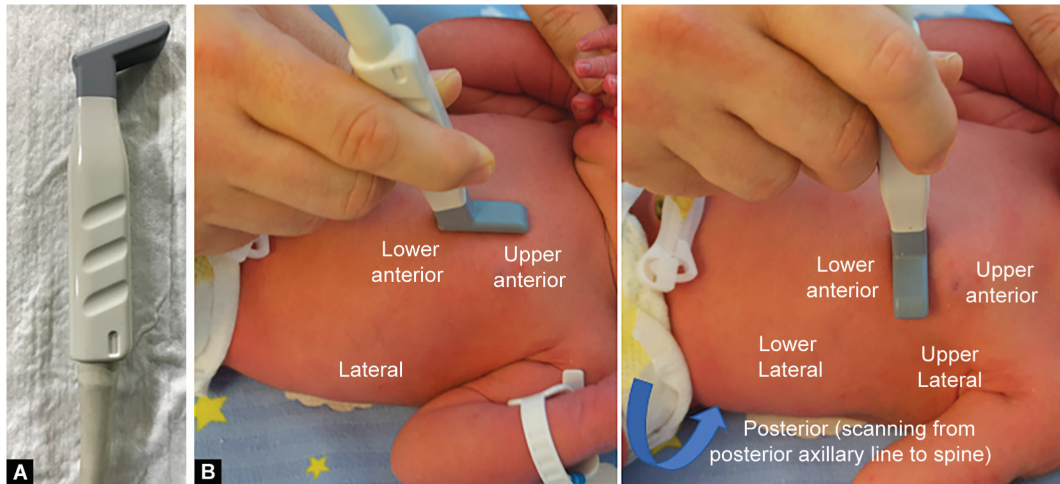


Figs 1A and B: (A) A hockey stick transducer (15 Mhz) used for lung ultrasound imaging; (B) Positioning of the probe parallel and perpendicular to the ribs and the blue boxes show various zones scanned in lung sonography. Left: 6-region approach showing anterior and lateral views of both the lungs. Right: 12-region approach can show upper anterior, lower anterior, upper lateral, lower lateral, upper posterior, and lower posterior views

Source: Corsini I, Parri N, Ficial B, et al. Lung ultrasound in the neonatal intensive care unit: Review of the literature and future perspectives. Pediatr Pulmonol 2020;55(7):1550–1562. DOI: 10.1002/ppul.24792.

Introduction

Ultrasound of the lung is emerging as an important tool in the clinical care of newborn infants.[1–8] This is an interesting development because sound waves are not known to penetrate air very well and consequently, the aerated lungs show many artifacts.[9–11] In neonates, the relatively under-aerated lungs permit better sonic evaluation of various pathologies with higher sensitivity and specificity than in adults.[12–15] Lung ultrasound can help in bedside diagnosis/ evaluation of respiratory distress syndrome (RDS), pneumonia, transient tachypnea of the newborn (TTN), pleural effusion, and pneumothorax.[1,5,16–22] Compared to chest radiographs, LUS is more convenient as it can be readily available at the bedside, is safer as there is no radiation exposure, and has the potential to improve care by allowing quick interpretation and repeated testing to monitor the course of disease and response to therapy.[22,23–32] Ultrasound can help evaluate the response to recruitment maneuvers during high-frequency ventilation.[33–36] There may be less inter-observer variability, and consequently, higher reliability.[1,37,38]

Frequently Used Terms and Scanning Methods

Lung ultrasound is commonly done using a high-frequency linear transducer or a hockey-stick transducer (10 Mhz or higher), in supine, lateral, and prone positions. The transducer is placed either parallel or perpendicular to the ribs. Each lung is evaluated in three regions:

1. The anterior area between the sternum and the anterior axillary line;
2. The lateral area between the anterior and posterior axillary lines; and
3. The posterior area between the posterior axillary line and the spine.

Ultrasound evaluation of the lung may be performed in a 6- or 12-region approach (Fig. 1).

Pleural Lines

These hyperechogenic, thin, regular, and smooth horizontal lines are typically located 0.5 cm below the rib line.[1,37,39] These lines are an echo-reflection of the interface of the pleural lung surface.[40–42] When viewed together, the ribs and the pleural line have been described as the 'bat' sign.[2,40,43]

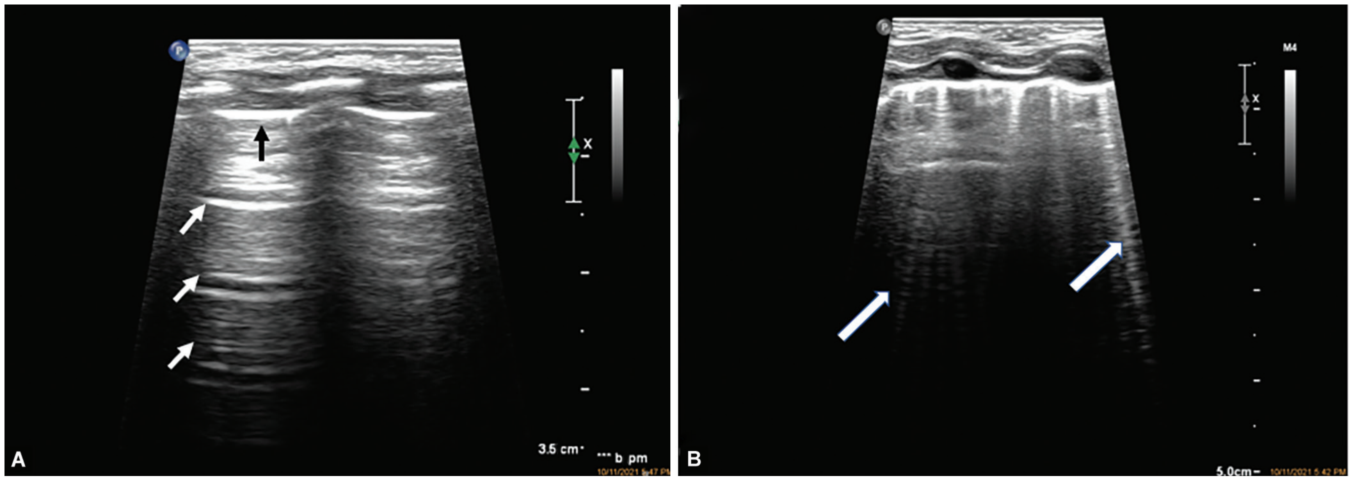
- **A-lines:** These reverberation artifacts are a series of linear, hyperechoic structures seen starting from just below the skin all the way to the pleural line.[1,40,44] These parallel lines are separated from each other at near equal distances, and may be associated with a set of parallel rods in a 'bamboo sign' (Fig. 2).[7,32,40,45]
- **B-lines:** These laser-like vertical hyperechoic artifacts arise from the pleural line and may continue up to the edge of the screen without fading (Fig. 2).[46] B-lines movesynchronously with lung–sliding breathing movements.[2,40,43] These lines indicate decreased aeration and accumulation of fluid in the interstitium. B-lines can be seen for 24–36 hours, even up to 48 hours in normal newborns.[31,47] In many, both A and B lines are seen.[40] The presence of fluid in the interstitial and alveolar space may be seen as confluent B-lines.[48]

Confluent or fused B-lines can fill the entire intercostal space and erase the A-lines.[49] However, the rib shadows are not obscured.[32] The presence of more than two confluent B-lines in the lung field(s) has been described as alveolar-interstitial syndrome (AIS).[6,48,50,51]

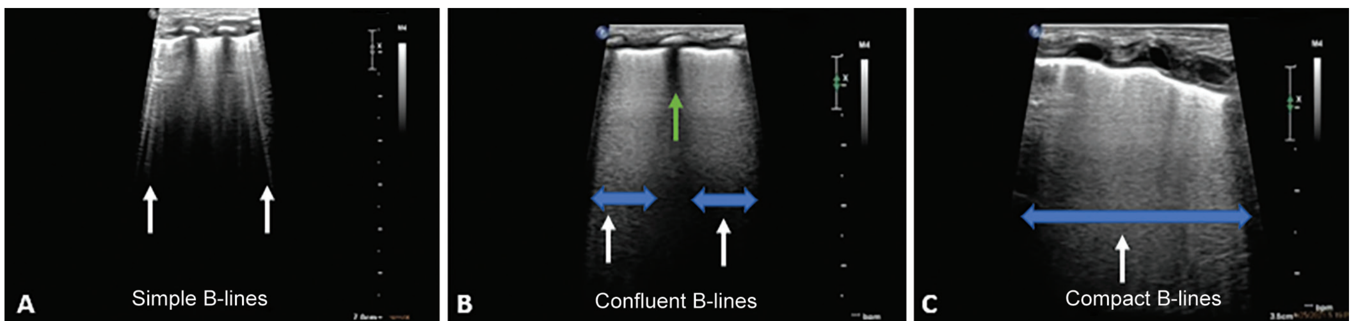
White-out or opacified lungs: The lung fields are filled with compact B-lines (Fig. 3).[12,51,52]

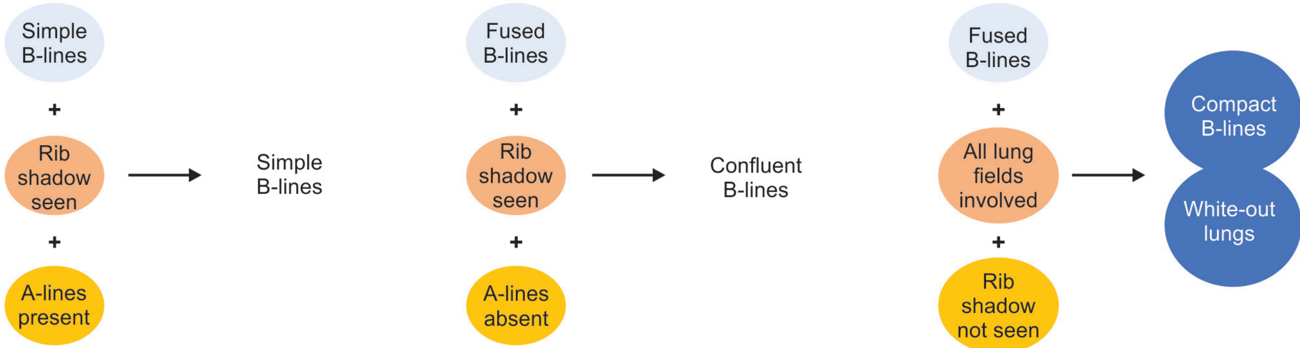
Lung sliding: The pleural line moves with respiration, which represents the relative movement between the parietal and visceral pleura during the respiratory cycle.[40] In a normal lung, the pleural line slides horizontally from one side to another during respiration and has been described as lung sliding.[53]

Lung consolidation: The lung tissues show air- and/or fluid bronchograms and "hepatization" with a density resembling that of the liver tissue on sonography.[54] Consolidated areas larger than 1 cm, not the smaller micro-consolidated areas, are likely to be seen on radiographs.[1,31,55,56]



Figs 2A and B: (A) Sonogram shows the normal pleural line (black arrow) and the normal pattern of A-lines (serial white arrows). Seen together, the A-lines have been viewed as a set of parallel rods and described as the 'bamboo' sign (serial white arrows); (B) Sonogram shows multiple simple B-lines (white arrows)





Figs 3A to C: Evolution of B-lines as shown in the flowchart and ultrasound images. (A) Sonographic appearance of multiple simple B-lines. A- and simple B-lines can be seen in normal lungs; (B) Confluent B-lines seen covering the entire intercostal space (blue arrow) with the rib shadow in between (green arrow); (C) Compact B-lines representing the fused confluent B-lines with no rib shadows (blue arrow). The detection of compact B-lines in all the lung fields is suggestive of white-out lungs

Air bronchograms: Dense, snowflake like areas seen in (severe) RDS and pneumonia.[57]

Lung Points

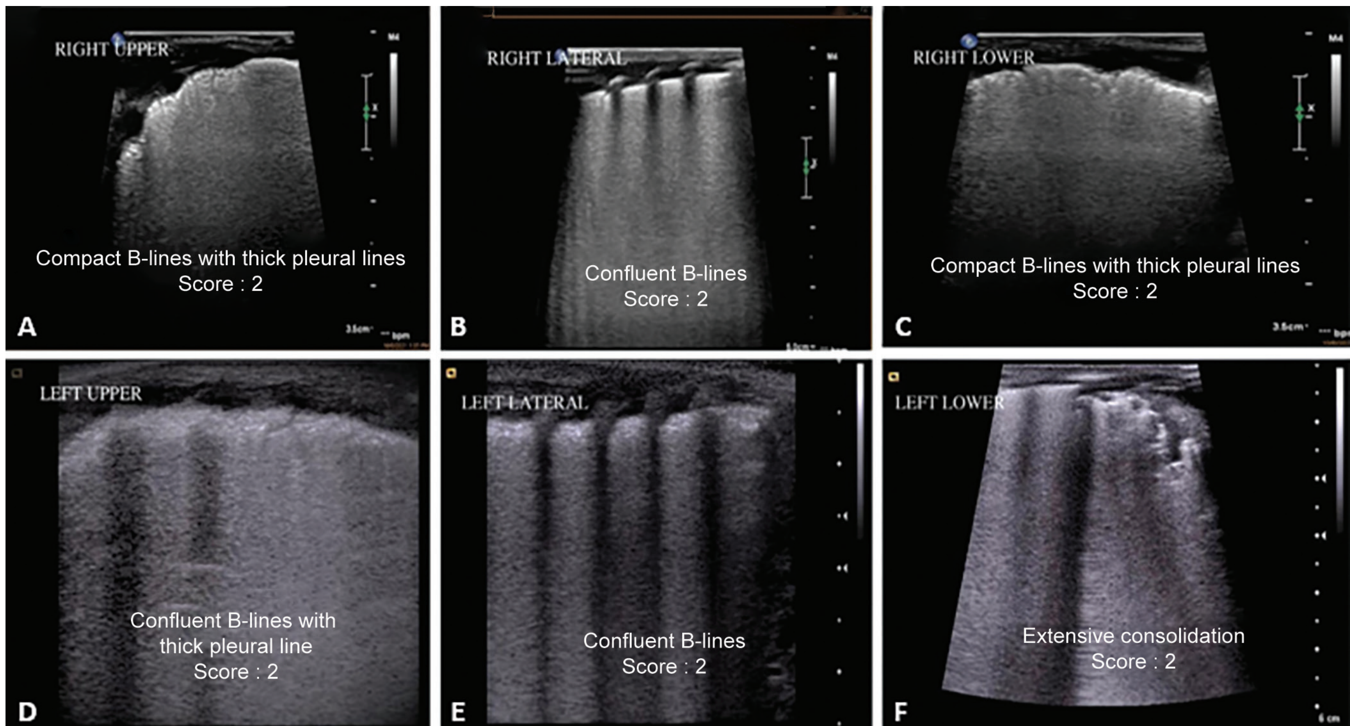
A specific sign of pneumothorax locates in the position of the gas boundary in cases of mild-to-moderate pneumothorax.[40,58] Real-time ultrasound shows transition points between areas that show lung sliding and others that do not.[25,59] A straight linear array high-frequency probe (5–13 MHz) may be most helpful in analyzing superficial structures such as the pleural line and providing better resolution.[58,60] Double Lung Point

In infants with varying severity or differing nature of pathological changes in different areas of the lung, such as between the upper and lower lung fields, a sharp cut-off point called the double lung point can be seen.[47,61]

Clinical Applications

Assessment of the Severity of RDS and the Need for Surfactant

The major features of a preterm baby with RDS on LUS include pulmonary consolidation, air bronchograms, alveolar interstitial syndrome, pleural line abnormalities, and diffuse white-out lungs.[1,39,62] Together, these findings raise the sensitivity and



Figs 4A to F: Respiratory distress syndrome. A preterm infant born at 28-week gestation with a birth weight of 1100 grams had severe respiratory distress. The Silverman-Andersen respiratory severity score was 7/10. A lung ultrasound was done at birth. (A, C) Compact B-lines with a thickened pleural line; (B) Confluent B-lines; (D, E) Confluent B-lines with thick pleural lines and absent A-lines; (F) Consolidation with the shred sign. The overall score at birth was 12 which correlated with the increasing requirement for FiO_2 and the need for surfactant

specificity of LUS to approach nearly 100%.[63,64] Traditional chest radiographs for RDS generally show pan-opacified lungs, where it might be difficult to differentiate pulmonary edema from pleural effusion.[55,65] Lung ultrasound can add to chest radiographs by defining various pathologies such as atelectasis, consolidation, pulmonary edema, and pleural effusion.[66,67] These findings in sonography correlate with the difference in the response of each infant to surfactant and RDS management.[67] To grade the severity of the RDS and the response to clinical management of RDS, LUS scoring has been proven to be a consistent and useful modality (Fig. 4).[1]

A 2020 systematic review and meta-analysis showed lung ultrasound (LUS) cut-off scores of > 5–6 to give pooled sensitivity and specificity rates of 88 and 80%, respectively.[68] The study concluded that LUS scores can be useful for decision-making on surfactant replacement therapy and assisted ventilation.[69] Similarly, the 2019 echography-guided surfactant therapy (ESTHER) trial showed that lung ultrasound performed within 3 hours after birth accurately identified infants who eventually developed severe RDS and needed surfactant.[70] The use of LUS also reduced the number of ventilator days and the need for high FiO2 requirements.

Differentiation of RDS from TTN

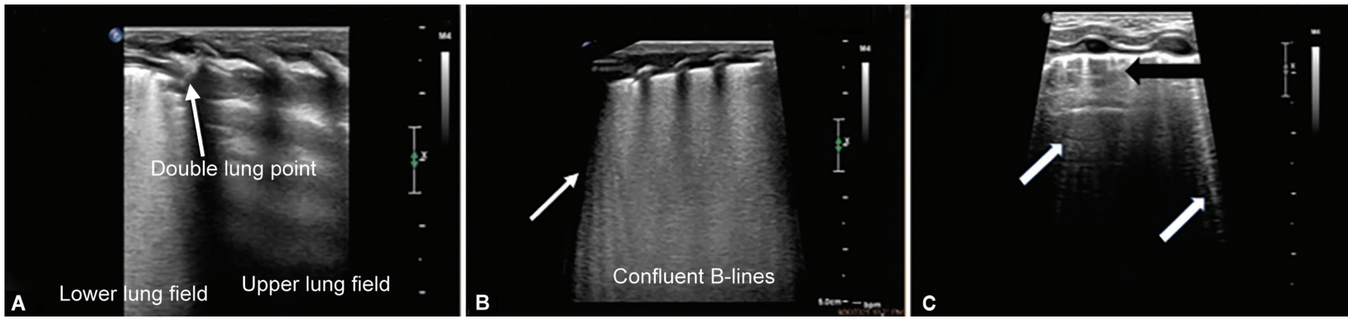
Respiratory distress syndrome has characteristic features such as consolidation and thickened pleural lines, which are not seen in TTN.[71–73] Infants with the double lung point and AIS patterns are more likely to have mild TTN,[47,61] whereas those with compact B-lines and severe AIS without the double lung point and consolidation may have severe disease (Figs 4 and 5).[57,72] Even though lung ultrasound might not always reliably differentiate between RDS and TTN just by detecting confluent /compact B-lines or AIS pattern and white-out,[57,71] sonography would still be more useful than chest X-rays to differentiate between the two groups.[74]

Diagnosis of Pneumonia

Bacterial pneumonia is a major cause of neonatal mortality.[75] It is also one of the most common hospital-acquired infections in neonatal intensive care units (NICUs).[76–80] There has been a high degree of discrepancy in the interpretation of chest X-rays in diagnosing pneumonia between radiologists and neonatologists.[81,82] Computed tomography (CT) is a well-accepted gold standard for diagnosing pneumonia, but it is not a good first-line investigation in neonates because of high radiation exposure, issues with feasibility, and high costs.[83,84] Lung ultrasound is emerging as a good first-line, non-invasive bedside modality for diagnosis and monitoring.[31,57]

The major diagnostic features of pneumonia in lung ultrasound include large areas of consolidation, air bronchograms, the disappearance of lung sliding, presence of lung pulse, abnormal pleural lines, the disappearance of A-lines, and AIS patterns.[25,51] Lung consolidation due to pneumonia shows includes large-sized, variably-shaped hypoechoic areas with irregular margins, which are frequently seen in the subpleural regions and are classically associated with the presence of dynamic air bronchograms within the regions of consolidation.[2,25,46,85,86] The liver-like appearance of the echotexture in consolidated areas has been described as hepatization of the lung tissue.[87,88]

The hypoechoic consolidated lung frequently contains hyperechoic linear elements that represent air in the bronchioles, called air bronchograms.[89,90] There are two types of these air bronchograms, named static and dynamic.[91,92] Dynamic air



Figs 5A to C: Transient tachypnea of the newborns. Lung ultrasound at birth in a male newborn born at 37-week gestation with respiratory distress. (A) Double lung point; (B) Confluent B-lines present in all lung fields, showing alveolar interstitial syndrome; (C) Simple B lines (white arrows) and a comet tail sign (black arrow)

Table 1: Features of lung consolidation in RDS, pneumonia, and in atelectasis

Differentiating features	*RDS*	*Pneumonia*	*Atelectasis*
Timing of the lesion	Acute phase	Acute phase	During recovery
Static air bronchograms	Punctiform arrangement	Punctiform arrangement	Linear parallel arrangement
Dynamic air bronchograms	May be present	Present (high PPV)	Absent
Margins	Regular/irregular margins	Irregular margins only	Sharp, clearlydefined margins
Pleural effusion	Rare	Small, parapneumonic	Large effusion
Fluid bronchograms	Less likely	Less likely	Classical for focal atelectasis
Shred sign	Less likely	Strong association	Less likely

bronchograms show centrifugal air movement in the bronchi in real-time lung ultrasound.[85,91,93] These are of diagnostic importance in differentiating pneumonia from resorptive atelectasis, which frequently tends to be static.[85,92,93]

Lung pulse is another diagnostic feature seen in real-time lung ultrasounds.[94] It is evocative of the apex beat and can be explained by the presence of a consolidated lung transmitting the vibrations of the beating heart.[53,94] Lung pulse is also seen in atelectasis, but the pulse seen in consolidation due to pneumonia is associated with the disappearance of lung sliding.[25,40] Therefore, the lung pulse can be visualized as a replacement of the normal lung sliding by pulsations that coincide with the heart rate.[94] Neonatal pneumonia has been recorded to be associated with the loss of lung sliding in 75%, lung pulse in 30%, and dynamic air bronchograms in 52.5% of the cases.[95]

In pneumonia, several non-specific lung findings can be seen. These include abnormalities of pleural lines ranging from fine lines with a coarse appearance to prominent irregularities, disruption, to the disappearance of pleural lines.[96,97] These abnormalities correlate with the severity of the inflammatory reaction.[96] Some cases show associated pleural effusions, compact and confluent B-lines, and AIS patterns.[96,98]

Diagnosis of Pulmonary Atelectasis

Atelectasis is frequently seen in criticallyill infants.[99] These lesions can prolong the duration of ventilation and hospitalization.[15,100] Atelectatic areas have been classified as obstructive or non-obstructive, unilateral or bilateral, and segmental or lobar.[101] On ultrasound, atelectasis can be viewed as focal or occult.[102] Focal atelectasis shows as an area of consolidation that is delimited by clear margins and contains air and/or fluid bronchograms.[41]

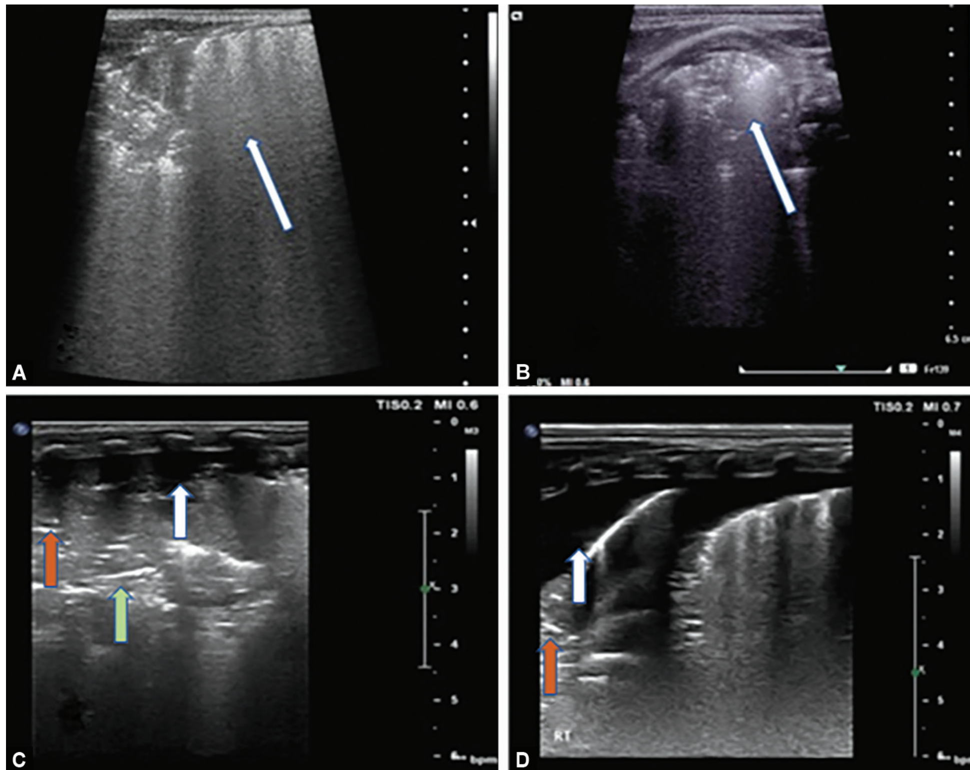
In atelectatic foci, the lung pulse is regularly visible. There are air bronchograms, which have a static, hyperechoic appearance.[25,103] There may be pleural line abnormalities, loss of A-lines, and findings of AIS around the atelectatic areas.[1,31,103,104] Occult lung atelectasis is so-named because these lesions are difficult to see on routine radiographs.[105] Lung ultrasound is useful for detecting these lesions; there are small areas of consolidation with irregular edges and punctate air bronchograms.[106] Lung sliding is present, but there is no lung pulse.[106] Identification of occult atelectasis is important in infants who are being weaned off assisted ventilation but still show limited lung function.[24]

As described above, regions affected by pneumonia show fluid bronchograms. Unlike the hyperechoic air bronchograms seen in atelectatic foci, these hypoechoic lesions can be linear or dendritic in pneumonic patches.[103,104] Lung ultrasound is useful for differentiating lung consolidation due to RDS, pneumonia, and atelectasis.[107] Lesions related to RDS are seen in premature infants or infants of diabetic mothers in the first few days after birth, pneumonia, and those due to atelectasis during high-severity illness or during assisted ventilation.[15,57,108] The differentiating features are summarized in Table 1.

Detection of Pulmonary Hemorrhage

Pulmonary hemorrhage is one of the most critical emergencies in criticallyill neonates.[109] It is frequently seen in extremely preterm with hemodynamically-significant patent ductus arteriosus, hypoxic-ischemic encephalopathy, and fulminant sepsis with disseminated intravascular coagulation. Chest X-ray usually reveals fluffy opacities, focal ground-glass, and sometimes, complete white-out opacities. Lung ultrasound can help in the early identification of pulmonary hemorrhage for emergency bedside management.[110] Alongside supportive clinical history, supportive sonographic signs include the shred sign and pleural effusions.[110]

Some sonographic features overlap between infectious pneumonia and pulmonary hemorrhage.[111] In pulmonary



Figs 6A to D: Pneumonia and pulmonary hemorrhage. (A, B) The shred sign of consolidation. Shred sign is the transition between the normal lung and the areas with consolidation, seen as hyperechoic broken lines; (C, D) pulmonary hemorrhage with air bronchograms (green arrows). Fluid bronchograms (red arrow) in images C and D are hypoechoic linear lines seen below the hemorrhage

hemorrhage, large areas of consolidation can be associated with massive pleural effusions.[2] In these effusions, floating fibrinous strands can be the signs of incomplete coagulation.[110] The volume of the effusion may reflect the severity of bleeding and that of the overall illness.[112] Lung consolidation may reflect the primary disease such as pneumonia or RDS, but some areas may be atelectatic due to airway blockage from secretions or thrombi.[112]

Shred sign is another classical feature of pulmonary hemorrhage (Fig. 6).[113] It is characterized by thick, irregular, broken hyperechoic lines that separate the aerated and consolidated segments of the lung.[114] Some non-specific features may include the absence of A-lines, abnormal pleural lines, and alveolar interstitial pattern.[40] The lung ultrasound features can sometimes overlap with other differentials like pneumonia with parapneumonic effusion, RDS, and pulmonary atelectasis.[25] A careful assessment of the clinical features can be helpful.

Evaluation for Pneumothorax

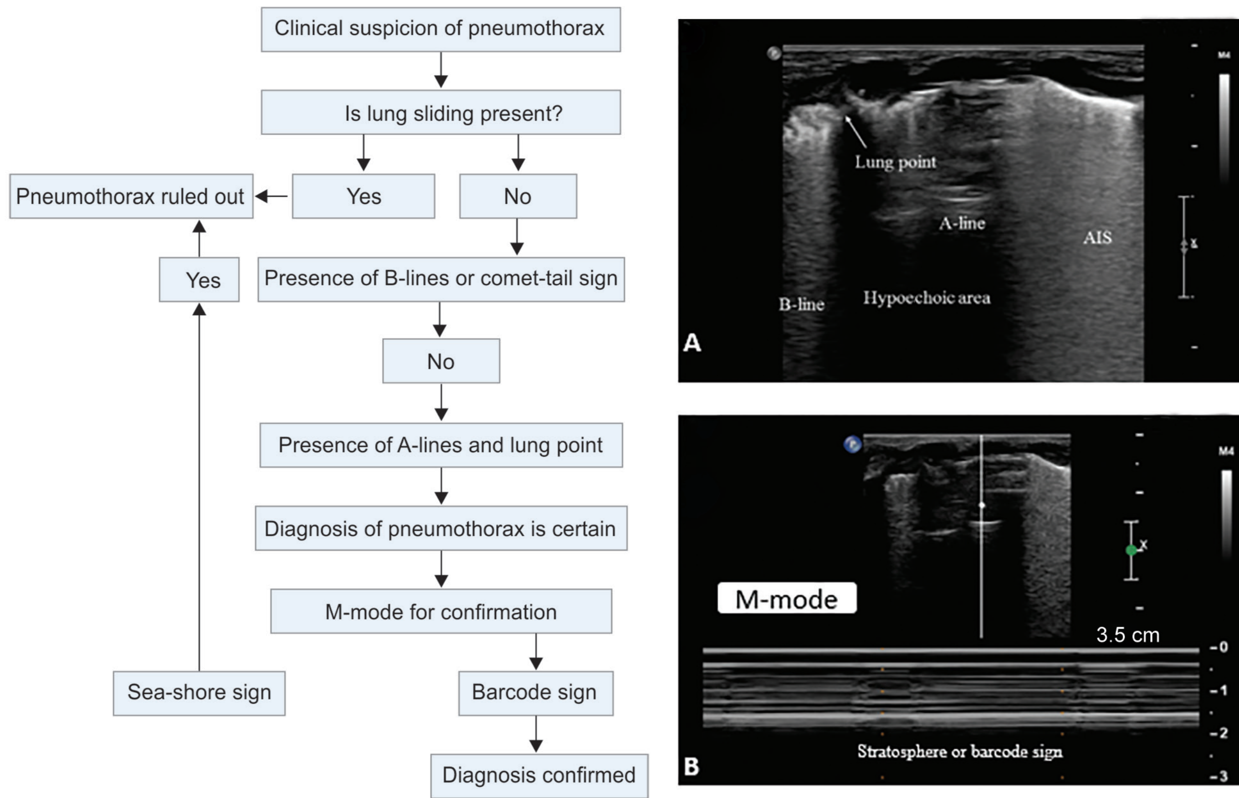
In infants with a pneumothorax, lung ultrasound can help determine the need for needle thoracostomy and chest tube drainage.[14,72,115] In 2019, a systematic review and meta-analysis showed that the overall specificity of lung ultrasound in diagnosing pneumothorax in neonates was 96.7% (95% confidence interval 88.3–99.6%).[14] Lung sliding is an important sign; its presence practically rules out pneumothorax. [116] Its absence has a sensitivity of 87.2% and a specificity of 99.4%. If lung sliding is absent, the comet tail sign can provide additional important information.[117] Comet tails are short-path vertical reverberation artifacts that fade rapidly and appear like a comet tail. The presence of the comet tail sign also rules out pneumothorax. These findings are shown in Figure 5. To summarize these findings, the absence of lung sliding, B-lines, and comet tail sign may be suggestive of a pneumothorax.[58] The lung point is the second most specific sign of pneumothorax; it is the transition point between the presence/absence of lung sliding and indicates the point of gas boundary in cases with mild-moderate pneumothorax (Fig. 7).

The systematic review of 2019 showed a specificity of 100% and sensitivity of 82% in diagnosing pneumothorax.[14] The lung point and presence of only A-lines with no lung sliding increase the specificity to 100% and sensitivity to 80%. Lung point denotes mild-to-moderate pneumothorax. However, the absence of lung point with absent lung sliding with A-lines and no comet tail signs or B-lines should be considered as a possible severe pneumothorax.[14]

The M-mode ultrasound can provide useful information in the diagnosis of pneumothorax.[58] In the normal lung, a series of high echodense wavy lines above the pleural line and uniform granular dot echoes below the pleural line together giving a beach-like appearance known as a sea-shore sign.[40,58] In cases with pneumothorax, the M-mode granular echo dots below the pleural line are replaced by a series of horizontal parallel lines.[58] These horizontal parallel lines are diagnostic of pneumothorax and this ultrasound sign is known as the barcode or stratosphere sign.[40,58,90,118] The diagnostic approach to evaluate for pneumothorax is outlined in Figure 7.

Evaluation of Chronic Lung Disease

Lung ultrasound is a promising new modality in the prediction of the risk and severity of chronic lung disease (CLD).[119] Sonography has been increasingly used in NICUs worldwide in diagnosing the need for ventilation, surfactant, and the differential diagnosis for



Figs 7A and B: Pneumothorax. Flow diagram showing a systematic approach for diagnosing pneumothorax. (A) A-lines with lung point, hypoechoic spare area, and absent lung sliding; (B) Classical barcode sign of pneumothorax on M-mode

Conventional Lung Ultrasound Scoring System

Scanning regions	A- lines	B-lines	Lung sliding	Consolidation	Pleural effusion	LUS score
Right Upper						
Right Lower						
Right Lateral						
Left Upper						
Left Lower						
Left Lateral						

Score 0	Score 1	Score 2	Score 3
• Only A-lines • Lung sliding present • No B-lines: Simple/compact consolidation; pleural effusion	• ≥ 3 well-spaced B-lines • A-lines present/absent • Lung sliding present • No consolidation/pleural effusion	• Severe B pattern: confluent/ compact B-lines • Subpleural consolidation <1 cm (not an essential criteria) • Minor consolidation/pleural effusion	• Extensive consolidation > 1 cm • With or without pleural effusion.

Fig. 8: Conventional lung ultrasound scoring system. Scoring system for respiratory distress in the upper anterior, lower anterior, and lateral regions on both sides. Each area can be scored from 0 to 3, as shown in the colored boxes below the scoring sheet. Maximum scores can be as high as 18

newborn respiratory distress.[51] Lung ultrasound imaging can be used as a scoring tool to predict the future risk of CLD.[120] A recent study used lung sonography at 1 week, 2 weeks, and 4 weeks after birth to predict CLD.[121] The study used the classical lung ultrasound score which included upper anterior, lower anterior, and lateral view on both sides of the ling field (Fig. 8).[37]

A lung ultrasound score of ≥ 5 at 1 week after birth had a sensitivity of 71% and specificity of 80% and the area under the

curve (AUC) was 0.8 on a receiver-operating curve. At 2 weeks, the sensitivity and specificity were 74% and 100% with an AUC of 0.93. The study concluded that very-low-birth-weight infants without CLD showed lower LUS scores beyond the first week after birth, whereas those at higher risk of CLD had persistently elevated LUS scores for 4 weeks and beyond.

A recent study in 2022 reported a new prediction method based on a modified lung ultrasound score.[122] The investigators modified the classical lung ultrasound score by including sagittal scans of the liver and spleen using a convex probe from the lower end of the ribs.[1,123–127] These datapoints were potentially useful because CLD affects the posterior and lower part of the lung more than the anterior region. The study showed that at 36 weeks post-menstrual age, this new model was a better predictor of moderate-to-severe CLD and oxygen dependence. In addition, the use of standardized lists for data collection was also useful.[103,128] Overall, lung sonography seems to be a promising modality for predicting CLD, which can help in early intervention with postnatal steroids in the second week of life to improve outcomes.[129,130]

Conclusion

This review sums up the potential importance of bedside lung ultrasound in diagnosis, assessment of the severity of illness, response to therapy, and prediction of outcomes of neonatal lung disease. Lung ultrasound can help in deciding the need for surfactant, in the differentiation of TTN vs RDS, confirming the presence of pneumothorax, and the assessment of the severity of atelectasis in neonates who are difficult to extubate. It also seems to be a promising modality for predicting CLD and deciding about the need for early intervention and modalities for follow-up. There is a need for the development of expertise and standardization of lung ultrasound in neonatal ICUs.

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REVIEW ARTICLE

Congenital Zika Virus Infections

Yahya Ethawi[1], Gangajal Kasniya[2], Nibras Al Baiti[3], Rehab Mohammed[4], FatimaElzahara Taha Mohammad[5], Roya Arif Huseynova[6]

Received on: 28 February 2023; *Accepted on:* 22 March 2023; *Published on:* 06 April 2023

Abstract

Zika virus (ZIKV) is an arthropod-borne flavivirus transmitted through bites of the *Aedes* mosquitoes. Infected mothers can vertically transmit ZIKV to their fetuses, particularly during the first and second trimesters. Infections beginning during early gestation can cause congenital Zika virus syndrome (CZS), which may be marked by arrested development and/or altered healing in the nervous system. There can be microcephaly, craniosynostosis, intracranial calcifications, ventriculomegaly, low brain volume and/or cortical atrophy, and hypoplasia/altered myelination in the corpus callosum, cerebellum, and brainstem. There may also be altered development with polymicrogyria, pachygyria, and lissencephaly. Clinically, infants with CZS may show facial dysmorphism, pulmonary hypoplasia, altered growth and development, hypertonia, hyperreflexia, limb contractures, and arthrogryposis multiplex. Perinatal infections can present with irritability, seizures, eye involvement, and sensorineural hearing loss (SNHL). Congenital zika virus syn and perinatal infections contrast with those acquired after birth, which usually have a relatively milder course. Overall, the mortality rate can reach 4–6%. Laboratory evaluation can include polymerase chain reactions on serum, cerebrospinal fluid, and urine; testing for immunoglobulin M (IgM); and plaque reduction neutralization tests (PRNTs) to confirm the specificity of these Zika virus IgM (ZIKV IgM) antibodies. Unfortunately, no specific treatment is available; most measures are largely supportive.

Keywords: Congenital Zika syndrome, Newborn, Real-time reverse transcription-polymerase chain reaction, Magnetic resonance imaging, Zika virus infection.

Newborn (2023): 10.5005/jp-journals-11002-0055

Introduction

Zika virus (ZIKV) was first isolated from a sentinel primate in Uganda in 1947.[1] It is a mosquito-borne virus named after the Zika Forest in Central Africa.[2,3] It circulated unnoticed in some regions in Africa and Southeast Asia until 2007, until an outbreak was recorded in the Yap Island in Micronesia.[4,5] The virus has since spread to parts of Central and South America and the Caribbean.[6–8] A major epidemic was seen in Brazil in 2015.[9,10] The incidence has gradually risen with new cases now having been reported from nearly 80 countries worldwide.[11–14]

The term congenital zika virus syndrome (CZS) has been used to describe the complicated clinical course seen in neonates born to mothers infected with ZIKV.[15–17] Several prospective cohort studies have shown that fetal ZIKV exposure *in utero* is associated with adverse birth outcomes and neurologic sequelae.[18–20] Unlike postnatal ZIKV infections after birth and in adults, congenital infections tend to be more severe and may be associated with neurological and multi-system complications.[13,21] In this article, we have focused on these vertically transmitted ZIKV infections.[22]

Zika Virus: Classification and Structure

Zika virus belongs to the Flaviviridae family of positive-strand RNA viruses that includes human pathogens such as the mosquito-transmitted dengue virus, West Nile virus, Japanese encephalitis virus, yellow fever virus, and the tick-borne encephalitic virus.[23–31] Flaviviruses are enveloped viruses containing an RNA genome of about 11 kilobase (kB).[32] There are multiple copies of a capsid protein, which is surrounded by an icosahedral shell consisting of 180 copies each of the envelope glycoprotein (about 500 amino acids) and a membrane protein (about 75 amino acids its precursor of about 165 amino acids); both are anchored in a lipid membrane.[32–36] There are seven non-structural proteins that are needed for replication, assembly, and for antagonizing the host innate immune responses.[37–40]

Flaviviruses evolve through three stages, including immature, mature, and fusogenic.[41,42] These are non-infectious, infectious, and host membrane–binding states, respectively.[39] The immature "spiky" immature particle is assembled in the ER and is non-infectious.[43] It matures through conformational changes of the surface

[1]Department of Neonatology, Saudi German Hospital Ajman (SGHA), Sharjah, United Arab Emirates

[2]Department of Neonatal–Perinatal Medicine, Cohen Children's Medical Center, New Hyde Park, New York, United States of America

[3]Department of Obstetrics, Saudi German Hospital at Sharjah (SGHS), Sharjah, United Arab Emirates

[4]Department of Pediatrics, Al Batool Teaching Hospital, Mosul, Iraq

[5]Department of Pediatrics, SGHA, Ajman, United Arab Emirates

[6]Neonatal Intensive Care Unit (NICU), King Saud Medical City, Riyadh, Saudi Arabia

Corresponding Author: Yahya Ethawi, Department of Neonatology, Saudi German Hospital Ajman (SGHA), Sharjah, United Arab Emirates, Phone: +971 505448203, e-mail: yahyaethawi@yahoo.com

How to cite this article: Ethawi Y, Kasniya G, Al Baiti N, *et al*. Congenital Zika Virus Infections. Newborn 2023;2(1):91–101.

Source of support: Nil

Conflict of interest: Dr. Yahya Ethawi is associated as the Editorial Board Member of this journal and this manuscript was subjected to this journal's standard review procedures, with this peer review handled independently of this Editorial Board Member and his research group.

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glycoproteins into a "smooth" particle in the low-pH environment of the trans-Golgi network.[44] The fusogenic stage is marked by an endosomal fusion loop seen in conditions with acidic pH.[45]

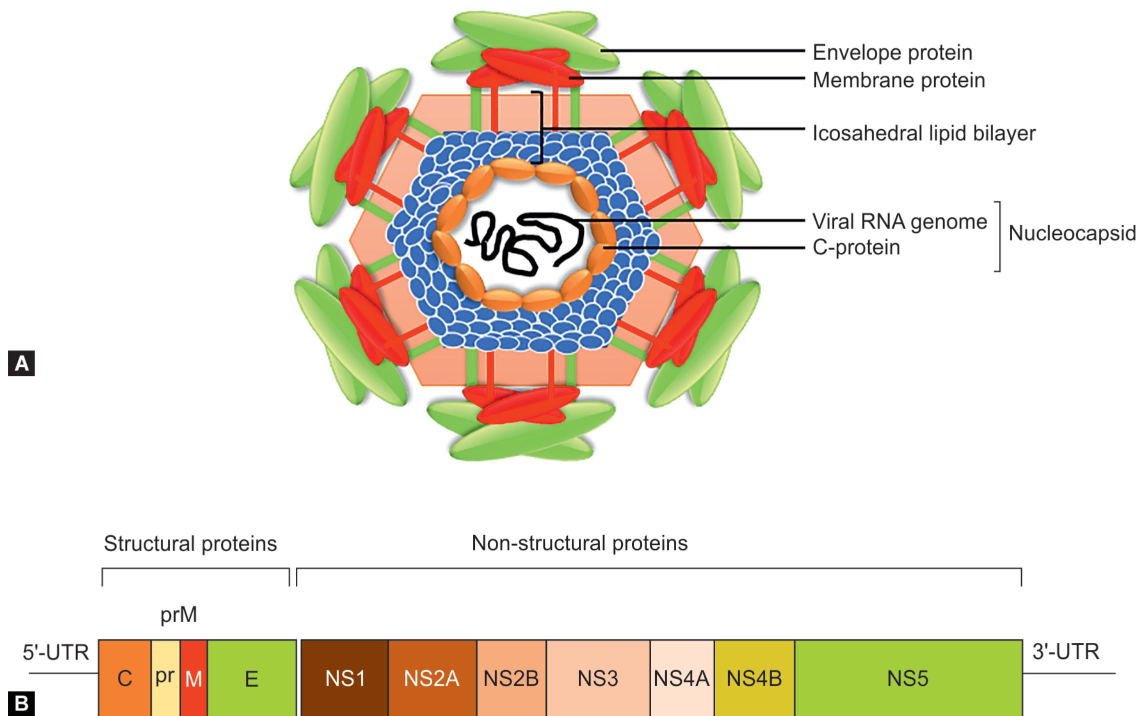
In this group of viruses, ZIKV specifically contains a typical flavivirus genome that is 10.8 kB long (Fig. 1).[44] The RNA is translated into a single polyprotein (3,423 amino acids) that is processed into the 3 above-mentioned structural proteins.[46] The capsid contains four α helices with a long pre-α1 loop and forms dimers; the pre-α1 loop contributes to the tighter association of dimeric assembly.[35,36,47,48] The membrane protein contains two loops that anchor it to the membrane.[43] Finally, the envelope protein is comprised of four domains; the stem-transmembrane domain anchors the protein into the membrane.[39] The seven non-structural proteins are labeled NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Interestingly, some of these proteins regulate viral replication.[49] The structural proteins form the virus particle, whereas the non-structural proteins assist in the replication and packaging of the genome.[50] The generation of the 10 individual proteins from the polyprotein is regulated by viral and host proteases, and the efficiency of furin, a host protease that cleaves the viral targets.[51,52]

Epidemiology

ZIKV is transmitted to humans primarily through the bites of infected *Aedes* mosquitoes, particularly those of the species *Ae. aegypti* and *Ae. albopictus*.[53] These mosquitoes live near human habitations and frequently get infected with viruses such as Zika, chikungunya, and dengue after biting infected persons who are viremic such as during the first week of infection.[54] These mosquitoes lay eggs in standing water such as near the edges of lakes and ponds, in plants in swamps and marshes, or in containers that hold water such as buckets, bowls, and animal dishes.[55] These mosquitoes bite humans and can transfer the viruses to other hosts.[56]

A pregnant woman can pass ZIKV to her fetus during pregnancy or the perinatal period.[57] Zika virus has also been found in mother's own milk, although viral transmission through breast milk has not been confirmed yet.[58] Flavivirus nucleic acid has been detected in breast milk.[59] However, we do not know the long-term effects of postnatal ZIKV transmission.[60] The benefits of breastfeeding may outweigh the risk of transmission through breast milk, and the Centers for Disease Control and Prevention (CDC) continue to encourage mothers to breastfeed even if they lived/traveled to endemic areas or were infected with ZIKV.[61]

Zika virus can be sexually transmitted from an infected person to his or her partners.[62] Many individuals with minimal symptoms can be infectious; studies suggest that ZIKV can be passed from an infected persons before the onset of symptoms, during acute illness, or after apparent clinical recovery.[63] Studies are on to determine



Figs 1A and B: (A) Schematic illustration of ZIKV: Zika virions are enveloped, 18–45 nanometer icosahedral structures. The genome is a positive strand RNA enclosed in a capsid and surrounded by a membrane. The RNA contains 10,794 nucleotides encoding 3,419 amino acids. (B) The ZIKV genome. The 10.8 kB long genome is translated into a single polyprotein, which is then processed into a capsid protein (C), an envelope glycoprotein (E) and membrane protein (proM, processed to M), and 7 non-structural proteins that are labeled NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. UTR = Untranslated region; prM = uncleaved pro-membrane protein; C = capsule; E = envelope; NS = non-structural protein. The mature ZIKV particle is about 50-nm in diameter. It has 180 copies of the E and M proteins embedded in the membrane. The E protein is comprised of four domains: A stem-transmembrane domain pair and three ectodomains I, II, and III seen outside of the membrane. The E protein exists as a dimer and predominates on the surface of the virion with the smaller M protein residing underneath. The M protein has a smaller extracellular region and a stem-transmembrane domain. Three E proteins dimers lie parallel to one another in a raft configuration, with the virion having a total of 30 rafts. The non-structural proteins assist in replication and packaging of the genome.

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the duration for which ZIKV remains detectable in semen and vaginal fluid of infected individuals, and their infectivity.[64] The virus may remain detectable in semen longer than in other body fluids such as vaginal fluids, urine, blood, conjunctival fluid, and amniotic fluid.[65,66]

Reports from Brazil and other countries have documented the presence of ZIKV in blood donated for transfusions.[67] During the French Polynesian outbreak, 2.8% of blood donors tested positive for ZIKV.[68] There are some reports of laboratory-acquired ZV infections, although the route of transmission could not always be established.[69] There is a need to investigate these reports because ZIKV diagnostic testing and laboratory research have expanded with increased risk of occupational exposure to laboratory workers and biomedical researchers.[70] The emergency committee of the World Health Organization (WHO) announced ZIKV disease as a Public Health Emergency of International Concern in 2016 and triggered the exploration of global involvement to define the pathophysiology and deal with the related clinical challenges.[71]

Pathophysiology

The mechanisms of the ZIKV passage across the placental barrier, the association between viremia and the development of CZS, and the exact timing of placental and fetal infection with maternal viremia are still not clear.[72] ZIKV can infect placental macrophages, trophoblasts, and endothelial cells, and then enter the fetus from these cells.[73] In infected fetuses, ZIKV has been isolated from the brain and cerebrospinal fluid.[21] However, the impact of placental infection in defining the syndrome's severity has not been confirmed yet.[74]

ZIKV infections of the fetal brain can damage the neuronal progenitor cells and interrupt neuronal proliferation, migration, and differentiation.[75] These events may slow or interrupt brain growth beginning at 20 weeks of gestation.[76,77] The risk of neurodevelopmental abnormalities in infected fetuses is the highest when maternal infection appears during the first and second trimesters of pregnancy because it is a crucial period for brain development.[78] Interestingly, some neonates who were exposed to ZIKV *in utero* did not show obvious abnormalities at birth but developed impairments over time.[79,80] In these infants, ZV replication may have continued after birth and interrupted brain growth.[79] Clearly, ZIKV-exposed fetuses need continued, comprehensive follow-up after birth.[81]

Histopathology

ZIKV is a neurotropic virus that specifically attacks neural progenitor cells.[82,83] Electron micrographs show ZIKV as dense particles in the damaged endoplasmic reticulum (ER) in these cells. This ER stress/unfolded protein response not only suppresses the proliferation of cortical progenitor cells but also damages mature neurons in the cerebral cortex.[83,84] Specific groups of enveloped structures with a bright interior resembling the residue of replication complex also support ZIKV replication in the neonatal brain.[39]

Zika infections in the developing brain may manifest with diffuse arachnoiditis with ependymitis and vasculitis.[85] Some foci show meningoencephalitis, ventriculomegaly or an *ex vacuo* hydrocephalus, microcephaly with lissencephaly, and cerebellar hypoplasia.[86] An additional spectrum of parenchymal lesions was observed involving the whole hemispheric wall namely the cortical plate (CP), the intermediate, and the ventricular zones. The CP lesions consisted in a loss of lamination with radial glia disruption, focal polymicrogyria, neuronal loss, chromatin fragmentation with numerous apoptotic residues and mineralization.[86] The loss of lamination can disrupt radial glia and cause a diffuse loss of neurons.

Necrotic lesions can be seen in the subcortical region in the vicinity of damaged vessels.[86] The loss of cortical neurons has been linked with ZIKV-associated microcephaly.[87] Several neurobiological studies have shown increased cell death and the impaired cell cycle leading to a decreased neural progenitor cell proliferation, causing a decrease in the number of cortical neurons.[88] In addition to ER stress, ZIKV infection can cause chromatin change and necroptosis.[89] Viral particles have been observed in basal/apical progenitor cells, neurons in the cortical plate, and in the ventricular and subventricular zones.[90] The loss of callosal fibers and longitudinal tracts has been identified as a cause of the cerebral atrophy and the ventricular enlargement.[91] The disruption of the hypothalamic and pituitary axis can cause adrenal gland atrophy.[92]

Immunohistochemical studies may show T-lymphocytic and histiocytic meningitis with abundant cerebral astroglial and macrophagic reactions.[85] Vasculitis is marked by the presence of swollen endothelial cells surrounded by active microglia and astrocytes.[93,94] In the cerebellum, the width of the external and the internal granular layers was reduced.[85] The neurons were shrunken and contained fragmented chromatin (karyorrhexis).[95,96] Macrophages and numerous hypertrophic astrocytes were present.[96] In the spinal cord, the astrocytic and macrophagic reaction was mild and neurons were spared.[83] The longitudinal tracts were missing. Glial fibrillary acidic protein-reactive antibody confirmed the astroglial nature of the gliosis seen close to the necrotic regions in the subventricular and in the intermediate zones.[21]

In situ hybridization shows ZIKV particles within the cerebral parenchyma mainly in the ventricular/subventricular zone and in the cortical plate.[77] The neuronal precursor cell is the main target for ZIKV leading to cell death, although, neuronal cells in all stages of maturity can be affected.[82] These changes can explain the microcephaly and poor cortical gyration.[97] Moreover, viral cerebritis can affect cerebral embryogenesis and result in microcephaly or other central nervous system abnormalities.[85,98]

There may be inflammatory changes in other organs. The placenta may contain a Hofbauer cells hyperplasia with signs of inflammation.[99] Truncal vessels may show fibromuscular hypertrophy causing a narrowing of the lumen.[85] Some cases may show features of acute chorioamnionitis, villitis, and funisitis.[100] Some studies have shown an interstitial lymphocytic infiltrate in the testes.[101]

Clinical Manifestations

The full CZS spectrum is evolving with the recognition of the following subtle manifestations in growing infants:

- Fetal growth restriction.[102–107]
- Congenital anomalies in 7–40% of infants.[108–111] Central nervous system findings include large ventricles, microcephaly, and intracranial calcifications.[112] Some infants show craniosynostosis, low brain volume and/or cortical atrophy (Fig. 2), and hypoplasia/altered myelination in the corpus callosum, cerebellum, and brainstem. There may also be altered structural development with polymicrogyria, pachygyria, and lissencephaly. Clinically, infants with CZS may show facial dysmorphism, pulmonary hypoplasia, altered growth and development, hypertonia, hyperreflexia, limb contractures, and arthrogryposis multiplex.

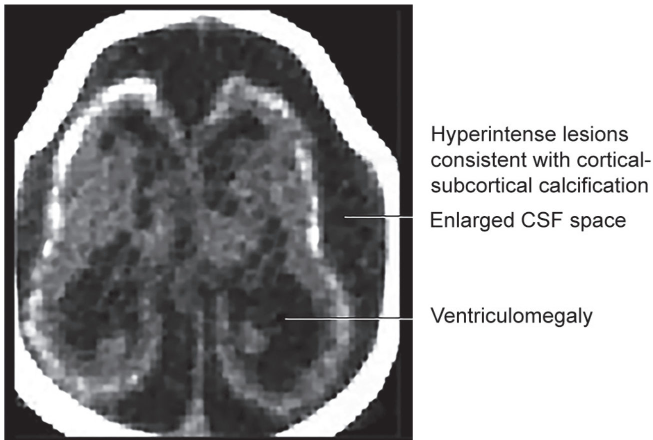

Fig. 2: Axial CT image of an infant with congenital ZIKV infection and severe microcephaly shows cerebral atrophy with ventriculomegaly, prominent cerebrospinal fluid space, and extensive, punctate cortico–subcortical calcifications.

- Perinatal infections can present with irritability, seizures, eye involvement, and sensorineural hearing loss (SNHL). Congenital Zika syndrome and perinatal infections contrast with those acquired after birth, which usually have a relatively milder course.
- Fetal/perinatal death.[113]

There are five signs that have recorded frequently in infants with CZS as follows:

- Decreased brain tissue with subcortical calcifications.
- Microcephaly.
- Hypertonia with limitations of body movement seen shortly after birth.
- Congenital joint contractures such as arthrogryposis and clubfoot.
- Eye lesions, such as focal retinal pigmentary mottling and macular scarring.

The following findings are relatively more specific to CZS:[114] (a) Partially collapsed skull with severe microcephaly, (b) subcortical calcifications with thin cerebral cortices, (c) focal pigmentary retinal mottling with macular scarring, (d) congenital contractures and arthrogryposis, and (e) severe early hypertonia.[115]

Microcephaly

The incidence of microcephaly has varied across studies. In some small cohorts, up to 90% of cases of CZV had microcephaly, and most cases have severe congenital microcephaly.[98,116] Other studies have shown lower incidence figures, with only 5–9% of infants with CZS having a small head circumference.[117] In a large cohort, Cauchemez et al.[103] estimated the frequency of microcephaly to be about 95 per 10,000 women infected during the first trimester. Severe microcephaly has been noted in 7–9% of all infants with CZS.[100,108,113,118–123] About 10% had moderate microcephaly.[106]

Microcephaly has been traditionally defined as an occipital head circumference (OHC; measured between occipital protuberance and glabella) that is 2 standard deviations (SDs) less than the average for gestational age (GA) or corrected GA. Severe microcephaly is defined as an OHC below 3 SDs.[124] It can be a primary abnormality seen at birth or a secondary failure of head growth that develops over time.[121,125] Proportionate microcephaly is a restriction of head circumference similar to that of length and weight. Infants with disproportionate microcephaly have a restricted head circumference but a normal weight and length.[126]

Infants with CZS frequently show disproportionate craniofacial dimensions where the face appears larger compared to a small head.[127] Up to 78% of infants with CZV infections develop craniosynostosis;[107,114] many show *cutis gyrata* where the continuously growing redundant scalp tissue begins to show folds over the cranium that is not growing any further.[115] A CZS-associated microcephaly may reflect a less-than-normal number of gray matter neurons with reduced brain volume. Microcephaly is usually seen when ZV infections occur early in pregnancy; however, proportionate microcephaly has been observed in the offspring of women infected as late as the third trimester of pregnancy.[128,129] In rare instances, microcephaly has been noted to resolve over time.[129]

Infants with CZV-related microcephaly frequently have seizures, cerebral palsy, and neurodevelopmental abnormalities. Many infants have abnormal facies, thin cerebral cortex on cranial imaging, macular scarring, focal pigmentary retinal changes, SNHL, irritability, hypertonia, hyperreflexia, and congenital contractures and talipes equinovarus due to decreased movements *in utero*.[97,130] In one cohort, 6% of infants had congenital anomalies, and 9% had neurodevelopmental abnormalities such as developmental delay, hearing loss, and seizures.[103] Neuroimaging showed major structural lesions in 42% and minor abnormalities in 24%. The physical (neurological) examination was abnormal in 21%. Nine percent were small-for-gestational age (SGA). Eye abnormalities were recorded in 7%, dysphagia in 3%, hearing defects in 3%, clinically evident or subclinical seizures (abnormal electroencephalogram) in 3%, and minor abnormalities in 10%.[100]

Ocular Manifestations

About 25% of infants with CZS showed eye abnormalities, which was considerably higher than the 6–7% incidence in the general population.[131,132] These findings included macular abnormalities; focal pigmentary retinal changes; chorioretinal atrophy, and optic nerve abnormalities such as optic nerve hypoplasia, increased cup-disk ratio, and pallor.[133–136] Other changes included pigmentary clumping, coloboma, subretinal hemorrhages, vascular tortuosity, and abnormal retinal vessels with focal vascular dilation.[137–140] Iris colobomas, microcornea, microphthalmia, lens subluxation, cataracts, intraocular calcification, congenital glaucoma, strabismus, and nystagmus were also seen in some infants.[141–145] The eye findings in CZS were not progressive.[132] Cortical visual impairment was the most likely cause of the loss of vision in infants with CZS.[146,147] Major visual impairment in CZS was seen in 30%. However, the rate of visual impairment was as high as 84% when the associated eye findings were included.[148]

Other Abnormalities

Sensorineural hearing loss is seen in 7–12%.[104] Arthrogryposis and club foot have been reported and are likely neurogenic in origin due to fixed posture *in utero*.[149] Other clinical signs of CZS include hypertonia, hyperreflexia, irritability, feeding difficulties, and dysphagia.[150] Seizures may occur due to underlying brain malformations, but may also be present in children without apparent CNS abnormalities with a median age of onset of a seizure is 4 months.[106,151] The seizures are usually refractory with poor initial control with medical therapy. Notably, 30–40% of infants with CZS are SGA.[107,130] Congenital heart disease (CHD) occurs in 10–15% and is mostly non-severe, such as secundum atrial septal defect (ASD), patent ductus arteriosus (PDA), and small

muscular or peri-membranous ventricular septal defect (VSD) and few had hemodynamically significant CHD defect such as large membranous VSD.[152,153]

Perinatal Infections

Infants infected around the time of birth develop acute encephalopathy and can present with irritability, seizures, eye involvement, and SNHL.

Postnatal Infections

Most patients remain asymptomatic. A small minority develops a mild course of fever, rash, and conjunctivitis.[58,154,155]

Neuroimaging

Imaging can detect neurological abnormalities such as intracranial calcifications, ventriculomegaly, low brain volume, delayed myelination, polymicrogyria, pachygyria, lissencephaly, corpus callosum, brainstem, cerebellar thinning or hypoplasia, large cisterna magna, and increased extra-axial fluid spaces.[156,157]

Intracranial calcifications due to ZIKV are seen the most frequently at the junction of the cortical and subcortical white matter. Notably, these lesions differ from the punctate lesions caused by cytomegalovirus. However, calcification may occur in the periventricular region, basal ganglia, thalamus, brainstem, and cerebellum.[158] These calcifications may diminish in number, size, or density with age in most children,[159] although these changes do not correlate with clinical improvement as most patients; these patients may still develop severe neurological sequelae. Notably, 40% of infants with hydrocephalus may require a ventriculoperitoneal shunt. Cranial ultrasound is an important screening tool but it often needs to be followed up with magnetic resonance imaging (MRI) for detailed evaluation. CT scans can detect intracranial calcifications while MRI is better for structural brain disease. A negative sonographic examination in infants who have seizures, microcephaly, and tone abnormalities should be followed by a more extensive neurological evaluation by specialists and a specific imaging evaluation.

Evaluation

A detailed evaluation as detailed in the following list is needed for infants with ZIKV infections confirmed by maternal laboratory test and clinical evidence of CZI such as microcephaly and/or other congenital anomalies:[160]

- Physical examination including anthropometric measurements (head circumference, length, and weight), neurologic abnormalities, and dysmorphic findings assessment.
- Laboratory testing, including complete blood counts, and a metabolic panel with liver function tests.
- Head ultrasound.
- Hearing test using auditory brainstem response to assess hearing.
- Eye examination by an experienced ophthalmologist before or shortly after discharge from the hospital.
- Other specialties consultation (a) neurologist; (b) infectious disease specialist; (c) clinical geneticist; (d) early intervention and developmental specialists; and (v) family and supportive services.
- Other optional consultations (a) orthopedist, (b) physiatrist, (c) physical and/or occupational therapists, (d) lactation specialist, (e) nutritionist; (f) gastroenterologist; (g) speech or occupational therapist; (h) endocrinologist for evaluation; (i) pulmonologist; (j) otolaryngologist; and (k) cardiologist.

The WHO and CDC define microcephaly as occipitofrontal circumference (OFC) above 2 SDs below the mean or below the third percentile for gender, age, and GA at birth.[124,161,162] Severe microcephaly is a HC below 3 SDs below the mean.[161] Both CDC and WHO recommend detailed clinical assessments before making a diagnosis of microcephaly to decide the plans for follow-up.[124]

Laboratory Evaluation

The following infants should be tested:[160]

- The mother has evidence of ZIKV infections during pregnancy.
- There are clinical or neuroimaging findings suggestive of CZS with maternal or paternal possible exposure, regardless of maternal ZIKV laboratory status.

The postnatal laboratory tests include the following:[160,163]

- Serum and or urine for ZIKV RNA using real-time reverse transcriptase-polymerase chain reaction (rRT-PCR).
- Serum Zika virus immunoglobulin M (IgM) using enzyme-linked immunosorbent assay (ELISA).
- Cerebrospinal fluid (CSF; if available) for ZV RNA by rRT-PCR and ZIKV IgM.[160,163] Early samples can distinguish between congenital, perinatal, and postnatal infection. Cord blood should not be used as it may yield false-positive results.[160]
- Plaque reduction neutralization test (PRNT) detects specific neutralizing antibodies of the Zika and dengue viruses which is not available for routine use. It can confirm the specificity of IgM antibodies against the ZV, which can rule out a false-positive IgM test. For a positive or presumptive positive or possible positive or equivocal result without PRNT of the mother's sample, a ZV PRNT on the infant's initial sample is of great help. If the neonatal PRNT is initially positive, a repeat PRNT test should be done after the age of 18 months to differentiate true initial fetal infection from maternal passive transfer of antibodies, at which time maternally acquired antibodies will have waned.

Maternal serum should be checked for ZIKV IgM and its neutralizing antibodies. To distinguish from other arboviruses, the infants should be tested for dengue virus IgM and its neutralizing antibodies. The interpretation of these results is complex because of the cross-reaction between Zika and dengue antibodies. Neutralization assays can confirm or exclude the result. Histopathologic assessment of the placenta and umbilical cord can add more information.

Interpretation and Diagnosis

Confirmed diagnosis: In the first few days of life, ZIKV RNA present in the serum, urine, or CSF are collected, regardless of IgM antibodies being positive or negative.[160]

Probable diagnosis: A negative PCR while IgM against ZIKV is positive which indicates probable ZIKV infection. The IgM result may be false-positive due to cross-reacting IgM antibodies or may be a result of a non-specific reactivity.[164] Mother testing results are very important in this regard. Therefore, a positive IgM in the infant makes congenital ZIKV infections very likely if the maternal ZVI is confirmed. While the presence of CSF IgM is very suggestive of congenital ZIKV infections.[164]

Diagnosis unlikely: The congenital infection is unlikely if both PCR and IgM are negative.[160] A negative PCR result alone cannot rule out

congenital infection transient viremia as it is not known the period of postnatal viral shedding of *in utero* infected newborns. Some authors suggest the viremic period can reach up to 67 days after birth.[165] There is a need for evidence to definitively excluded CZV infection based on negative rRT-PCR and IgM, in infants with known ZV exposure. A negative newborn PCR test may be due to the absence of virus shedding in the urine despite confirmed maternal infection exposure. Moreover, a negative IgM test may be due to delay IgM antibodies release as in congenital rubella and CMV infection.

Differential Diagnosis

Infants suspected to have ZIKV infections should be evaluated for rubella, cytomegalovirus, and toxoplasmosis. Infections other than ZIKV infections frequently show hepatosplenomegaly, thrombocytopenia, and skin lesions.[10,81,128] A detailed evaluation of other causes of microcephaly is also required.

Management

The management is supportive as there is no specific antiviral treatment for CZS. The supportive care needs to focus on (a) seizures; (b) feeding difficulties; (c) hypertonia; and (d) hearing loss.

Parents should be provided with key sets of information. Maternal transmission of ZIKV to the fetus may occur during labor and delivery. There are reports of two cases of intrapartum ZIKV transmission from mothers infected within 2–3 days of delivery to the infant. However, these infants were not symptomatic, while the others showed thrombocytopenia and a widespread rash.[58,166,167] ZIKV has been detected in breast milk, but there is no documented evidence of transmission in breast milk.[154]

Testing both the mother and the baby is indicated during the first 14 days after birth if the mother is exposed to ZIKV within 14 days of delivery with ≥2 of the following; (a) rash, (b) conjunctivitis, (c) arthralgia, (d) fever.[166] If either or both newborn's or mother's symptoms developed within the first week of birth, both newborn serum and urine ZIKV using real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) should be obtained. However, if available, urine from both the mother and newborn should be obtained in the 2nd week and should be evaluated by Zika rRT-PCR. A positive laboratory test confirms the diagnosis.

If the rRT-PCR is negative 3 days after maternal symptoms, test for ZIKV IgM and neutralizing antibody titers. A positive test is suggestive of the diagnosis. Maternal ZIKV IgM and neutralizing antibody titers should be assessed if the newborn is symptomatic, and the mother is asymptomatic. Possible ZIKV exposure is not an indication of lumber puncture, but if the CSF is available for other reasons, a testing for ZIKV RNA using rRT-PCR is appropriate action.[166]

Follow-up

- The general pediatrics services should focus on (a) monitoring growth parameters such as weight, length/height, and HC; (b) routine immunizations; (c) anticipatory guidance; (d) psychosocial support; (e) other necessary testing services; and (f) consultations with other specialist services as needed.[160]
- Follow-up with experts in (a) hearing and vision; (b) neurology, focusing on seizures, tone abnormalities, and *ex vacuo* hydrocephalus; (c) developmental services; (d) feeding difficulties, breathing difficulties, choking, or coughing with feeding and assessment for dysphagia; (e) nutrition; and (f) continued supportive services and palliative care.

Prognosis

The prognosis of newborns with CZI is uncertain. The reported mortality rate among live-born infants with confirmed and probable CZI in Brazil is 4–6%.[150]

The prognosis of severe CZS with microcephaly and severe other cerebral abnormalities is very poor. However, the prognosis of milder forms is not known.[112] Nearly 1/3 of the children are either below average developmental scores or have neurosensory abnormalities such as abnormal eye examinations and/or hearing assessments during the second and third years of life.[129] Approximately 29% scored below average in a minimum of one developmental assessment, especially language assessments and 2% of children may be in the autism spectrum disorder during the second year of life.

The presence or absence of structural and functional neurologic abnormalities at birth may not predict later neurodevelopmental outcomes.[129,133] Approximately ½ of abnormal neurologic examination or abnormal neuroimaging findings at birth may develop normally in the follow-up assessments in their second or third years of life. About 25% of patients who appeared asymptomatic at birth may have delayed neurodevelopmental outcomes with or without abnormal hearing or ophthalmologic outcomes on follow-up.

Prevention

Protection against Zika virus infections during pregnancy:

- Avoid travel to areas with mosquito transmission of ZV.[168–176]
- Protection against mosquito bites.[177,178]
- Protection against sexual transmission for a partner who traveled to or lives in an area with a risk of ZV.
- Adherence to standard infection-prevention precautions.

Guidance for couples planning pregnancy:

- Reproductive-age couples in the affected areas should know the risks of transmission of ZV, the consequences of ZVI during pregnancy, and they should consider the possibility of delaying pregnancy.[171,174]
- Partners planning to conceive better to avoid or may postpone travel to areas where mosquito transmission of ZVI is likely unless the travel is very essential.[179]
- Wait for a minimum of 3 months after a potential exposure prior to a trial of conception with the use of abstinence or condoms during this period.[180]
- Those with infertility treatment who require to use of donor sperm or donor egg should only obtain these gametes from laboratories following FDA recommendation for screening guidelines and avoid donors traveling to risky places within 6 months of donation.[180] If they are using their own gametes same testing and timing recommendations of the FDA should be followed.[113]

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REVIEW ARTICLE

Pathophysiology of Enteropathogenic *Escherichia coli*-induced Diarrhea

Prabhdeep Kaur[1], Pradeep K Dudeja[2]

Received on: 04 March 2023; *Accepted on:* 25 March 2023; *Published on:* 06 April 2023

Abstract

Enteropathogenic *Escherichia coli* (EPEC) are important diarrheal pathogens of infants and young children. Since the availability of molecular diagnosis methods, we now have new insights into the incidence and prevalence of these infections. Recent epidemiological studies indicate that atypical EPEC (aEPEC) are seen more frequently than typical EPEC (tEPEC) worldwide, including in both endemic diarrhea and diarrhea outbreaks. Therefore, it is important to further characterize the pathogenicity of these emerging strains. The virulence mechanisms and pathophysiology of the attaching and effacing lesion (A/E) and the type-three-secretion-system (T3SS) are complex but well-studied. A/E strains use their pool of locus of enterocyte effacement (LEE)-encoded and non-LEE-encoded effector proteins to subvert and modulate cellular and barrier properties of the host. However, the exact mechanisms of diarrhea in EPEC infection are not completely understood. From the clinical perspective, there is a need for fast, easy, and inexpensive diagnostic methods to define optimal treatment and prevention for children in endemic areas. In this article, we present a review of the classification of EPEC, epidemiology, pathogenesis of the disease caused by these bacteria, determinants of virulence, alterations in signaling, determinants of colonization vs. those of disease, and the limited information we have on the pathophysiology of EPEC-induced diarrhea. This article combines peer-reviewed evidence from our own studies and the results of an extensive literature search in the databases PubMed, EMBASE, and Scopus.

Keywords: Attaching and effacing lesion (A/E), Epidemiology, Ion transporters, LEE pathogenicity island, Type III secretion system (T3SS), Tight junctions.

Newborn (2023): 10.5005/jp-journals-11002-0056

Key Points

- Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea worldwide and particularly in developing countries.
- Global prevalence of atypical EPEC (aEPEC) is higher than typical EPEC (tEPEC).
- Enteropathogenic *E. coli* strains adhere to intestinal epithelial cells (IECs) in two patterns; the first one is of localized adherence (LA), where bacteria adhere in discrete microcolonies, and the second one is of diffuse adherence in which bacteria adhere uniformly over the cell surface.
- Enteropathogenic *E. coli* employs its type III secretion system and effector proteins to modulate cellular and barrier properties of the host intestinal milieu.
- Enteropathogenic *E. coli* infection leads to extensive disruption of microvilli on IECs and consequent loss of absorptive surfaces and altered electrolyte transport that may be secondary to both altered expression of ion/solute transporters and the loss of mucosal surface area.

[1]Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois at Chicago, Illinois, United States of America

[2]Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois; Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois, United States of America

Corresponding Author: Pradeep K Dudeja, Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois; Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois, USA, Phone: +(312) 996-6651, e-mail: pkdudeja@uic.edu

How to cite this article: Kaur P, Dudeja PK. Pathophysiology of Enteropathogenic *Escherichia coli*-induced Diarrhea. Newborn 2023;2(1):102–113.

Source of support: Supported by work done as part of the NIH projects DK054016, DK92441, the Veterans Affairs Senior Research Career Scientist Award 1IK6BX005242, VA-BCCMA award IO1BX005862 and the VA Merit Award BX002011 (to Pradeep K. Dudeja).

Conflict of interest: None

Introduction

Escherichia coli (*E. coli*) is the predominant facultative anaerobic species in the intestine. Most strains are non-pathogenic and play an important role in maintaining intestinal physiology.[1,2] This organism was first described by German pediatrician Theobald Escherich in 1885, under the name "*Bacterium coli commune*" as a short rod that had initially been isolated from normal infant feces.[3] The current classification systems of *E. coli* consider many strains (Flowchart 1).[4]

Enteropathogenic *E. coli* (EPEC) is a major cause of infantile diarrhea in developing countries.[5] EPEC strains were epidemiologically associated with outbreaks in 1940s and 1950s and were first described in 1955.[6] These strains currently account for 1.3 million deaths every year.[7] The incidence is now being noted more accurately since the development of molecular diagnostic methods. In this study, we have reviewed the epidemiology of EPEC infections in infants and children and our studies in animal models to understand the pathophysiology of EPEC-associated diarrhea.

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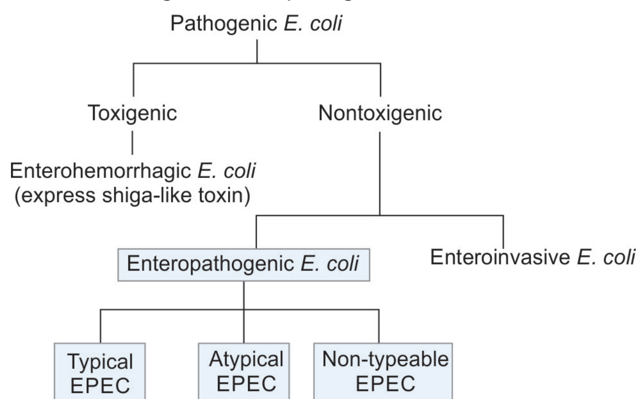
Flowchart 1: Categorization of pathogenic *E. coli*



Epidemiology of EPEC Infections

Although most strains of *E. coli* are avirulent commensals in the gastrointestinal tract, many can cause diarrhea, urinary tract infection, and sepsis/meningitis. Several *E. coli* pathogens have been implicated in public health problems worldwide.[8] The incidence of EPEC-related disease seems to have decreased over the last several decades. It is unclear if this reduced incidence is due to interventions such as the promotion of breastfeeding, or whether earlier studies based on O:H-serotyping overestimated the relative contribution of these organisms compared to newer molecular methods and/or adherence assays.[7]

Enteropathogenic *E. coli* was the first strain of *E. coli* identified as the cause of infantile diarrhea in the 1940s and 1950s. These outbreaks of "summer diarrhea" were frequent in developed countries until the 1950s and had high mortality.[6] EPEC strains were first shown to be pathogenic in human volunteer studies carried out by Levine et al.[9] in 1978. They tested classic EPEC strains (O127 and O142) associated with infant diarrhea that had been stored for 7–9 years. These isolated strains did not express LT and ST enterotoxins or show invasiveness. Enteral administration to healthy young adult volunteers caused a notable diarrheal illness.

In a systematic review of 266 studies published between 1990 and 2002, EPEC was identified with a median prevalence of 8.8% (inter-quartile range, IQR of 6.6–13.2) in the community setting, 9.1% (IQR 4.5–19.4) in the outpatient setting, and 15.6% (IQR 8.3–27.5) in the inpatient setting. Enteropathogenic *E. coli* may be the second most frequently seen cause of diarrhea after rotavirus (25.4%) in the inpatient setting. However, there are important regional and temporal variations.[10] Investigators from Peru combined data on six different diarrheagenic strains of *E. coli* from eight different studies of children <3 years of age. Multiplex real-time PCR showed that the average EPEC prevalence in diarrheal stool samples ($n = 4{,}243$) was 8.5% (95% CI: 7.6–9.3), second only to enteroaggregative *E. coli* (EAEC; 9.9%). Enteropathogenic *E. coli* prevalence increased with age; these strains were found in 3% of diarrheal samples in children <6 months, in 11% of children 6–12 months, and in 16% of children 13–24 months. In these cohorts, exclusive breastfeeding was more frequent than in other studies (>80% for infants younger than 6 months), and hence young infants may have been protected from symptomatic infection. Among asymptomatic controls ($n = 3{,}760$), EPEC was detected in 10.9% (95% CI: 9.4–11.4).[11–13] Similarly, in a recent study in India, EPECs were identified in 3.2% of 648 children <5 years of age who were hospitalized for diarrhea.[14] In another study, EPEC has been noted to be the most prevalent pathotype with an average prevalence of 10.9% (95% CI: 9.4–11.4), followed by EAEC (10.4%).[7] A study reported that more than 20% of all episodes of persistent diarrhea in the pediatric population; aged >14 days are mainly caused by diarrheagenic *E. coli* such as aEPEC. Another study identified specific *E. coli* strains from patients of infantile gastroenteritis. This study reported that serogroups O111 and/or O55 were more putative in causing diarrhea in recipients, and disease outcome in terms of severity of symptoms was largely dependent on the size of the dose.[15–17]

Enteropathogenic *E. coli* is known to be an important cause of infantile diarrhea in Brazil, Chile, Peru, and Iran.[18] Studies in Brazil, Mexico, South Africa, and Bangladesh have shown that EPEC infections cause 30–40% of infant diarrhea with high mortality rates.[19–23] In several studies conducted in Latin America, tEPEC was found to be the main cause of endemic diarrhea in children < 1 year of age. The frequency of tEPEC infection drops with an increase in age group, and adults rarely experience tEPEC episodes.[18] This may be due to development of immunity or the loss of receptors interacting with some specific adhesins. Although tEPEC were major agents of acute diarrhea in infants until the 1990s, a clear decline in many of these countries was seen in the global enteric multicenter study, a population-based case–control study including seven countries in Africa and Asia with the goal to identify the etiology, burden, and mortality of acute moderate-to-severe pediatric diarrhea.[24,25] The reasons for the decline are unclear but may be linked to improved public health with active interventions, therapy, sanitary conditions, and control of hospital infections.[24,26] However, tEPEC infections remain associated with a 2.8-fold higher risk of death among infants aged 0–11 months.[25]

Atypical EPEC continues to be frequently detected in various parts of the world.[7] Thirteen studies from peri-equatorial/tropical countries showed aEPEC isolates in 78% (131/169) of all EPEC cases in children.[18] Wheeler et al.[27,28] reported the identification of 142 aEPEC strains with only one tEPEC in 2774 samples from symptomatic children from the UK. A study from Australia identified 61 EPEC strains from a stool samples of symptomatic patients and highlighted the higher frequency of aEPEC at 95.1% (58/61).[29] In 2009, the aEPEC strain O76 was reportedly responsible for a nursery outbreak in Finland.[30]

In another study, Sakkejha et al.[31] detected 109 EPEC isolates in England from 2010 to 2012, with 93% of the patients with diarrhea; aEPEC were seen more frequently than tEPEC. Overall, according to 266 studies published between 1990 and 2002, EPEC remains major pediatric pathogen.[7] As such, in 2014 a European, multicenter, prospective quarterly point-prevalence study of community-acquired diarrhea (EUCODI) showed a high frequency of EPEC.[32]

For unknown reasons, EPEC disease is becoming less frequent in infants in developed countries in developed/temperate climate zones of the world. However, day care centers and pediatric wards of hospitals are still prime breeding grounds for EPEC outbreaks.[33–35] Globally, EPEC is responsible for infantile diarrhea in under-developed nations with nearly 30% mortality.[36]

Even though EPEC is strongly associated with infant diarrhea, many studies have also found EPEC, particularly aEPEC, in asymptomatic controls.[18] There may be multiple possible reasons for this apparent anomaly: (a) host susceptibility.[37] There may be genetic variability in specific mucosal receptors, including proteins and carbohydrate moieties; (b) individual variability in non-specific host barriers such as the gut microbiome, mucus layer, and epithelium. The variability in the strength of these barriers may influence bacterial overgrowth and susceptibility to disease.[38] (c) Immune

status of the host, which may limit bacterial flora to colonization but not cross numerical thresholds needed to cause disease.[39] In addition, secretory immunoglobulin A (sIgA) in the intestine and in human milk can limit/prevent enterocyte colonization/mucosal invasion by enteropathogens.[40] Human milk also contains other non-specific defense factors such as lactoferrin and enterotoxin-binding oligosaccharides. In endemic areas, colostrum contains specific sIgA against EPEC.[41] In addition, children may acquire natural immunity with age. Opintan et al.[42] showed that EPEC carriage, not disease, is frequently seen in healthy children in endemic areas after 2 years of age. Bacterial factors are also important in asymptomatic carriage of EPEC. Some strains are more likely to not cause symptoms, such as those with the phylogenetic marker gene *yhaA*. Children without diarrhea frequently carried aEPEC strains that were OI-122 *efa1/lifA*-negative and *yhaA*-positive. There is considerable variability in the severity of disease between individual strains.[43]

The variability in diagnostic tests also needs attention. In this regard, the bacterial load is an important consideration. Barletta et al.[44] compared children with diarrhea *vs*. asymptomatic controls. When a quantitative real-time PCR assay was used, the bacterial load was significantly higher in the symptomatic infants than in age-matched controls. Other factors may also need consideration. For instance, the collection of control samples and sample size are pivotal factors.[45] The transmission of EPEC from controls to other patients needs further consideration. Finally, environmental factors such as poor hygiene and fecal contamination may also increase the bacterial load in control groups.[7]

EPEC Definition and Classification

Escherichia coli serotypes were first classified based on the Kauffmann system in the 1940s. The three antigen systems included the somatic O, flagellar H, and the capsular K surface antigens.[46,47] In 1955, the term EPEC was coined to describe strains that were primary intestinal pathogens but were rarely encountered in the feces of healthy individuals and in infections other than diarrheal diseases.[4] Formally, 187 O serotypes were documented, but currently 176 are considered as true O serotypes. Six (O31, O47, O67, O72, O94, and O122) are no longer considered as O serotypes, some being duplicate names for an O antigen and others were in organisms that were reclassified into other genera and three (O34, O89, and O144) strains are also removed from this classification which are incapable of producing O antigens and are removed from these O serotypes. For O serotypes, the most variable cell component is O antigen because of existence of variations in sugar moieties and the linkages present within as well as between O units. Due to the existence of these variations, there is diversity of various clones in the species. Each expresses different surface antigen on the cell surface which offers selective advantages in varied environments. The O antigen is one of main virulence factors and its loss can severely impair the pathogenicity and virulence. O antigens play vital roles, including protection against phagocytosis and clearance via neutrophils and monocytes, as well as have inhibitory effects on the bactericidal activity of lysozyme, a key player in host innate immunity.[48] The major O serogroups, including O55, O86, O111, O119, O125, O126, O127, O128ab, and O142, are considered to contain EPEC serotypes.[49,50] The variability in O surface antigen provides basis for typing of the bacterial species for taxonomical as well as epidemiological purposes. It is most widely utilized to signify the presence of enteropathogens and considered as a basic tool for bacterial outbreak investigations and surveillance.[48] O55 serotype is most rarely found in healthy individuals. However, varied pathogenicity levels are exhibited within O serotypes as all serotypes are not equally pathogenic, and only a limited number of H serotypes are incriminated within O serotypes.[49] Another antigen, H (flagellar) is also expressed by EPEC strains. H2 and H6 are predominantly expressed flagellar antigens, and the least frequent ones include H7, H8, H9, H12, H21, H27, H25, and H34. However, some EPEC strains lack H flagellar antigens and are, therefore, classified as H-negative. These strains are non-motile.[51]

Several EPEC serogroups may share characteristics with the Shiga toxin-producing *E. coli* (STEC).[52,53] Both can induce attaching-and-effacing (A/E) lesions on intestinal epithelial cells (IECs), and the bacteria attach to IECs and efface the microvilli on the cell surface (Fig. 1).[54] There is a need to identify specific virulence genes to distinguish between the two bacterial genera as these differ in pathogenicity. EPEC pathotypes do not produce the Shiga toxin (*stx*$^-$), but some aEPEC strains such as the O55:H7 resemble the LEE-positive Shiga toxin-producing *E. coli* such as the STEC O157:H7 in their genetic and virulence characteristics.[4] Most tEPEC and aEPEC strains may differ in adherence patterns; tEPEC strains show localized adherence (LA) patterns, but the aEPEC can produce a localized-like adherence, a diffuse adherence (DA), or an aggregative adherence (AA) pattern.[24]

Enteropathogenic *E. coli* binds IECs by an outer membrane protein called intimin, which is encoded by the gene *eae*. The genetic elements needed to produce the A/E lesions are encoded on a genomic pathogenicity island, the locus of enterocyte effacement (LEE).[55,56] Another pathogenicity factor is the plasmid *E. coli* adherence factor (pEAF).[4,57] Enteropathogenic *E. coli* is classified as typical or atypical based on the presence of pEAF, which contains two important operons.[24] These include a type IV bundle-forming pilus *(bfp)* and a plasmid-encoded regulator (*per*). The *bfp* promotes bacterial adherence and formation of compact microcolonies. *Bfp* and *per* are important transcriptional activators for LEE pathogenicity island.[4,58]

The plasmid pEAF imparts important characteristics to EPECs. Three subgroups can be seen:

(1) Typical EPECs (tEPECs) are *eae*$^+$ *bfpA*$^+$ *stx*$^-$. Most belong to classical O:H serotypes and express *bfp* to show the localized adherence (LA) phenotype.[59] The expression of EPEC virulence genes on classical EPEC serogroups is not universal. However, tEPEC strains are more homogeneous in their virulence traits than aEPEC. Most of the typical strains produce the virulence factors encoded by the LEE region and EAF plasmid.[24]
(2) Atypical *E. coli* (aEPEC) strains lack the EAF plasmid and hence are bfpA negative and are defined as *eae*$^+$ *bfpA*$^-$ *stx*$^-$. The lack of Bfp, makes atypical EPEC strains exhibit localized-like (LAL) pattern, which is mainly characterized by the presence of bacterial microcolonies. LAL is the most common pattern, but atypical EPEC strains also exhibit diffuse (DA) or aggregative adherence (AA) patterns.[58,60] LAL$^+$ aEPECs show pili and other known adhesins. Some aEPECs express the enteroaggregative heat-stable toxin (EAST1) and other potential virulence factors not encoded in the LEE, such as a hemolysin.[60–62]
(3) Non-typeable EPECs, which are identified among aEPECs and do not belong to classical EPEC serogroups. There are >200 of these strains.[63,64]

Virulence Factors and Signaling

For successful infection and formation of an A/E lesion, two major virulence factors are needed, the type IV bundle-forming pilus (BFP) and LEE.

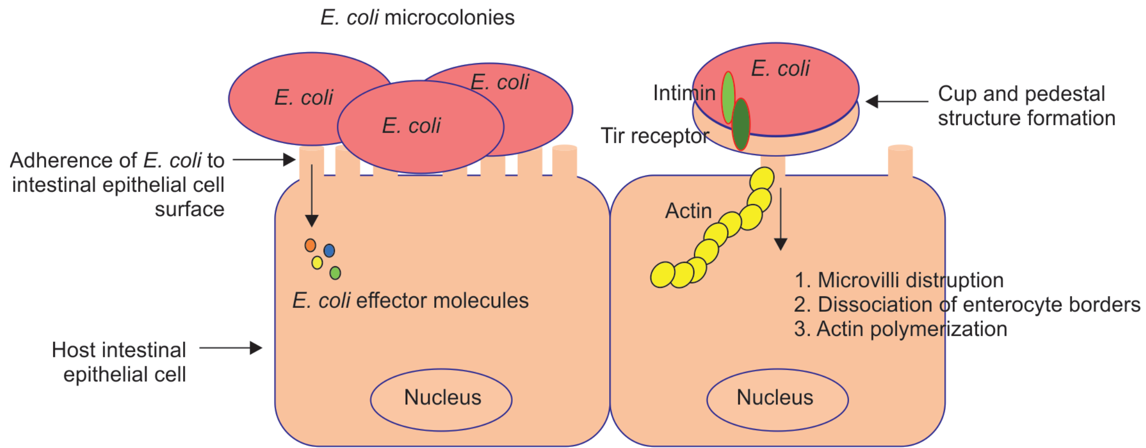


Fig. 1: Attachment of EPEC to the host epithelial cells results in the formation of cup and pedestal structures in the A/E lesion. A/E lesions exhibit intimate bacterial adherence to intestinal epithelial cells, extensive disruption of microvilli and enterocyte borders, and alterations in F-actin arrangement with accumulation of cytoskeletal proteins beneath adherent microcolonies resulting in the formation of a typical cup and pedestal-like structure

Type IV Bundle-forming Pilus (BFP)

Type IV BFP is a dynamic fibrillar organelle responsible for the initiation of initial non-intimate attachment of EPEC to the host IECs. Further, BFP recruits individual EPEC together as aggregates and leads to the formation of microcolony on the host cell membranes, typically known as a localized adherence (LA) phenotype. The ~80 kb plasmid (pEAF) encodes 14 genes, which are required for the biogenesis of BFP and consequently in the formation of the EPEC adherence factor (EAF). The strains lacking pEAF are incapable of forming typical LA phenotype.[57,65,66] Activation of BfpA is mediated by the plasmid-encoded regulator A (PerA). The activated form is a major pilus subunit and is called pre-bundlin. Further, pre-bundlin is acted upon by the prepilin peptidase, BfpP, and is then converted to the mature forms.[67,68]

Two nucleotide-binding proteins, BfpD and BfpF, further mediate the extension of the pilus and retraction, respectively. Aggregation of EPEC is promoted by BfpD, whereas BfpF facilitates the separation of EPEC from cellular aggregates that are maintaining a constant supply of bacterial cells for further infectious steps. BfpF-mediated dissociation of bacterial cell aggregates permits the intimate attachment of individual EPEC to the gut epithelium, resulting in efficient activation of T3SS and successful translocation of effector molecules into the host cells (Fig. 2).[65] In addition to filamentous actin, cytoskeletal proteins such as α-actinin, talin, ezrin, myosin-light chain, vasodilator-stimulated phosphoprotein (VASP), the Wiskott–Aldrich syndrome protein (WASP), and the actin-related protein 2/3 (Arp2/3) complex are also observed in EPEC-induced A/E lesions. Additionally, many proteins involved in focal adhesion such as α-actinin and vinculin were found to be recruited to sites of A/E lesions.[69–73] After EPEC attachment to the host surface, kinases encoded by Ab1/Arg, Src, and Tec families lead to phosphorylation of tyrosine residues in the cytoplasmic domain of translocated intimin receptor (Tir). Phosphorylated Tir interacts with two adaptor proteins (Nck1 and Nck2). This interaction results in the recruitment of actin nucleation-promoting factor, N-WASP, which further activates the Arp2/3 complex that assembles actin beneath EPEC (Fig. 2). These signaling events lead to the formation of actin-rich pedestals on host cell luminal membrane, along with inflammatory response and diarrhea.[65]

Locus of Enterocyte Effacement

Once the bacterial aggregates dissociate from the host cell membranes via BfpF, EPEC expresses the LEE for further intimate attachment to intestinal epithelial cells (Fig. 2). Enteropathogenic *Escherichia coli* contains a 35,624 base pair LEE pathogenicity island (LPI), which contains 41 open reading frames (ORFs) of more than 50 amino acids arranged in five major polycistronic operons (LEE1 to LEE5).[74,75] Locus of enterocyte effacement pathogenicity island encodes for the majority of EPEC effector proteins. Locus of enterocyte effacement encodes for the T3SS machinery (Esc and Sep proteins), outer membrane adhesin (intimin), translocators (EspA, EspB, and EspD), chaperones (Ces proteins), effector proteins (EspF, EspG, EspH, Map, and EspZ), translocated intimin receptor (Tir), regulatory proteins Ler (LEE-encoded regulator), repressors including GrlR (global regulator of LEE proteins), and activators such as GrlA (global regulator of LEE proteins).[76] Various factors influence the regulation of LEE, including Ler, GrlR, and GrlA; and *E. coli* global regulators such as the H^+NS, IHF, and FIS.[77,78] These genes are separated into three functional domains – a region encoding intimate adherence (Tir and intimin), a region encoding the EPEC-secreted proteins (including espA, espB, espD, and espF) and their putative chaperones, and the region encoding a type III secretion system.[79]

LEE1, LEE2, and LEE3 encode for the genes involved in the production, assembly, and regulation of T3SS. Locus of enterocyte effacement-encoded structures are comprised of three vital components: (a) outer membrane needle complex (EscC, EscD, EscF, EscI, and EscJ); (b) inner membrane, which contains an export apparatus (EscRST, EscU, and EscV); and (c) a cytoplasmic sorting platform (EscA, EscK, EscL, EscN, and EscQ). The gene of translocation apparatus, the extracellularly secreted proteins of T3SS are encoded via LEE4 genes (EspA, EspB, and EspD). The role of EspB is implicated in the effacement of microvilli on the intestinal surface. The EspABD translocon apparatus of T3SS is responsible for the translocation of six LEE-encoded effectors (Tir, Map, EspF, EspG, EspZ, and EspH). These effectors are involved in the sequential events during EPEC infection which include disruption of tight junctions, mitochondrial dysfunction, and formation of filopodia in host intestinal epithelial cells. The genes for adhesin (intimin), 94 kDa

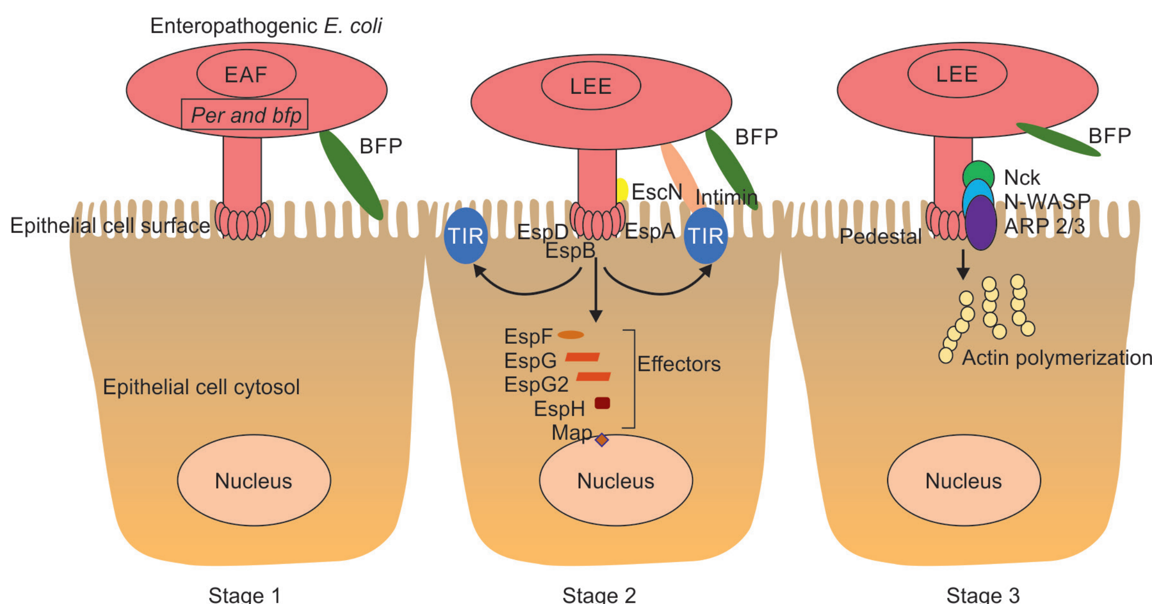


Fig. 2: Schematic representation of localization of virulence factors type IV BFP and LEE of EPEC on the small intestine and other interacting proteins involved during A/E lesion formation: Stage 1: Initial adherence and microcolony formation of EPEC on intestinal epithelial cells induced via type IV BFP and its activator Per. Stage 2: Effacement of microvilli mediated by activation of LEE operons via EspABD complex and translocation of T3SS effector proteins into intestinal epithelial cells. EPEC utilizes a type III secretion system (T3SS) to inject bacterial virulence factors directly into host cells. The T3SS apparatus is composed of several key protein components, including EspA, EspB, and EspD. EspA forms a needle-like channel and EspB and EspD cap this structure to form a pore that allows direct translocation of secreted effector molecules known as *E. coli* secreted proteins, EspF, EspG, EspH, Tir, and Map into the host cytosol. Translocated intimin receptor (Tir) is inserted into the plasma membrane, where it serves as a receptor for intimin, with Tir-intimin interaction triggering signaling events leading to pedestal formation. Stage 3: Intimate attachment of EPEC on the surface of host epithelial cells mediated by the interaction of adhesin intimin with Tir. This is followed by phosphorylation of Tir and recruitment of host cellular proteins and other adaptor proteins (Nck, N-WASP, and Arp2/3 complex) resulting in induction of actin polymerization beneath attached EPEC (BFP, bundle forming pilus; LEE, locus of enterocyte effacement; Tir, translocated receptor; EPEC, Enteropathogenic *Escherichia coli*; EAF, EPEC adherence factor; PER, plasmid-encoded regulator; A/E, attaching and effacing; Nck, Non-catalytic tyrosine kinase; WASP, Wiskott–Aldrich syndrome protein and Arp2/3, actin-related protein 2/3 complex)

outer membrane protein of EPEC, and its translocation receptor (Tir) are encoded via LEE5.[65] The gene encoding for intimin, *eae* (*E. coli* attaching-and-effacing), is comprised of four distinct intimin subtypes (*α*, *β*, *γ*, and *δ*).[80] Different intimin subtypes are expressed in different tissues; the small intestinal mucosal layer expresses intimin-*α* clones, and the Peyer's patches exhibit expression of intimin-*γ*.[81] Different intimin types could bind to the host cell protein nucleolin, which then colocalizes with adherent bacteria.[82] Chaperone proteins have also been discovered in T3SS in EPEC and are essential for secretion of espD, espA, and espB.[76]

Effectors encoded outside the LEE pathogenicity island have been described in all A/E-producing pathogens.[24] Scattered across the whole genome, six pathogenicity islands harbor the clusters of non-LEE-encoded (Nle) effectors.[75,83] These Nle effectors include NleA-H, EspG2/Orf3, Cif, EspJ, and EspL. NleA (also called EspI) suppresses protein secretion; EspJ inhibits phagocytosis; and NleE and NleH activate innate immune responses. A/E *E. coli* strains utilize both LEE-encoded and non-LEE-encoded effector proteins to subvert and modulate cellular and barrier properties of the host for successful infection in a well-controlled manner.[7]

Pathogenesis

Enteropathogenic *E. coli* is generally considered to be a noninvasive pathogen but can cause subclinical to fatal diarrhea.[4] Studies with adult volunteers reported that 12–24 hours post infection with tEPEC (10^9–10^{10} of bacterial inoculum) can induce diarrhea.[9] As discussed before, EPEC strains attach to IECs in two different patterns – localized adherence (LA) in which bacteria adhere in discrete microcolonies and diffuse adherence (DA) in which bacteria adhere uniformly over the cell surface. Localized adherence was highly correlated with specific EPEC serogroups in strains isolated from patients with diarrhea.[66] The BFP is usually seen as the initial EPEC attachment factor.[84] The major pilin subunit of BFP is identified as the *bfp*A. Bundle-forming pilus is encoded by a cluster of 14 genes on the EAF plasmid and mediates LA phenotype, which is further responsible for antigenicity, biofilm formation, autoaggregation, and compact microcolony formation.[51,57,85] Genes external to the *bfp* gene cluster were also necessary for full expression of BFP. This included the global regulator element of EPEC pathogenesis *per*ABC (*bfp*TVW) and the chromosomal *dsb*A gene encoding for a disulfide isomerase.[86]

The BFP-mediated interbacterial interactions may allow the dispersal of individual bacteria from autoaggregates and colonization to other epithelial sites, contributing to the spread of infection within the gut. In addition to BFP, additional fimbrial structures have also been characterized and could have roles in EPEC-host cell adhesion. There may be rod-like fimbriae and fibrillae, suggesting that the bacterial–host cell interaction is a multifactorial process. More recently, flagella have been implicated in EPEC adherence to IECs.[87] However, there is some uncertainty because a flagellated strain that lacked BFP, intimin and EspA failed to adhere to IECs in *ex vivo* studies. The term EPEC adherence factor

(EAF) refers to the plasmid-mediated adhesion. *Escherichia coli* strains isolated from outbreaks of infantile gastroenteritis almost invariably possess the EAF plasmid.[88] EPEC adherence factor plasmid generally promotes non-intimate cell adhesion. For A/E lesion formation, chromosomally encoded factors were required for the A/E phenotype, and the genes on the plasmid may play a secondary role.[89] Localized adherence (LA) pattern is exhibited by various EPEC serogroups including O55, O86, O111ab, O119, O125, O128ab, and O142.[90] The existence of 60 MDa plasmid (denoted as pMAR2) is responsible for localized adherence pattern exhibited by EPEC strain E2348/69 (O127:H6).[91]

Mucosal adhesion by EPEC may involve two distinct stages: (a) initial attachment of EPEC promoted by plasmid-encoded adhesins; and (b) effacement of brush border microvilli leading to intimate EPEC attachment. Although the second stage could occur without the first, the presence of plasmid-encoded adhesin enhanced mucosal colonization.[89] A/E lesions exhibit association of bacterial cells to IECs followed by extensive disruption, loss of brush borders and microvilli, alterations in F actin rearrangements, and ultimately cup and pedestal formations.[7] These structures may provide a strong attachment of EPEC to the cell surface, preventing dislodgement in the ensuing diarrheal response. Many affected bowel segments show depletion of glycocalyx. Some areas show a mucous pseudomembrane coating on the mucosal surface. There are characteristic cytoskeletal alterations with disruption of the brush border cytoskeleton and proliferation of filamentous actin beneath the foci where bacteria adhered to the host cell surface. There are at least three prominent changes: (a) adherence to IECs; (b) delivery of 25–50 virulence factors into the host cell using a type III secretion system (T3SS)[51]; and finally, (c) firm adherence to the cell surface with the formation of pedestals (Fig. 2).[86] The T3SS is one of the five most important secretion systems utilized by Gram-negative bacteria, besides the T4SS, T5SS, T6SS, and T7SS, to inject effector proteins into the host cells to promote colonization and virulence. It is important because it is exclusively involved in virulence.[92,93]

The T3SS, intimin, and the translocated intimin receptor (tir) are all essential virulence determinants of the intimate adherence, a process that requires the T3SS to inject tir into the host cell. Tir acts as a receptor for bacterial binding via tir–intimin interaction. These trigger many signaling cascades such as phosphorylation of a host phospholipase and recruitment of cytoskeletal proteins beneath the adherent bacteria. Intimin can also subvert cellular processes independently of tir.[86] Mitochondrial-associated protein (Map) targets host cell mitochondria and also contributes to the disruption of the epithelial barrier.[94]

The hallmark of EPEC infection is A/E lesion which marks the intimate attachment of the bacteria to the host enterocytes and results in the effacement of the microvilli. The IEC membrane in these foci can also be raised locally in a characteristic pedestal shape that may extend up to 10 µm outwards from the cell to form pseudopod-like structures.[95] This near-complete destruction and extensive loss of intestinal epithelial surface with villus atrophy and thinning of the mucosal layer is frequently seen during severe EPEC infections.[96] The extensive loss of microvilli on the infected IECs alters the expression and function of ion transporters, channels, and tight junctions. The pathogenesis of microvillus effacement is seen as a 2-step process that requires synergistic action of three effectors (Map, EspF, and Tir) on intimin, and retention of the detached microvillar material. Other studies have focused on the type III secretion system and its effectors including tir, map, espF, and espG.[65] Enteropathogenic *E. coli* also rapidly inactivates the sodium-D-glucose cotransporter (SGLT-1) by multiple mechanisms. SGLT-1 plays a crucial role in the daily uptake of fluids from the intestinal lumen.[97] Calcium signaling may also be important; it may activate actin-severing proteins, resulting in cytoskeletal rearrangement and brush border effacement. However, all these possibilities need further confirmation.[98,99] Some aEPEC is strongly associated with acute disease, whereas others have been noted in persistent diarrhea.[100] Clinically, aEPEC outbreaks may cause mild but prolonged non-dehydrating, non-inflammatory diarrhea. There is usually no fever, vomiting, or abdominal pain.

Pathophysiology of Diarrhea

We now understand EPEC pathogenesis at cellular and genetic levels, but the pathophysiology of the resulting diarrhea remains elusive. The extensive loss of microvillus and subsequent reduction in absorptive surface certainly contributes to diarrhea. However, the rapid onset of diarrhea remains unexplained and appears to be multifactorial in nature. Enteropathogenic *E. coli* can alter epithelial permeability by activating signaling cascades that phosphorylate Ser/Thr residues on the myosin light chains.[101] This might contribute to diarrhea through increased permeability and disruption of tight junction integrity (Fig. 3). Recently, another EPEC effector molecule, the espF, was shown to be translocated by the T3SS into host cells, where it disrupts host IEC tight junctions and could contribute to diarrhea.[102] Enteropathogenic *E. coli* can also activate NF-κB in host cells and induce host inflammatory responses, which, in turn, could increase paracellular permeability and cause tissue damage.[103] The stimulatory effects of EPEC infection have been implicated on NF-κB activation and downstream enhancement of Cl secretion and fluid accumulation in the colon.[104,105]

Prolonged EPEC infection leads to inflammation and disruption of structure and barrier function of tight junctions (Fig. 3).[5,106,107] Enhanced paracellular permeability, inflammation, and disruption of tight junctions have been implicated in EPEC-mediated chronic diarrhea.[108] Studies have highlighted the downstream effects of prolonged inflammation in terms of increased influx of neutrophils, resulting in the release of 5-AMP that is further converted into secretagogue adenosine.[109,110]

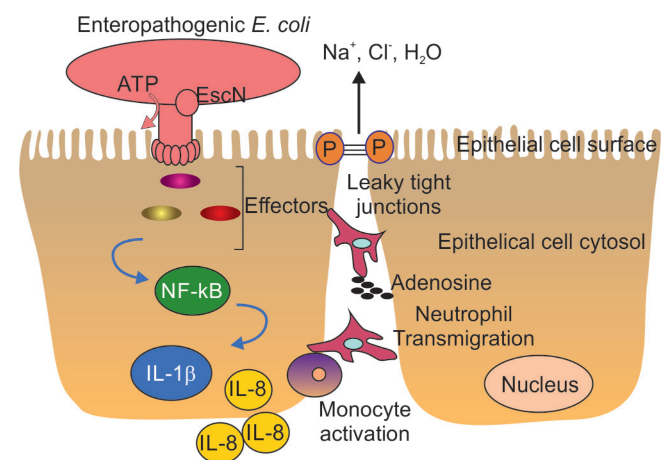


Fig. 3: EPEC infection induces inflammation and disrupts the epithelial barrier resulting in leaky tight junctions. Bacterial overgrowth, cytokine expression, biofilms, and leukocyte infiltration all create positive-feedback loops of inflammatory changes. Cytokines such as interleukin (IL)-1*β* and chemokines such as IL-8 activate regulatory factors such as the nuclear factor-*κ*B and progressively enhance the inflammatory changes and dysfunction of the epithelial barrier

Enteropathogens such as EPEC likely cause diarrhea by altering electrolyte transport.[111] Impairment of ion and solute transport may directly or indirectly influence the fluid transport processes and barrier integrity in gut epithelial cells.[112] Recent advances indicate that EPEC infection can directly influence ion transport mechanisms involving $Cl^-/HCO_3^-/OH^-$ exchange, Na^+/H^+ exchange, serotonin transporter, and short-chain fatty acids transporters. The following section will review the potential mechanism(s) involved in the regulation/alteration of ion and nutrient transporters on the gut epithelial cells during EPEC-induced diarrhea.

(a) Effect of EPEC Infection on Na^+/H^+ Exchanger Type 3 (NHE3)

Diarrhea caused by enteric pathogens may involve decreased NaCl absorption, enhanced Cl^- secretion, or both.[5] In early onset diarrhea, decreased intestinal NaCl may be pathophysiologically more important than the rise in Cl^- secretion.[113,114] The effector proteins of EPEC namely, NleA and Map, interact with Na^+/H^+ exchanger regulatory factor 2 (NHERF2) and alter its function and ultimately leading to decreased Na^+ uptake.[115] In intestinal epithelial cells, the expression of NHE2 and NHE3 is restricted to the apical surface, whereas NHE1 is expressed on the basolateral membranes. Our group has shown that EPEC infection in *in vitro* models activated NHE2 but inhibited the NHE3, the key Na^+ absorbing transporter (Fig. 4).[116] In *in vitro* models, EPEC infection leads to inhibition of the $Cl^-/HCO_3^-/OH^-$/exchange activity critical for intestinal chloride absorption.[116] Also, as stated above the activity of NHE3, which is a major Na^+ absorbing isoform, is inhibited.[114] These findings may be a source of uncertainty in the relative pathophysiological importance of NHE2 vs. NHE3; NHE3 here could very well be the more important of these two as a regulator of Na^+ absorption and determinant of the onset of diarrhea.[114] Also, prolonged EPEC infection contributes to inflammation and disruption of the structure and barrier function integrity of tight junctions and could contribute to diarrhea.[117]

(b) Effect of EPEC Infection on Downregulated in Adenoma (DRA/SLC26A3)

Intestinal epithelial cells express an integral membrane Cl^-/HCO_3^- transporter, the downregulated in adenoma (DRA/SLC26A3).[115] EPEC suppresses the function and apical expression of DRA/SLC26A3, and may thus contribute to the pathophysiology of diarrhea. Studies from our group demonstrated an increased endocytosis and decreased apical expression of DRA/SLC26A3 in EPEC-infected cells (Fig. 4).[118] Other studies suggest that reduced exocytosis may also play a role. The virulence factors EspG1 and EspG2 may alter DRA/SLC26A3 expression on epithelial cells via mechanisms involving microtubule disruption.[118]

(c) Effect of EPEC Infection on Absorption of Short-chain Fatty Acids (SCFAs)

Short-chain fatty acids play a significant role in sustaining colonocyte health and metabolism, integrity of epithelial lining, and in the maintenance of colonic fluid and electrolyte balance. Butyrate, a key SCFA, has been shown to play an important role in fluid balance by enhancing electroneutral NaCl absorption[119] and reducing Cl^- secretion.[120] Our group has shown that EPEC infection can significantly reduce butyrate uptake by intestinal epithelial cell lines (Fig. 4).[117] EPEC infection reduced the expression of monocarboxylate transporter 1 (MCT1), the primary SCFA transporter in gut epithelial cells. Butyrate also plays an anti-inflammatory role,[121] and decreased availability of butyrate has been noted both in acute and chronic inflammatory conditions.[122]

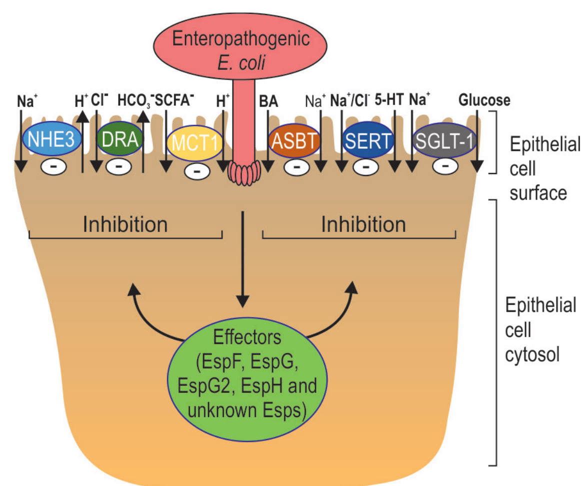


Fig. 4: Schematic representation of transporters affected during EPEC infection. EPEC infection affects intestinal epithelial barrier and leads to reduced expression/function of ion and solute transporters and results in the development of diarrhea. Type III secretion system of EPEC is responsible for the release of *E. coli*-secreted proteins (Esps) into the infected host cells. EspF exhibits inhibitory impact on Na^+/H^+ exchange isoform 3 and EspG disrupts the microtubules, which further leads to decreased apical expression of DRA resulting in reduction of apical Cl^-/OH^- (HCO_3^-) exchange activity and inhibition of electroneutral NaCl absorption in the intestinal milieu. EPEC inhibits butyrate absorption by reducing the plasma membrane expression of monocarboxylate transporter 1 (MCT-1). EPEC also inhibits the function of serotonin transporter (SERT) and increases 5-HT availability by activating protein tyrosine phosphatases (PTPases), which can further modulate the ion absorption and contribute to the onset of diarrhea. EPEC-induced inhibition of SGLT-1 also promotes fluid accumulation with similar effects. (NHE3, Na^+/H^+ exchanger type 3; DRA, downregulated in adenoma; MCT-1, Monocarboxylate transporter 1; ASBT, Apical sodium-dependent bile acid transporter; BA, Bile acid; SERT, Serotonin transporter; SGLT-1, D-glucose transporter)

(d) Effect of EPEC Infection on Apical Sodium-dependent Bile Acid Transporter (ASBT)

Apical sodium-dependent bile acid transporter (ASBT) is a putative transporter responsible for stimulating the intestinal absorption of bile acids. Reduced ASBT expression/function has been implicated in the pathogenesis of diarrhea. Annaba et al.[123] have shown the negative impact of EPEC infection on ileal ASBT expression/function in various *in vitro* models.

(e) Effect of EPEC Infection on the Serotonin Transporter (SERT)

Serotonin transporter is a key regulator of the extracellular availability of serotonin (5-HT), and its function was inhibited in response to EPEC infection in intestinal epithelial cells. Serotonin transporter activity is reduced via activation of the Src-homology-2 (SH2) domain containing protein tyrosine phosphatase (PTPase). In the absence of SERT, 5-HT circulates in the extracellular *milieu* resulting in the activation/sensitization of its cognate receptors.[124] In this study, SHP2 is associated with SERT during EPEC infection due to dephosphorylation at tyrosine residues and thereby inhibiting its function and activity. High luminal serotonin levels (due to

inhibition of SERT) have been linked to fluid accumulation in the gut lumen.

(f) Effect of EPEC Infection on Sodium D-glucose Transporter (SGLT-1)

In addition to SERT and other transporters outlined above, EPEC has also been shown to inhibit the function of the sodium-D-glucose transporter (SGLT1), which is a major contributor of fluid uptake in the small intestine[112] and hence could contribute to diarrhea.

Effect of EPEC Infection on Tight Junctions

Enteropathogenic *E. coli*-mediated disruption of the gut epithelial barrier also contributes to the onset of diarrhea. Epithelial cells are normally bound together by a network of tight junctions. The membrane barrier is selectively permeable for the passage of ions and solutes across the paracellular space. It also serves as a boundary that prevents the coalescence of apical and basal plasma membrane proteins to maintain the polarity of the epithelial cells and prevents the backflow of fluids into the lumen.[115] The cell–cell adhesion is maintained by the transmembrane proteins which are associated with the cytoskeleton via the adaptor proteins. The members of claudin family and the transmembrane proteins of the marvel-domain containing protein families, such as occludin, tricellulin/marvelD2, and marvelD3, are key regulators of paracellular permeability. Tight junction-associated exchange factors for Rho GTPases also modulate the actin cytoskeleton and membrane permeability.[115,125–127] During EPEC-induced diarrhea, leakages are observed in the tight junctions; studies suggest the potential role(s) of effector proteins EspF, Map, EspG1/G2, and NleA in disrupting the host cell tight junctions.[128–130] The *N*-terminus of EspF contains mitochondrial- and nucleolus-targeting sequences that can alter the function of these organelles. The *C*-terminus of EspF contains three proline-rich repeats that interact with the eukaryotic sorting nexin 9 (SNX9) and neuronal Wiskott–Aldrich syndrome protein (N-WASP), and are ultimately involved in the activation of the Arp2/3 complex and regulation of actin polymerization.[128–132] EspF may recruit zonula occludens (ZO-1 and ZO-2) into actin pedestals.[133] In murine models, EspF can disrupt tight junctions via internalization of claudin-1, 3, and 5.[115]

Another EPEC effector protein, Map, interacts with EspF and is involved in the disruption of tight junctions. Similar to EspF, Map is recruited to mitochondria where it modulates the mitochondrial processes and functions. Map acts as a guanine-nucleotide exchange factor (GEF) for Cdc42 GTPase and promotes its activation leading to the formation of transient filopodia. A Thr-Arg-Leu motif is present at the C-terminus of Map, which interacts with the Na^+/H^+ exchanger regulatory factor 1 (NHERF1). This complex links with ezrin and then promotes the interaction between Map and actin cytoskeleton.[128,134,135] The tight junction proteins, the zonula occludens-1 and occludin, are disrupted by NleA leading to increased paracellular permeability.[130]

Conclusions

Studies show aEPEC to be more prevalent than tEPEC worldwide. Therefore, it is important to further characterize the pathogenicity of these strains, virulence mechanisms, and the pathophysiology of these infections. While there is strong evidence showing that EPEC-induced diarrhea is multifactorial in nature and involves compromised gut barrier integrity and decreased absorption of fluid, which is contributed by decreased NaCl and solute absorption. However, the exact mechanisms of diarrhea in EPEC infection are still evolving. From the clinical perspective, there is a need for fast, easy, and inexpensive diagnostic methods to define optimal treatment and prevention for children in endemic areas.

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